UNIVERSIDADE FEDERAL DE MINAS GERAIS

Instituto de Ciências Biológicas

Programa de Pós-Graduação em Microbiologia

Jaqueline Silva de Oliveira

AVALIAÇÃO DE ROTAS ALTERNATIVAS DE TRANSMISSÃO DO VACCINIA VIRUS (VACV) EM ÁREAS URBANIZADAS DO ESTADO DE MINAS GERAIS, BRASIL.

Belo Horizonte

2021

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Tese apresentada ao Programa de Pós-graduação em Microbiologia da Universidade Federal de Minas Gerais como requisito parcial para obtenção de título de Doutor (a) em Microbiologia.

Orientadora: Profa. Dra. Giliane De Souza Trindade
Co-Orientador: Dr. Galileu Barbosa Costa

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ATA DE DEFESA DE TESE

ATA DA DEFESA DE TESE DE **JAQUELINE SILVA DE OLIVEIRA**

Nº REGISTRO: **2017665775**

Às 14:00 horas do dia **26 de JULHO de 2021**, reuniu-se, por via remota, a Comissão Examinadora composta pelos Drs. Erna Geessien Kroon (Departamento de Microbiologia/ICB/UFMG), Maria Isabel Maldonado Coelho Guedes (Escola de Veterinária da UFMG), Danilo Bretas de Oliveira (Faculdade de Medicina da Universidade Federal dos Vales do Jequitinhonha e Mucuri (UFVJM), Jane Megid (Faculdade de Medicina Veterinária e Zootecnia, Botucatu, UNESP) e a Profa. Dra. Giliane de Souza Trindade - Orientadora para julgar o trabalho final "Avaliação de rotas alternativas de transmissão do vaccinia virus em áreas urbanizadas do estado de Minas Gerais, Brasil", da aluna **Jaqueline Silva de Oliveira**, requisito final para a obtenção do Grau de **DOUTOR EM CIÊNCIAS BIOLÓGICAS: MICROBIOLOGIA**. Abrindo a sessão, a Presidente da Comissão, Profa. Dra. Giliane de Souza Trindade, após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra à candidata, para a apresentação de seu trabalho. Seguiu-se a arguição pelos Examinadores, com a respectiva defesa da candidata. Logo após, a Comissão se reuniu, sem a presença da candidata e do público, para julgamento e expedição de resultado final. A candidata foi considerada **APROVADA**. O resultado final foi comunicado publicamente à candidata pela Presidente da Comissão. Nada mais havendo a tratar, a Presidente encerrou a reunião e lavrou a presente ata, que será assinada por todos os membros participantes da Comissão Examinadora.

Membros da banca:

Dra. Profa. Erna Geessien Kroo
Dra. Maria Isabel Maldonado Coelho Guedes
Dr. Danilo Bretas de Oliveira
Dra. Jane Megid

De acordo:

Profa. Dra. Giliane de Souza Trindade

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Profa. Danielle da Glória de Souza

(Coordenadora)

Belo Horizonte, 26 de julho de 2021.

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Obrigada!

Jaqueline Silva de Oliveira, Julho de 2021.

RESUMO

A vaccínia bovina (VB) é uma zoonose viral emergente associada ao vírus vaccínia (VACV), caracterizada principalmente pela presença de lesões ulcerativas na pele e nas membranas mucosas de bovinos e ordenhadores. No ambiente rural, o VACV é transmitido através do contato direto entre trabalhadores e animais infectados. Após 20 anos da emergência da VB no Brasil, aspectos relacionados à origem e transmissão do VACV ainda são desconhecidos e pouco se sabe sobre a potencial circulação viral em ambiente urbano. Neste estudo, foi avaliada a hipótese de exposição humana através do consumo de queijo artesanal produzido em Minas Gerais (MG). A análise de 71 queijos artesanais produzidos em diferentes regiões do estado, entre 2015 e 2018, detectou o material genético do VACV em 28,1% dos queijos, cuja amostragem contemplou todos os anos de coleta e todas as bacias leiteiras. Na área urbana da bacia leiteira do Serro-MG, foi realizado um estudo populacional para avaliar os fatores de exposição humana. Participaram 372 indivíduos, cuja pesquisa molecular do vírus em swab orofaríngeo detectou o VACV em 25 amostras (6,7%). Foi realizado um inquérito soroepidemiológico que detectou uma prevalência global de 16,9% (63/372) de anticorpos neutralizantes, com título entre 100 e 800 UN/mL. Os anticorpos IgG foram detectados em 26 indivíduos (7%), enquanto o IgM foi detectado em 2,7% da população estudada (10/369). A presença de IgG e anticorpos neutralizantes, foram correlacionados estatisticamente à idade > 40 anos. As ocupações de “aposentado” e “trabalhador rural” foram associadas à presença de anticorpos neutralizantes, enquanto, para a presença de IgG apenas “trabalhador rural” mostrou associação. Neste estudo, indivíduos vacinados contra a varíola tiveram aproximadamente quatro vezes mais chances de ter anticorpos neutralizantes, IgG e IgM, em relação àqueles não vacinados; a manipulação de leite cru durante a produção de derivados do leite, também demonstrou associação a detecção de anticorpos neutralizantes (OR=2.45; IC 95% = 1.17-5.16) e IgG (OR=3.6; IC 95% = 1.18-9.8). Esses dados são indicativos da exposição ao VACV em área urbana, fora do contexto de surto e transmissão clássica e reforçam a hipótese da existência de rotas alternativas para a transmissão do VACV no Brasil.

Palavras-chave: vaccínia bovina, vaccinia virus, rotas alternativas de transmissão, soroprevalência, epidemiologia.

ABSTRACT

Bovine vaccinia (BV) is an emerging viral zoonosis associated with the vaccinia virus (VACV), characterized mainly by the presence of ulcerative lesions on the skin and mucous membranes of cattle and milkers. In the rural environment, VACV is transmitted through direct contact between workers and infected animals. Twenty years after the emergence of BV in Brazil, aspects related to the origin and transmission of VACV are still unknown and little is known about the potential viral circulation in an urban environment.In this study, the hypothesis of human exposure through the consumption of artisanal cheese produced in Minas Gerais (MG) was evaluated. The analysis of 71 artisanal cheeses produced in different regions of the state, between 2015 and 2018, detected the genetic material of the VACV in 28,1% of the cheeses, whose sampling included every year of collection and all milk basins. In the urban area of the Serro-MG dairy basin, a population study was carried out to assess human exposure factors. A total of 372 individuals were sampled, whose molecular investigation of the virus in an oropharyngeal swab detected VACV in 25 samples (6.7%). A seroepidemiological survey was carried out which detected an overall prevalence of 16.9% (63/372) of neutralizing antibodies, with a titer between 100 and 800 UN/mL. IgG-type antibodies were detected in 26 individuals (7%), while the IgM was detected in 2.7% of the studied population (10/369). The presence of IgG and neutralizing antibodies were statistically correlated with age > 40 years. The occupations of “retiree” and “rural worker” were associated as a risk factor with the presence of neutralizing antibodies, while for the presence of IgG, only “rural worker” showed an association. In this study, individuals vaccinated against smallpox were approximately four times more likely to have neutralizing antibodies, IgG and IgM, compared to those who were not vaccinated; handling raw milk during the production of dairy products also behaved as a risk factor for detection of neutralizing antibodies (OR=2.45; 95% CI = 1.17-5.16) and IgG-type antibodies (OR=3.6; 95% CI = 1.18-9.8) . These data are indicative of exposure to VACV in urban areas, outside the context of an outbreak and classical transmission, and reinforce the hypothesis of the existence of alternative routes for the transmission of VACV in Brazil.

Key words: bovine vaccinia, vaccinia virus, serro, alternative routes of transmission, soroprevalence, epidemiology.

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μL – Microlitro

ATCC – *American Type Culture Collection*

ATI – Proteína formadora do corpúsculo de inclusão do tipo acidófilo

BSA – (*Bovine seric albumine*) Albumina sérica bovina

CDC – *Centers for Disease Control and Prevention*

CEF – Complexo fusão-penetração

CEV – Vírus envelopado associado à célula CO2 – Dióxido de carbono

COEP – Comitê de Ética e Pesquisa

CPXV – *Cowpox virus*

DMEM - Meio Dulbecco MEM

DNA – Ácido desoxirribonucleico

dsDNA – DNA de dupla fita

ECOVIR – Ecologia de Viroses Emergentes

EEV – Vírus envelopado extracelular

ELISA - (*Enzyme Linked Immuno sorbent Assay)* Ensaio imunoenzimático

EMBRAPA – Empresa Brasileira de Pesquisa Agropecuária

GAG – Glicosaminoglicano

GP2 – Vírus Guarani propriedade 2

HA – Hemaglutinina

IBGE - Instituto Brasileiro de Geografia e Estatística

ICTV – Comitê Internacional de Taxonomia de Vírus

IEV – Vírus envelopado intracelular

IgG – Imunoglobulina do tipo G

IgM – Imunoglobulina do tipo M

IL – Interleucina

IMA – Instituto Mineiro de Agropecuária

IMV – Vírus maduro intracelular

INF – Interferon

ITR – Regiões terminais invertidas

kb – Quilobase

MEM – Meio mínimo essencial

mg – Miligrama

ml – Mililitro

mRNA – RNA mensageiro

MPXV – *Monkeypox virus*

NCPLV – Vírus gigantes núcleo citoplasmáticos de DNA

NK – Natural killer

nm – Nanômetro

OMS – Organização Mundial da Saúde

OPV – (*Orthopoxvirus*) ortopoxvírus

PAMP – Padrão molecular associado à patógeno

PBS – Tampão salina fosfato

PCR – (*Polimerase chain reaction*) Reação em cadeia da polimerase

pH – Potencial hidrogeniônico

pb – Pares de bases

PNH - Primatas não humanos

PRNT – (*Plaque Reduction Neutralization Test*) Teste de Neutralização por Redução de Placas

QA – Queijo artesanal

QMA – Queijo minas artesanal

RNA – Ácido ribonucleico

SFB – Soro fetal bovino

TCLE – Termo de Consentimento Livre e Esclarecido

TLR – (*Toll like receptor*) Receptor do tipo toll

TNF – Fator de necrose tumoral

UFMG – Universidade Federal de Minas Gerais

UN – Unidades neutralizantes

VACV – *Vaccinia virus*

VACV-Br – Vaccinia vírus brasileiro

VACV-WR – Vaccinia virus Western Reserve

VARV – *Variola virus*

VB – Vaccinia bovina

VGF – Fator de crescimento vira

SUMÁRIO

1. INTRODUÇÃO

1.1.Doenças emergentes associadas aos poxvírus

A emergência dos poxvírus representa uma das maiores ameaças à saúde humana e animal (DAMON et al., 2013). Esses vírus pertencem à família *Poxviridae,* a qual é composta por vírus geneticamente e morfologicamente complexos. Os poxvírus contém genoma de DNA e multiplicam-se no citoplasma das células, sendo capazes de infectar um amplo espectro de hospedeiros, incluindo humanos (MOSS et al., 2013).

Os poxvírus são classificados em duas subfamílias, a *Entomopoxvirinae* e *Chordopoxvirinae,* que infectam hospedeiros invertebrados e vertebrados, respectivamente (Tabela 1). A subfamília *Chordopoxvirinae* é dividida em 18 gêneros (ICTV, 2021; MOSS et al., 2013).

Tabela 1: Taxonomia dos poxvírus.

SUBFAMÍLIA	GÊNERO	ESPÉCIES	PROTÓTIPO
Chordopoxvirinae	*Avipoxvirus*	12	*Fowlopx virus*
	Capripoxvirus	3	*Sheeppox virus*
	Centapoxvirus	2	*Yokapox virus*
	Cervidpoxvirus	1	*Mule deerpox virus*
	Crocodylidposvirus	1	*Nile crocodilepox virus*
	Leporiposvirus	4	*Myxoma virus*
	Macropopoxvirus	2	*Eastern kangaroopox virus*
	Molluscipoxvirus	1	*Molluscum contagiosum virus*
	Mustelpoxvirus	1	*Sea otterpox virus*
	Orthopoxvirus	**12**	***Vaccinia virus***
	Oryzopoxvirus	1	*Cotia virus*
	Parapoxvirus	5	*Orf virus*
	Pteropoxvirus	1	*Pteropox virus*
	Salmonpoxvirus	1	*Salmon gillpox virus*
	Sciuripoxvirus	1	*Squirrelpox virus*
	Suipoxvirus	1	*Swinepox virus*
	Vespertilionpoxvirus	1	*Eptesipox virus*
	Yataposvirus	2	*Yaba monkey tumor virus*
Entomopoxvirinae	*Alphaentomopoxvirus*	7	*Melolontha melolontha entomopoxvirus*
	Betaeentomopoxvirus	16	*Amsacta moorei entomopoxvirus*

Gammaentomopoxvirus	6	*Chironomus luridus entomopoxvirus*
Diachasmimorpha entomopoxvirus	2	

Fonte: International Commitee on Taxonomy of Viruses (ICTV), 2021; disponível em: www.ictvonline.org/.

Orthopoxvirus (OPV) é o gênero mais importante e bem caracterizado da subfamília *Chordopoxvirinae.* O OPV se destaca por possuir espécies relevantes no contexto da saúde pública mundial. Dentre as espécies, apenas o *Variola virus* (VARV), agente etiológico da varíola, é um patógeno estrito a humanos, enquanto outras espécies como *Monkeypox virus* (MPXV), *Cowpox virus* (CPXV) e *Vaccinia virus* (VACV), também denominado *Buffalopox virus* (BPXV) em algumas regiões geográficas, são patógenos zoonóticos, podendo ser transmitidos de outros hospedeiros animais para humanos e amplamente detectados mundialmente (Figura 1) (FENNER et al., 1988; SHCHELKUNOV et al., 2013).

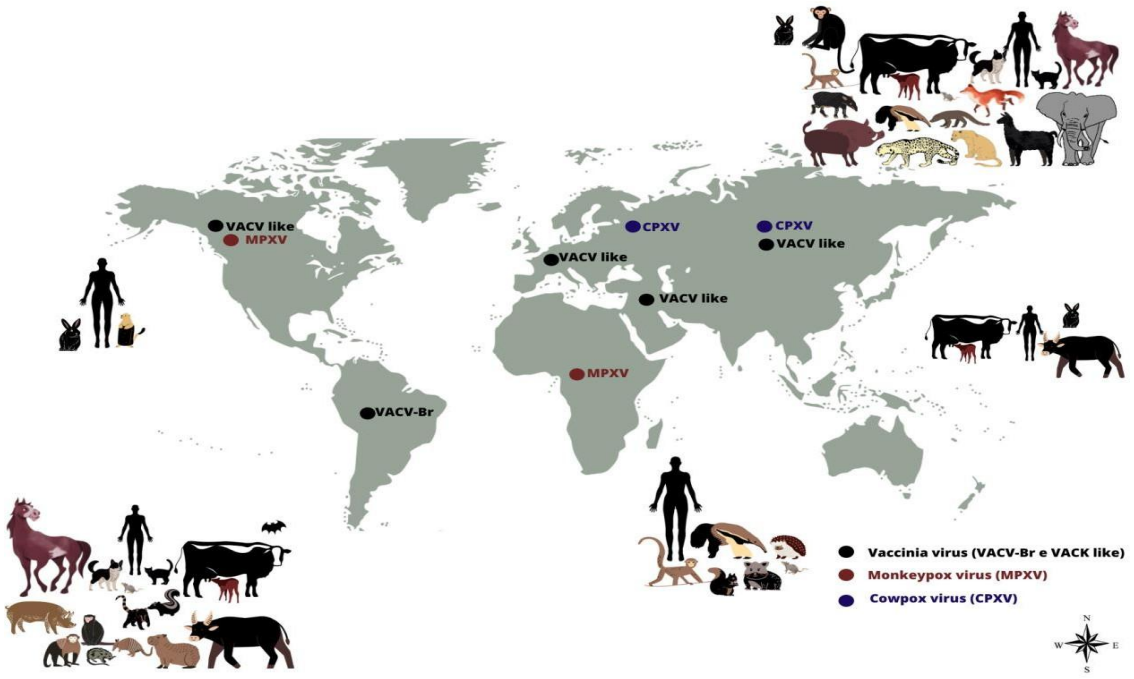


Figura 1. Distribuição mundial e espectro de hospedeiros dos OPV zoonóticos.

A imagem mostra o espectro de hospedeiros animais que foram associados a infecção natural por algumas espécies de OPV, de acordo com diferentes regiões do mundo (exceto pelo vírus MPXV nos Estados Unidos da América, associado a uma infecção importada). Infecções por OPV foram demonstradas em animais pertencentes a diferentes ordens, usando métodos diferentes (isolamento viral, detecção molecular de genomas virais ou pesquisa sorológica de anticorpos contra OPV). A ocorrência de alguns OPV zoonóticos já foi confirmada (por isolamento de vírus ou detecção molecular do genoma viral) em algumas regiões geográficas (indicado por pontos coloridos: azul: vaccínia vírus (incluindo BPXV e rabbitpox vírus) na América do Sul, Europa, Ásia e Oriente Médio; marrom: MPXV na África e na América do Norte; laranja: CPXV na Europa e na Ásia). Fonte: Silva et al., 2021– com modificações.

Os OPV compartilham similaridade genética, gerando uma resposta imune cruzada entre as diferentes espécies. Portanto, a infecção por um OPV é considerada protetiva contra a exposição a qualquer outra espécie do gênero (GUBSER et al., 2004; ESSBAUER et a., 201*0*).A alta similaridade antigênica entre os OPV favoreceu o sucesso da campanha de vacinação contra a varíola. O VARV foi responsável por ocasionar a morte de aproximadamente 500 milhões de pessoas no século passado e, em 1980, a varíola foi declarada erradicada devido à campanha mundial de vacinação conduzida pela Organização Mundial de Saúde (OMS) (FENNER et al., 1988).

Nesse contexto histórico, o VACV utilizado como vacina, ganhou notoriedade na medicina e, atualmente consiste no protótipo dos OPV sendo um dos vírus mais estudados na virologia e o primeiro vírus a ser visualizado em microscopia, cultivado em células, titulado, purificado e analisado quimicamente em laboratório (FENNER et al., 1988; ESPARZA et al., 2018; ICTV, 2021).

Outros OPV zoonóticos também são destaques na medicina mundial. Na África, o MPXV é endêmico em esquilos e roedores de florestas tropicais, tendo os primatas e os humanos como hospedeiros acidentais. Nos últimos anos, a frequência e distribuição geográfica dos casos na África Ocidental e Central aumentaram e houve relatos da importação de casos na América do Norte, Ásia e Europa (CDC, 2003; SKLENOVSKÁ & RANST 2018; VAUGHAN et al., 2018; PETERSEN et al., 2019; YONG et al., 2020).

O MPXV tornou-se o OPV com maior significância para humanos desde a erradicação da varíola, devido à sua associação com alta morbidade e letalidade. A infecção em humanos ocorre pelo contato com animais infectados ou a partir da exposição a tecidos e secreções destes animais. A doença apresenta-se de forma similar à varíola, sendo caracterizada pelo desenvolvimento de mal-estar, febre e o aparecimento de lesões maculopapulares generalizadas, podendo apresentar uma taxa de letalidade de cerca de 10% em surtos africanos (ESSBAUER et al, 2010; REYNOLDS et al, 2012;2019; SKLENOVSKÁ & RANST 2018).

A infecção causada pelo CPXV foi inicialmente documentada por Edward Jenner, é chamada popularmente de “varíola bovina”. A doença foi relatada até início de 1970 na Europa, sendo transmitida esporadicamente para os leiteiros que mantinham contato com os animais infectados. Atualmente, o CPXV circula principalmente em roedores silvestres, consideradoshospedeiros naturais, na Europa e Oriente Médio. Além dos roedores, o vírus infecta um amplo espectro de hospedeiros, vindo acidentalmente a infectar felinos, humanos e diversos outros mamíferos, dentre eles, animais domésticos e animais de criação mantidos em zoológicos (MARENNIKOVA, 1972; BAXBY, 1979; MEYER, 1998; PELKONEN, 2003; CORAS,

2005; KURTH et al., 2008; MÖSTL et al., 2013; MCINERNEY et al., 2015).

1.1.1. Poxvírus: morfologia da partícula, estrutura do genoma e ciclo de multiplicação.

Os poxvírus são vírus grandes núcleo-citoplasmáticos de DNA (NCLDV), possuindo partículas virais com dimensões de aproximadamente 250-270nm de diâmetro por 350-370nm de comprimento (Figura 2) (CYRKLAFF et al., 2005; IYER et al., 2006). As partículas, além de grandes, são pleomórficas, apresentando-se na forma oval ou semelhante a um tijolo (CYRKLAFF et al., 2005; MOSS et al., 2013).

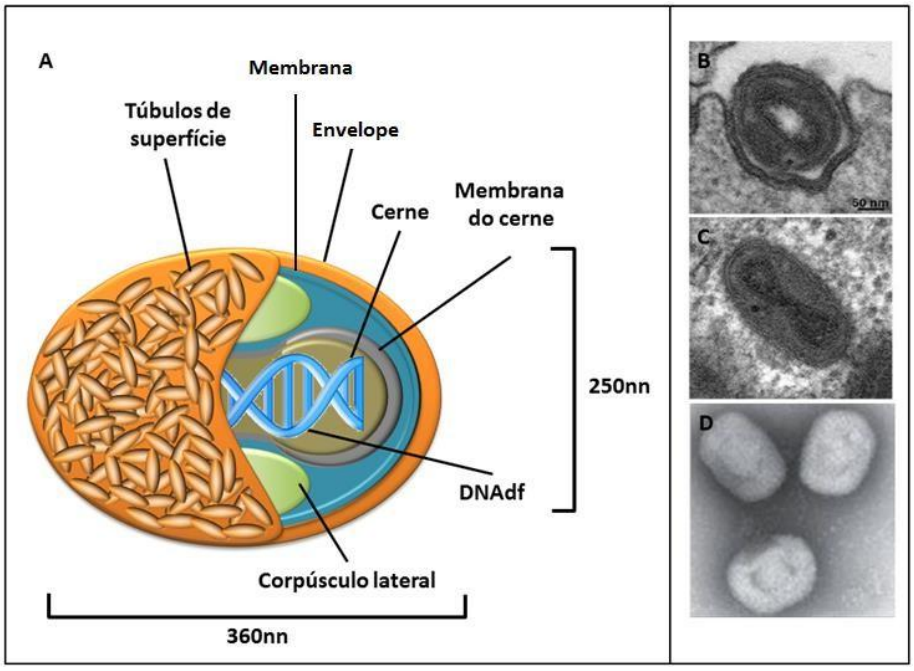


Figura 2. Morfologia e estrutura dos poxvírus.

Representação esquemática da partícula na forma de vírus envelopado extracelular (EEV) dos poxvírus (B) Microscopia eletrônica de transmissão (MET) do VACV na forma de EEV (C) MET do VACV na forma de vírus maduro intracelular (IMV) (D) Microscopia eletrônica de varredura mostra partículas em forma de tijolo. Fonte: Moss, 2001 – modificado por Laboratório do Vírus/ UFMG; Moss, 2013 – com modificações; Vora et al., 2015.

A partícula é composta por um cerne bicôncavo que envolve o genoma, enzimas e fatores de transcrição precoces; corpúsculos laterais constituídos por proteínas imunomoduladoras (DAMON, 2013; SCHMIDT et al., 2013); e uma membrana lipídica externa contendo proteínas de superfície tubulares ou globulares arranjadas de forma irregular nos OPV ou de forma helicoidal nos parapoxvirus (DAMON, 2013). O genoma dos poxvírus consiste em uma molécula de DNA fita dupla linear (dsDNA), com tamanho entre 140 a 300 kpb, variando de acordo com a espécie viral (Figura 3) (MOSS, 2013).

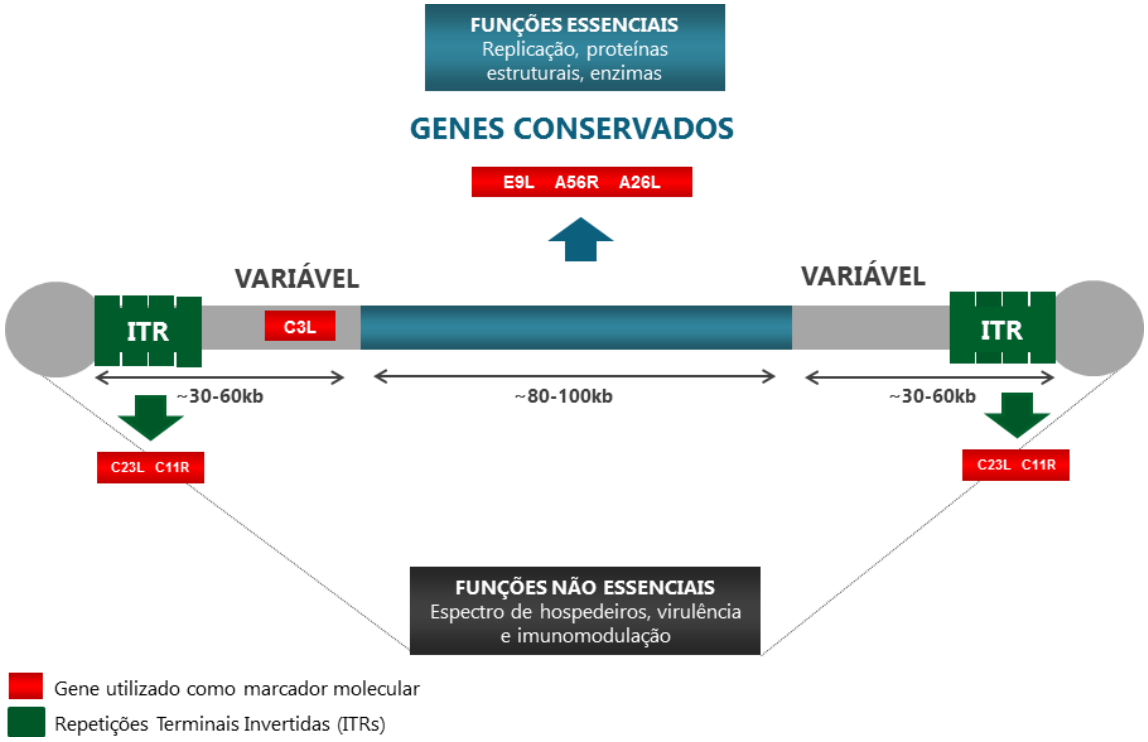


Figura 3. Representação Esquemática do Genoma dos Poxvírus

A região central do genoma dos poxvírus concentra genes conservados e são relacionados principalmente às funções essenciais do ciclo de multiplicação viral, como replicação do genoma e síntese de proteínas estruturais e não estruturais. Dentre esses, destacam-se o gene E9L (codifica a DNA polimerase), gene A56R (codifica a hemaglutinina viral) e o gene A26L (codifica a proteína do corpúsculo de inclusão). Nas extremidades do genoma concentram-se genes comumente mais variáveis entre as espécies de poxvírus e relacionados à funções como espectro de hospedeiros, virulência e imuno modulação. Dentre eles, o gene C11R que apresenta uma expressão precoce (codifica o fator de crescimento viral) e o gene C3L (codifica a proteína de ligação ao complemento). Em vermelho, têm-se os genes comumente utilizados como marcadores moleculares na detecção de OPV. Fonte: Smith & Mcfadden, 2002 – modificado por Oliveira, JS

Os elementos importantes para a multiplicação viral compõem a partícula madura, dentre eles a poli A polimerase, RNA polimerase dependente de DNA, enzima de metilação e fatores transcricionais. A região central do genoma dos chordopoxvírus possui aproximadamente 100 genes altamente conservados e essenciais para a replicação. Nas extremidades do genoma, localizam-se predominantemente os genes responsáveis pelas interações do vírus com o hospedeiro. Esses genes codificam proteínas associadas à virulência e imuno modulação, apresentando tamanho variável devido às deleções, repetições e transposições (GUBSER et al., 2004; HENDRICKSON et al., 2010; MOSS, 2013).

Alguns genes destacam-se como marcadores moleculares utilizados para a detecção dos OPV (Figura 3), dentre eles E9L (codifica a DNA polimerase) e C3L (*complement binding protein* – proteína de ligação ao complemento) (LI et al., 2006;2010; AITICHOU et al., 2008).

Os alvos comumente utilizados para detecção ou caracterização do VACV incluem o gene C11R (*viral growth factor* – fator de crescimento viral), A26L (*A type inclusion boddy protein* – proteína do corpúsculo de inclusão), A56R (gene da hemaglutinina) e C23L (*chemokine binding protein* – proteína de ligação a quimiocina) (ROPP et al., 1995; MEYER et al., 1997; LI et al., 2006; DRUMOND et al., 2008; TRINDADE et al., 2008; ABRAHÃO et al., 2010a; ASSIS et al., 2012; KROON et al., 2016; CALIXTO et al., 2018).

O ciclo de multiplicação dos poxvírus ocorre inteiramente no citoplasma da célula hospedeira, peculiaridade compartilhada com os asfarvírus e mimivírus (MOSS, 2013). Esse fenômeno ocorre devido à capacidade dos poxvírus em codificar os genes necessários para a sua própria multiplicação, conferindo certo grau de independência em relação à maquinaria nuclear da célula hospedeira (MOSS, 2013).

A penetração dos poxvírus na célula hospedeira pode ocorrer através da fusão do envelope viral com a membrana celular ou pela via endossomal (Figura 4) (ROBERTS & SMITH, 2008; SCHMIDT et al., 2011; MOSS, 2013). Após o desnudamento viral e liberação do nucleocapsídeo no citoplasma, ocorre a expressão gênica temporal e ativação da maquinaria de transcrição viral. Seguida do desnudamento secundário, dando início à replicação do DNA viral (BROYLES, 2003; GUBSER et al., 2004; DOWER et al., 2011; YANG ET et al., 2011).

Durante a morfogênese do VACV, partículas virais distintas podem ser formadas com divergência no número de membranas, nas proteínas de superfície e nas suas propriedades imunológicas e biológicas (ROBERTS & SMITH et al., 2008).

Os vírus imaturos (IVs), após clivagens proteolíticas de elementos do capsídeo e condensação do cerne viral, dão origem aos vírus maduros intracelulares (IMVs). A partícula IMV é liberada apenas através da lise celular, no entanto pode ser transportada até o complexo de Golgi, onde adquire duas membranas adicionais, originando o vírus envelopado intracelular (IEV), não infeccioso. Os IEVs são direcionados para a periferia celular, onde se fundem à membrana plasmática, gerando o vírus envelopado extracelular (EEVs) (SMITH et al., 2002; MOSS, 2013).

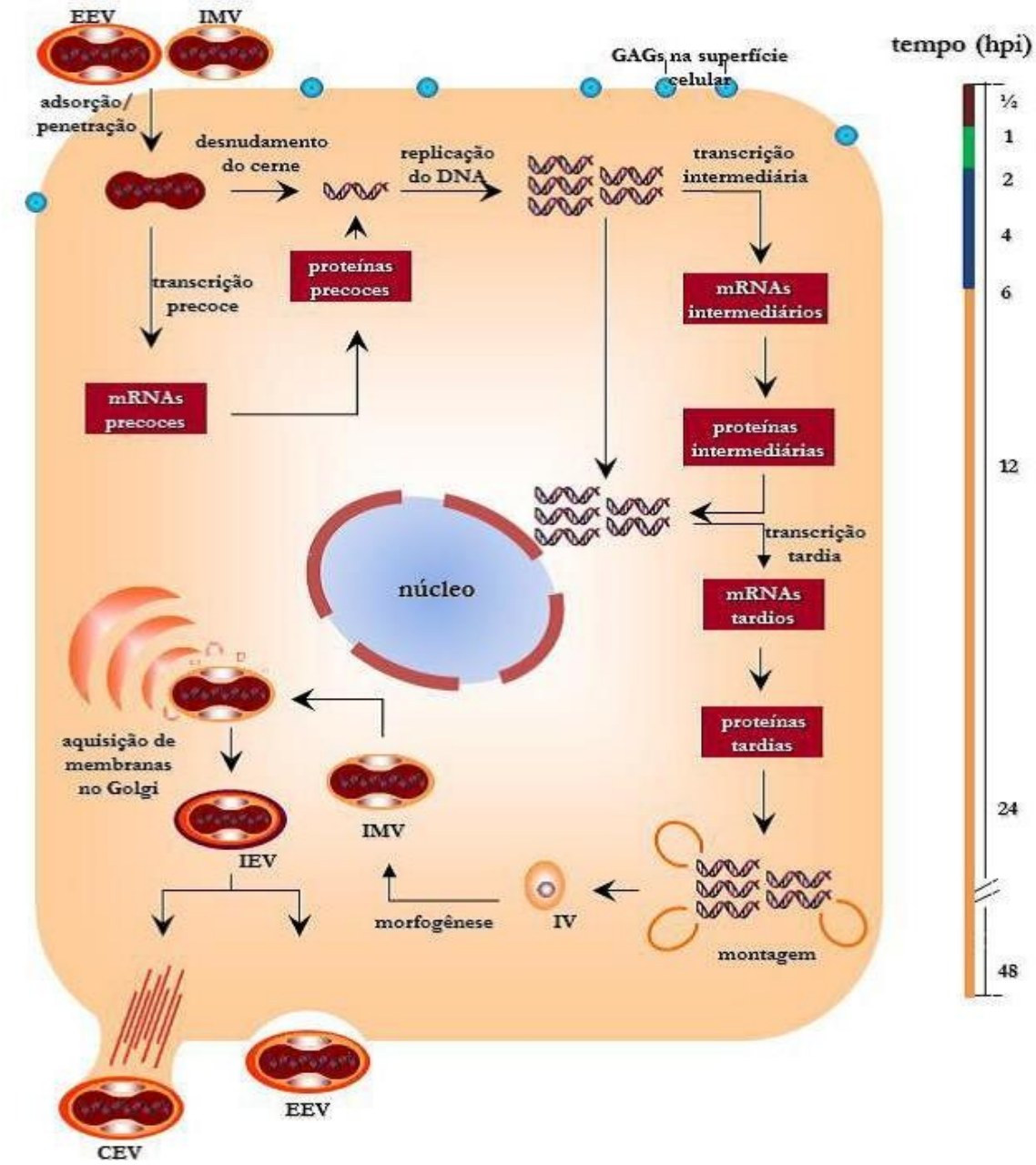


Figura 4. Diagrama do ciclo de multiplicação de VACV

O ciclo de multiplicação ocorre inteiramente no citoplasma da célula do hospedeiro. A penetração dos poxvírus na célula ocorre por via endossomal ou através da fusão de membranas. Após o desnudamento primário ocorre expressão gênica precoce, seguida de desnudamento secundário e liberação do genoma viral no citoplasma celular. Inicia-se o processo de replicação do DNA do VACV, expressão gênica tardia, seguida da morfogênese que ocorre em fábricas virais localizadas na periferia celular. Os IMV´s são liberados apenas através de lise da célula; o EEV é liberado por fusão de membrana. Durante a liberação, algumas dessas partículas podem permanecer associadas à célula, os CEV´s, e podem induzir a formação das caudas de actina favorecendo a propagação das partículas virais no organismo do hospedeiro. Fonte: Mcfadden, 2005 – modificado por Brasil, BSAF.

Os poxvírus possuem partículas virais estáveis em condições ambientais adversas. Estudos que utilizaram o VACV como modelo demonstraram a persistência dessas partículas em alimentos e em fezes de animais infectados experimentalmente. Essa estabilidade pode conferir aos poxvírus a capacidade de permanecer viável no ambiente, favorecendo sua

transmissão e apontando um risco para a saúde pública (ESSBAUER et al., 2007; ABRAHÃO et al., 2009; OLIVEIRA et al., 2010).

1.1.2. Rotas de infecção e patogênese

A patogênese e os sintomas decorrentes da infecção por OPV são influenciados pelo tropismo viral e pela resposta imune do hospedeiro. Portanto, nesse processo são determinantes alguns fatores como espécie de OPV envolvida na infecção, rota de entrada do patógeno e estado imunológico do hospedeiro (MCFADDEN, 2005; DAMON, 2013). A infecção por esses vírus geralmente é sistêmica, podendo manifestar-se na forma de erupção cutânea com lesões sistêmicas ou localizadas. Os dois perfis são visualizados nas erupções decorrentes da infecção por CPXV, podendo-se manifestar-se de forma localizada em humanos e bovinos ou de forma sistêmica em felinos (FENNER, 1988; ESTEBAN & BULLER, 2005).

A infecção por OPV, em geral, ocorre através da entrada do vírus por via percutânea, mucosa ou pelo trato respiratório. Sabe-se que o curso clínico da infecção varia de acordo com a rota de entrada do vírus. Esse fenômeno foi descrito em infecções por VARV e MPXV. A infecção humana por MPXV, por exemplo, pode ser adquirida por meio de exposição a gotículas respiratórias ou outros fluidos corporais; ou por contato direto com a secreção da lesão de infectados (HUTSON et al., 2011). A transmissão zoonótica pode ocorrer por inoculação direta através de mordidas e micro abrasões; ou contato direto com fluidos corporais de animais infectados ao realizar atividades de caça, preparo de carcaças de animais contaminados ou outro tipo de contato (REYNOLDS et al., 2006; DAMON et al., 2011).

Em 2003, o MPXV foi introduzido nos Estados Unidos, através de uma remessa de animais exóticos destinados à venda como animais de estimação, trazidos da África Ocidental. Neste episódio, foram relatados 47 casos humanos, entretanto, embora a idade dos pacientes e o histórico vacinal contra a varíola tiveram pouca influência na manifestação ou gravidade da doença, a provável via de infecção foi relevante para o desenvolvimento dos casos (REYNOLDS et al., 2006).

Os indivíduos que relataram uma exposição invasiva, através de mordida ou micro abrasões por animais infectados, foram mais propensos a desenvolver sintomas sistêmicos e serem hospitalizados durante a doença. Além disso, exposições invasivas foram associadas a um curto período de incubação, início dos sintomas na ausência de febre e desenvolvimento de lesão primária, antes do início da febre e erupção cutânea generalizada. Por outro lado, aqueles que tiveram exposição não invasiva (via respiratória ou mucosa) apresentaram alguns sintomas respiratórios superiores, mas tiveram menos sintomas sistêmicos gerais e uma progressão mais clássica da doença (REYNOLDS et al., 2006).

Através da rota respiratória, o vírus atinge o sistema respiratório superior e inferior, multiplicando-se em macrófagos alveolares e pequenos bronquíolos. Ao atingir os linfonodos regionais, dissemina-se pelo organismo do hospedeiro e manifesta-se na forma de erupções cutâneas (DAMON, 2013).

Na infecção por via percutânea ou através da mucosa, infecções experimentais têm demonstrado que após a entrada do vírus no organismo do hospedeiro, ocorre a multiplicação viral no sítio primário de infecção e o vírus migra dos linfonodos regionais, alcançando a corrente sanguínea. Isso resulta na viremia primária, onde o vírus pode disseminar-se no organismo associado a células sanguíneas ou livres no plasma. A disseminação via corrente sanguínea favorece a infecção de diversos órgãos, dentre eles fígado, baço e linfonodos regionais que se caracterizam pela alta vascularização. Cerca de cinco dias pós-infecção observa-se a viremia secundária com migração das partículas virais para a pele e desenvolvimento de lesões ulcerativas, caracterizadas pelo aparecimento de erupções maculopapulares que progride para pápulas, vesículas, pústulas e, posteriormente, lesões de crosta, iniciando-se a fase de cicatrização (Figura 5) (FENNER, 1988; ESTEBAN & BULLER, 2005; DAMON et al., 2013).

Para avaliação da patogênese do VACV, estudos experimentais em modelos animais foram realizados no Brasil, onde a circulação natural do vírus é endêmica e afeta principalmente os bovinos de leite (FERREIRA et al., 2009; GUEDES et al., 2013; RIVETTI JR et al, 2013; REHFELD et al., 2013). No modelo em bovinos, a exposição ao VACV foi realizada por via intradérmica. Os animais desenvolveram lesões limitadas ao local da inoculação, compatíveis com a infecção natural. As lesões surgiram entre o 2º e 4º dia pós-infecção (dpi) e cicatrizaram no tempo médio de 18 dias (RIVETTI JR et al, 2013).

O DNA viral foi detectável em úlceras na mucosa oral e no sangue dos animais de forma prolongada e intermitente, sugerindo que a infecção não se limita a área onde as lesões ocorrem e reforçando a existência de disseminação viral sistêmica (GUEDES et al., 2013*)*. Esta hipótese foi corroborada pela detecção viral por imuno-histoquímica e biologia molecular nos órgãos linfoides dos animais infectados (RIVETTI JR et al, 2013). Outros estudos consolidaram essa premissa, mediante a identificação da eliminação do VACV no leite de bovinos infectados, experimentalmente e naturalmente, incluindo animais com infecção subclínica (Figura 5) (ABRAHÃO et al., 2009C; OLIVEIRA et al., 2015; REHFELD et al., 2017A;2018; MATOS et al., 2018).

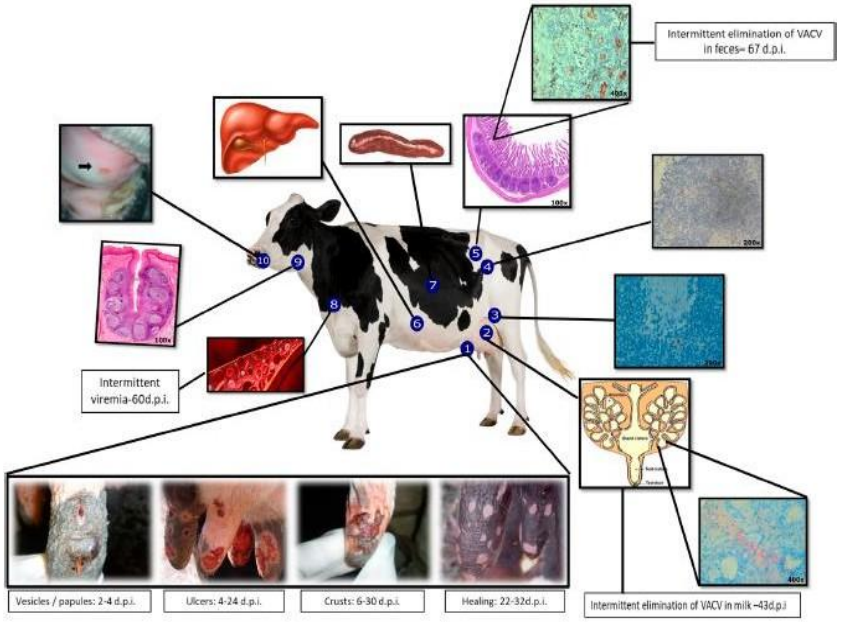


Figura 5. Sinais clínicos e o modelo proposto para a patogênese do VACV em bovinos

Multiplicação do VACV no epitélio dos tetos e disseminação por vasos linfáticos e rotas hematogênicas. 1. Tetas; evolução das lesões: local de inoculação primária; 2. Glândula mamária; 3. Linfonodo retro mamário; 4. Linfonodo mesentérico; 5. Íleo; 6. Baço; 7. Fígado; 8. Viremia intermitente; 9. Amígdalas; 10. Úlcera na mucosa oral. A eliminação intermitente do VACV foi detectada por um período de 67 d.p.i. nas fezes e 43 d.p.i. no leite de animais infectados experimentalmente. Fonte: Matos et al., 2018.

Adicionalmente, a infecção experimental revelou que as partículas virais infecciosas puderam ser detectadas nas fezes dos bovinos entre o 1º e 15º dpi, enquanto o material genético viral foi detectável até o 67º dpi, demonstrando que o período de infecção VACV não se limita à resolução das lesões porque a presença do vírus foi observada nas fezes, mesmo após a cicatrização das lesões, que ocorreu em média no 18º dpi. Esse achado demonstra que a eliminação viral nas fezes poderia contribuir para o ciclo epidemiológico do VACV, uma vez que a presença do vírus no ambiente poderia favorecer a exposição de outros indivíduos (RIVETTI JR et al., 2013).

1.1.3. Vacinas antivariólicas

A vacinação contra a varíola foi um marco na história da medicina mundial que culminou na erradicação de uma grave doença infecciosa, com alto índice de letalidade. Um importante fator que contribuiu para o sucesso da campanha de vacinação foi o tropismo apresentado pelo VARV. O fato de esse vírus infectar apenas hospedeiros humanos e não possuir outros hospedeiros como reservatórios possibilitou sua erradicação em 1980 (SMITH & MCFADDEN, 2002; MCFADDEN, 2005).

A vacinação foi iniciada pelo médico Edward Jenner em 1796 na Europa. Jenner utilizou o CPXV derivado de bovinos infectados, inoculado após escarificação da pele. Em 1939, a presença de VACV foi identificada na miscelânea viral que compunha a vacina. Posteriormente, o CPXV foi substituído por VACV e em 1967 a OMS padronizou a vacina a partir de quatro amostras de VACV. O VACV, cuja origem é desconhecida, ainda vem sendo utilizado em muitos laboratórios como um vetor vacinal expressando proteínas exógenas de vários agentes infecciosos (FENNER et al., 1989; WISER et al., 2006; JACOBS et al., 2009; SCHRICK et al., 2017).

A vacina utilizada na campanha mundial de vacinação contra a varíola correspondia a diferentes amostras de VACV, aplicadas em diferentes regiões do mundo, sendo elas a Dryvax® New York Board of Health [NYCBH], nas Américas; Líster, no Reino Unido e Índia; EM-63, uma variante derivada da NYCBH, utilizada na Rússia; Copenhagen na Dinamarca e Ankara, na Turquia. Essas amostras eram caracterizadas como vacina de primeira geração, sendo constituída do líquido extraído das lesões de bovinos infectados, entretanto, essa prática pouco refinada poderia favorecer a transmissão de outras doenças infecciosas (FENNER et al., 1988; SMITH E MCFADDEN, 2002; WISER et al., 2006; JACOBS et al., 2009).A vacinação era administrada através da via percutânea e frequentemente poderia resultar no desenvolvimento de sinais clínicos, como surgimento de uma pápula que evoluiu a vesícula, e subsequentemente uma pústula e crosta, acompanhadas de manifestações sistêmicas como febre, mialgia, linfadenopatia e cefaleia. A manifestação do quadro clínico era indicativa de uma vacinação bem-sucedida (FENNER et al., 1988; ROCK et al., 2004).

A vacina antivariólica apresentava taxas de efeitos adversos não aceitáveis para o atual padrão sanitário. Além disso, era composta por um conjunto de quasispecies do VACV e, portanto, geneticamente heterogênea. O desenvolvimento de novas tecnologias permitiu o aprimoramento das vacinas que evoluíram para vacinas de segunda e terceira geração, desenvolvidas a partir de amostras atenuadas do VACV. Um exemplo em desenvolvimento é o Vaccínia vírus Ankara Modificado (*Modified Vaccinia Ankara* – MVA). Atualmente, têm sido estudadas vacinas de quarta geração, caracterizadas por possuir modificações genéticas, incluindo deleções, inserções ou bloqueio de genes essenciais para a multiplicação viral (WISER et al., 2006; JACOBS et al., 2009; OVERTON et al., 2018).

Embora a vacinação em massa tenha sido suspensa após a erradicação da varíola, existem, ainda hoje, protocolos bem estabelecidos para vacinação de militares e profissionais de saúde em alguns países da Europa e nos Estados Unidos. Essa é uma medida preventiva a uma possível ação bioterrorista. Eventualmente é reportado o papel dos indivíduos vacinados como disseminadores do vírus (SMITH & MCFADDEN, 2002; VORA et al., 2008; LEDERMAN et al., 2009; TACK et al., 2013).

1.1.4. Vacinação antivariólica no Brasil

A propagação da vacinação antivariólica no Brasil data do início do século XIX. Em Minas Gerais, a introdução da vacinação ocorreu em 1805. A vacina havia sido encaminhada para a Bahia e o Rio de Janeiro (SILVEIRA & MARQUES, 2011). Na capital do império, em 1811, foi criada a Junta Vacínica da Corte, encarregada da propagação e conservação da vacina no Rio de Janeiro e nas demais províncias. No entanto, o serviço de vacinação passou a ser mais organizado após a criação do Instituto Vacínico do Império em 1846 e da Junta de Higiene Pública em 1850 (FERNANDES et al., 1999). O instituto, além de normatizar e fiscalizar promovia debates a respeito da técnica e da distribuição da vacina (SILVEIRA & MARQUES, 2011).

A vacinação era praticada após publicação de editais que informam a população os períodos de aplicação. Geralmente, o médico ou cirurgião encarregado, administrava a vacina na Câmara, nas igrejas ou nas fazendas espalhadas pelos municípios (GURGEL et al., 2011; SILVEIRA & MARQUES, 2011). Entretanto, diante do reduzido número de médicos e boticários, frequentemente o governo recrutava filantropos e até mesmo curiosos para atuarem como vacinadores. Mesmo assim, os cargos foram abandonados, permanecendo vago durante anos, o que desfavorecia o serviço na época (GURGEL et al., 2011).

Alguns documentos sobre a vacinação em Minas Gerais relatam a ausência dos efeitos esperados decorrentes da vacinação, como o surgimento das pústulas, das quais o material era coletado para realizar a propagação da vacina. Algumas explicações incluem a deterioração da linfa pelo calor; demora no transporte das amostras e danificação das lâminas e tubos capilares encaminhados (SILVEIRA & MARQUES, 2011).

Devido à metodologia utilizada e a reação gerada, a população passou a demonstrar aversão à vacina. Logo, o governo passou a punir os indivíduos que se recusassem a se submeter à prática. Essas punições consistiam em prisão, multa e inadmissão em escolas e serviços públicos. No entanto, embora houvesse resistência, à medida que as epidemias surgiam, a população espontaneamente partia em busca da vacina na tentativa de sobreviver (SILVEIRA & MARQUES, 2011).

Quase um século depois, em 1959 a OMS propôs a campanha mundial de erradicação da varíola, que foi reestruturada em 1965 e intensificada em 1967 (MUNIZ, 2010). No Brasil, a Campanha Nacional contra a Varíola (CNCV) foi instituída em 1962 e contribuiu para a consolidação das práticas educativas na área da saúde (FERNANDES et al., 2011).

A estratégia para a vacinação em massa nas áreas urbanas, ou com significativa

concentração de pessoas, foi a de mobilização da população para grandes encontros em lugares públicos. Os últimos casos de varíola no Brasil foram descritos no Rio de Janeiro em 1971. Dois anos depois a erradicação da varíola no Brasil foi certificada e a vacinação continuou obrigatória na rotina dos serviços de saúde até 1975. Em maio de 1980 a erradicação mundial da varíola foi declarada pela Assembleia Mundial da Saúde (HOCHMAN et al., 2011).

1.1.5. Perfil da resposta imune do hospedeiro

A presença de epítopos conservados nas proteínas de superfície dos OPV confere uma reatividade imunológica cruzada entre as espécies do gênero, o que possibilitou o uso do VACV como imunizante na campanha mundial de vacinação contra a varíola (JACOBS et al., 2009; HENDRICKSON et al., 2010; MILLER et al., 2011; MOSS, 2013).

Atualmente, é sabido que em uma infecção primária causada por OPV, a penetração da partícula viral inicia a ativação da produção de interferon (IFN), responsável pela indução do estado antiviral nas células vizinhas, e outras citocinas que promovem o recrutamento de leucócitos e inflamação no local da infecção (SMITH & KOTWALL, 2002; ESTEBAN & BULLER, 2005; TIAN et al., 2009; DAMON, 2013).

As células dendríticas e os macrófagos, componentes da resposta imune inata, são responsáveis pelo reconhecimento de padrões moleculares associados a patógenos (PAMPs), e a resposta imune inata à infecção consiste na produção de óxido nítrico e citocinas pró inflamatórias. Dentre elas, interleucina 1 (IL-1), IL-12, fator de necrose tumoral α (TNF-α) e IFN's do tipo I (α e β) que atuam estimulando a diferenciação das células T auxiliares (TCD4+) em células ativas produtoras de IFN-γ. Esse mecanismo promove o controle da multiplicação viral. A imunidade mediada pelas células TCD4 + e pelo MHC de classe II é essencial para eliminação do vírus durante a infecção aguda (SMITH & KOTWALL, 2002; TIAN et al., 2009; DAMON, 2013).

IFNs do tipo I e IL-12 também ativam células T citotóxicas (TCD8 +), importantes na eliminação das células infectadas. As células naturais killers (NK) atuam na resposta celular desencadeada contra o vírus. Ambas as células TCD4 + e TCD8 + mostraram-se essenciais na proteção contra doença primária induzida por VACV (HAMMARLUND et al., 2003; DAMON, 2013; SMITH et al., 2018).

Entretanto, os poxvírus, como o VACV, possuem genes imunomodulatórios que codificam proteínas relacionadas à evasão da resposta imunológica inata. Algumas dessas proteínas, como a VCP, são secretadas pela célula infectada e são capazes de neutralizar fatores do sistema complemento. Outras proteínas atuam inibindo a apoptose ou vias de sinalização que levam à produção de interferons, citocinas e quimiocinas pró-inflamatórias. Para isto,

durante a multiplicação viral, o VACV pode promover a minimização da produção de dsDNA, molécula que atua como PAMP e que ao ser reconhecida pelo hospedeiro, dá início à produção de interferon. Outra forma seria a inibição da síntese proteica do hospedeiro, que pode ocorrer através do aumento da degradação de mRNA do hospedeiro, mediada pelas proteínas virais. A remoção ou inativação dos genes imuno modulatórios poderia levar a diminuição da virulência no VACV (SMITH et al., 2013).

A resposta imune humoral mostra-se importante no processo de eliminação do vírus, sendo relatado que camundongos deficientes em linfócitos B não eliminam as partículas virais de maneira eficiente (SMITH & KOTWAL, 2002; XU et al., 2004; CHAUDHRI et al., 2006; MOYRON-QUIROZ et al., 2009).

Adicionalmente, a imunidade humoral induz uma forte resposta de anticorpos neutralizantes que demonstra ser o principal mecanismo efetor responsável pela proteção contra a infecção secundária em uma reexposição ao vírus (DAVIES et al., 2005; PUTZ et al., 2005).

Uma resposta imune robusta mediada por anticorpos foi identificada em indivíduos vacinados contra a varíola, havendo produção de anticorpos neutralizantes contra as diferentes formas IMV e EEV. Essa resposta geralmente torna-se detectável em testes sorológicos a partir do 14º dia após a vacinação, alcançando um pico no primeiro mês pós-vacina, declínio no primeiro ano de imunização e mantendo-se estável por um longo período. Quando indivíduos vacinados são submetidos ao reforço vacinal, apresentam uma rápida resposta de anticorpos, os quais podem ser detectados a partir do 4º dia pós-imunização (AMANNA et al., 2006; HAMMARLUND et al., 2003).

A imunidade desenvolvida após a vacinação é capaz de induzir uma resposta de células B específicas que, assim como os níveis de anticorpos, tendem a uma diminuição pós-imunização, mas podem permanecer detectáveis por até 50 anos (CROTTY et al., 2003). A resposta imune mediada por anticorpos tem um papel fundamental na proteção em longo prazo. O título de anticorpos pode permanecer estável durante décadas, enquanto a resposta imune celular (T CD4 e CD8+) diminui gradativamente ao longo do tempo (HAMMARLUND et al., 2003; BELYAKOV et al., 2003; EDGHILL-SMITH et al., 2005; PANCHANATHAN et al., 2010).

A resposta imune protetora anti-OPV parece persistir por muitos anos (KAREM et al., 2005; VINER & ISAACS, 2005; KIM et al., 2006; TAN et al., 2012; KWANCHUM et al.,2017). Hammarlund e colaboradores revelaram que mais de 90% dos voluntários vacinados analisados mantiveram resposta imune humoral e/ou celular detectável pelo período de 25-75 anos após a vacinação, permanecendo a resposta de anticorpos estável em alguns indivíduos por até 75 anos (HAMMARLUND et al., 2003). Ainda, uma população avaliada na China

apresentou resposta imune desencadeada pela vacinação detectável por 40 anos (LIU et al., 2012).

No Japão, um estudo semelhante demonstrou que a maioria dos vacinados antes do programa de vacinação ser encerrado na década de 70, mantiveram um alto percentual de positividade para a presença de IgG (HATAKEYAMA et al., 2005).

Por outro lado, estudos indicam que a ineficácia da imunidade protetora em longo prazo pode estar relacionada com a diminuição da população de linfócitos T de memória, que apresentam uma meia-vida de 8-15 anos e são responsáveis pela estimulação dos plasmócitos produtores de anticorpos (HAMMARLUND et al., 2003). Além disso, foi demonstrado que apesar da formação da marca vacinal característica da vacinação antivariólica e indicadora da "pega" da vacina, alguns indivíduos desenvolvem uma fraca resposta imune humoral e celular (KENNEDY et al., 2016).

Hammarlund e colaboradores (2003) relataram que indivíduos imunizados com uma dose de reforço, apresentaram o título médio de anticorpos significativamente maior do que os títulos detectados após apenas uma única vacinação. Entretanto, estudos realizados no Brasil, onde a circulação natural de OPV emergiu há 20 anos demonstraram que pessoas com histórico de vacinação foram infectadas quando expostos de forma natural ao VACV (SILVA-FERNANDES et al., 2009; COSTA et al., 2015).

1.1.6. Resposta imune às infecções zoonótica por VACV-Br

Os surtos de infecções zoonóticas por VACV no Brasil podem ajudar a compreender importantes aspectos das infecções naturais em uma escala populacional. Em infecções naturais causadas pelo VACV-Br observou-se um perfil de resposta humoral (IgG ou anticorpos neutralizantes) semelhante aos demais estudos conduzidos no mundo, sendo os anticorpos indetectáveis por pelo menos 10 dias após exposição ao vírus (TRINDADE et al., 2009).

Entretanto, embora a alta produção de IFN- γ por linfócitos CD8+ e CD4+ seja uma marca registrada das infecções por OPV, foi detectada uma produção surpreendentemente menor em um paciente não vacinado e infectado por VACV-Br zoonótico. Adicionalmente, o isolado viral associado à infecção apresentou genes imunomodulatórios funcionais, o que pode ser associado com a possível imunomodulação negativa detectada durante a infecção natural (TRINDADE et al., 2009).

Entretanto, ao expandir a avaliação e o número de infectados, o padrão de deficiência na produção de IFN- γ não se sustentou. Isso pode ser explicado com o fato da produção de citocinas poder variar em função de outros fatores, intrínsecos de cada paciente. Relatos de

surtos zoonóticos no Brasil identificaram que células imunes específicas são moduladas durante uma infecção aguda em humanos (SILVA-GOMES et al., 2012). De maneira geral, os poxvírus podem codificar genes responsáveis pela ação imunomodulatória no hospedeiro infectado, dentre eles, os genes E3L, K3L, B18R e B8R, que podem interferir na ativação de células B e T, e na produção de IFN (JOHNSTON et al., 2003).

Nas infecções naturais documentadas no Brasil foi observada diminuição das células apresentadoras de antígeno, linfócitos B e macrófagos logo após dez dias de infecção. Além disso, em indivíduos infectados foram identificadas células CD4 + e células B menos ativas quando comparadas a indivíduos não infectados. Assim como, uma menor quantidade de células CD8 + regulatórias (SILVA-GOMES et al., 2012).

A avaliação da resposta imune mediada pelas células CD4 + e CD8 + após infecção natural pelo VACV sugere que essas células estão envolvidas na resposta de memória imune contra a infecção natural pelo vírus (MEDEIROS-SILVA et al., 2013). Medeiros-Silva e colegas compararam, através de estimulação viral *in vitro*, células de indivíduos previamente infectados e não infectados. Pôde-se demonstrar que indivíduos vacinados infectados e não infectados apresentam subconjuntos de células CD4 + de memória e maiores porcentagens de linfócitos T de memória, CD4 + e CD8 +, expressando IFN- γ e TNF-α, quando comparados a indivíduos não vacinados não infectados. Isso sugere que as células CD4 + e CD8 + estão envolvidas na resposta de memória imune contra a infecção natural pelo VACV-Br (MEDEIROS-SILVA et al., 2013).

Recentemente foi demonstrado que a virulência de diferentes isolados de VACV é diretamente proporcional à sua capacidade de modular negativamente a resposta imune mediada por células *in vivo*. Esse achado pode ajudar a esclarecer o porquê de indivíduos, mesmo vacinados, desenvolverem a doença em decorrência das infecções zoonóticas no Brasil (FREITAS et al., 2018). Para garantir a segurança da vacinação, as vacinas utilizadas no processo de imunização geralmente são compostas por VACV atenuados e avirulentos, o que pode estimular um diferente padrão de resposta imune, em relação à resposta desencadeada frente a uma infecção natural causada por VACV virulento (FONSECA & FLORES et al., 2014).

Ao comparar a resposta imune desencadeada pela infecção por uma amostra de VACV altamente atenuada e não replicativa (MVA), um vírus atenuado e replicativo (VACV-Lister) e uma amostra virulenta (VACV-WR), Freitas e colaboradores demonstraram que o VACV virulento foi capaz de modular negativamente a resposta imune celular (monócitos, macrófagos e CD4+) e a produção de citocinas pró inflamatórias (IFN- γ e TNF-α) em modelo murino,enquanto o VACV altamente atenuado induziu a resposta imune celular esperada e o

VACV intermediário induziu uma resposta moderada. Em relação à imunidade humoral, os animais infectados com as diferentes amostras virais desenvolveram anticorpos em níveis detectáveis, embora o vírus virulento tenha estimulado a produção de um maior nível de anticorpo, quando comparado às demais amostras virais (FREITAS et al., 2018).

Esses achados reforçam a hipótese de que a modulação da resposta imune durante as infecções zoonóticas poderia estar associada às infecções naturais em indivíduos vacinados no Brasil e poderia, ainda, contribuir para explicar a heterogeneidade de respostas frente às infecções por VACV zoonóticos. Entretanto, mais estudos que avaliam a dinâmica das infecções naturais por VACV-Br são necessários para apoiar tais suposições e esclarecer como o vírus interage com seu hospedeiro para alcançar o sucesso replicativo.

1.2. Vinte anos da emergência do VACV no Brasil: o que descobrimos até aqui?

1.2.1. Vaccínia bovina

O VACV emergiu no Brasil no final da década de 90, sendo detectado durante surtos de doença exantemática acometendo bovinos leiteiros e ordenhadores. Esses surtos foram localizados em propriedades rurais na região sudeste do país, apontada como epicentro da circulação do VACV. A zoonose viral emergente foi denominada vaccínia bovina (VB) por acometer principalmente bovinos e ordenhadores (TRINDADE et al., 2003; LEITE et al., 2005; LOBATO et al., 2005).

A VB é caracterizada pela presença de lesões ulcerativas na pele e nas membranas mucosas. Em bovinos, essas lesões surgem principalmente nas tetas e úberes e frequentemente são acompanhadas de mastite que pode evoluir para a perda definitiva das glândulas mamárias, resultando na diminuição da produção de leite com impacto econômico significativo (Figura 6). Além disso, em propriedades rurais onde os bezerros em fase de amamentação estão em contato direto com as vacas lactantes infectadas, é comum observar bezerros apresentando lesões no focinho e na boca, o que reduz a ingestão de alimentos, podendo levar à perda de peso nos animais (TRINDADE et al., 2003; LEITE et al., 2005; LOBATO et al., 2005; MATOS et al., 2018).

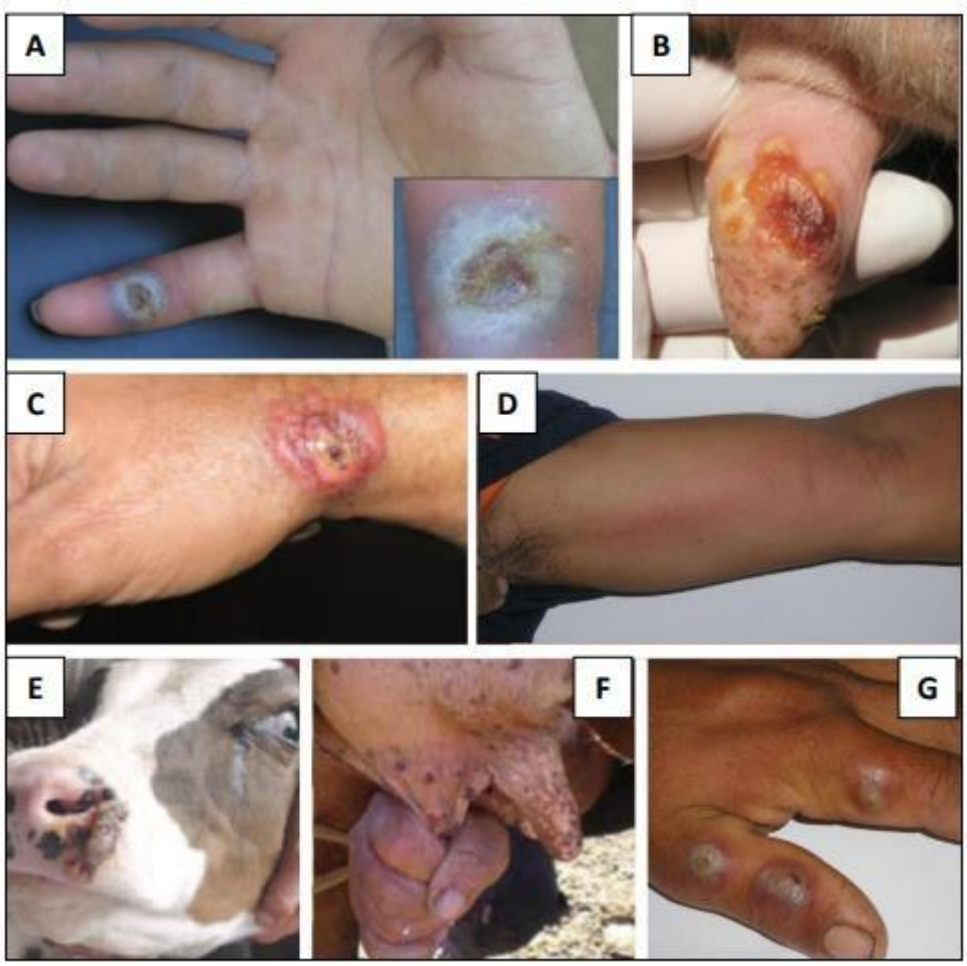


Figura 6. Lesões ulcerativas nos tetos das vacas, no focinho dos bezerros e em humanos infectados com VACV.

A e B) Lesão exantemática típica causada pelo VACV na mão do ordenhador e no teto de um bovino infectado C) Lesão ulcerativa típica na mão de um ordenhador D) Em humano, as lesões coincidiram com o aparecimento de linfangite periférica no braço esquerdo. E) Lesões ulcerativas no focinho de bezerros infectados com VACV F) Rota clássica de transmissão da VB, evidenciada pelo contato direto do ordenhador com o teto de bovino infectado, G) Pápulas e pústulas evoluíram para úlceras com tecido focal necrosado, evidenciando o padrão central umbilicado. Área de inflamação pode ser vista em torno da lesão. – Fonte: Grupo de Pesquisa em Ecologia de Viroses Emergentes (UFMG); Trindade et al., 2003; 2007;2014; Leite et al., 2005; Abrahão et al., 2010; Assis et al., 2013.

Embora não tenha sido associada à mortalidade, a infecção em humanos geralmente apresenta uma alta morbidade. O processo entre o surgimento dos sintomas e a cicatrização total das lesões leva aproximadamente 21 dias. As manifestações clínicas consistem principalmente em lesões localizadas no sítio primário da infecção, geralmente localizadas nos membros superiores de indivíduos que frequentemente apresentam histórico de contato com animais infectados apresentando lesões (Figura 7) (TRINDADE et al., 2003; 2007; 2009 LEITE et al., 2005; LOBATO et al., 2005; SILVA-FERNANDES et al., 2009; FONSECA et al., 2011).

No entanto, há relatos da presença dessas lesões características em áreas como a face, olhos, mucosa, abdômen e na região genital, possivelmente como resultado de autoinoculação e/ou transmissão por contato (SILVA et al., 2008; SCHATZMAYR et al., 2009; LIMA et al., 2018).

As lesões causadas por VACV surgem como pontos focais na pele acompanhados de prurido, seguido pelo aparecimento do edema local e a formação de vesículas. Os sintomas sistêmicos tais como febre, cefaleia, mal-estar, mialgia, linfadenopatia inguinal e cervical desenvolvem-se cerca de três dias após os sintomas iniciais. Posteriormente, as vesículas evoluem para pústulas umbilicadas com áreas focais de inflamação. Há o desenvolvimento de úlceras preenchidas com tecido necrótico, que tendem a cicatrizar, formando crostas negras e, finalmente, uma cicatriz permanente (SILVA-FERNANDES et al., 2009; ESSBAUER et al., 2010; KROON et al., 2011; TRINDADE et al., 2014; OLIVEIRA et al., 2017).

Os surtos de VB no Brasil são protagonizados por uma diversidade genética de isolados, que inicialmente foi evidenciada através da análise filogenética do gene A56R, possibilitando a divisão das amostras de VACV-Br em dois grupos, onde o grupo 1 caracteriza-se pela deleção de 18 nucleotídeos no gene A56R, que codifica a hemaglutinina viral. Enquanto no grupo 2, essa deleção não existe e as amostras são agrupadas junto à amostra protótipo VACV-WR (TRINDADE et al., 2007; DRUMOND et al., 2008). Posteriormente, revelou-se que a divergência genética observada nesses isolados resultava em alterações biológicas como diferença no perfil de virulência em modelo murino (FERREIRA et al., 2008).

Outros marcadores moleculares têm sido utilizados para caracterizar as amostras do VACV-Br. O gene da proteína solúvel de ligação a quimiocinas (C23L) apresenta uma deleção de 10 nucleotídeos em isolados do grupo 1, que classifica os isolados brasileiros em dois grupos, similar à classificação do gene A56R descrita acima, onde os vírus do grupo 2 são selvagens para a mutação (ASSIS et al, 2012).

O gene das proteínas de inclusões do tipo A (ATI) apresenta uma maior dicotomia e, pela análise deste marcador, os VACV-Br podem ser classificados em três grupos virais de acordo com o polimorfismo apresentado: o primeiro grupo não apresenta nenhuma deleção na região estudada do gene ATI; o segundo possui dois blocos de deleções; enquanto o terceiro grupo tem todo o gene ATI deletado (LEITE et al., 2007). Essa diversidade genética deve ser mais bem explorada para que seja compreendido quais propriedades biológicas podem conferir aos VACV-Br, como adaptação ao hospedeiro (TRINDADE et al., 2006; CAMPOS et al., 2011).

1.2.2.Perfil epidemiológico e principais áreas endêmicas do VACV-Br

A emergência do VACV no Brasil foi associada aos surtos de VB em 1999. Os estados do Rio de Janeiro e São Paulo sediaram os primeiros surtos descritos (Figura 7) (DAMASO et al., 2000; TRINDADE et al., 2003). A propagação do VACV no território brasileiro foi evidenciada a partir da detecção do vírus em surtos no sudeste (Minas Gerais, São Paulo, Rio de Janeiro), centro-oeste (Goiás e Mato Grosso) (NAGASSE-SUGAHARA et al., 2004; MEGID et al., 2008;2012; SCHATZMAYR et al., 2011; SANT'ANA et al., 2013; ABRAHÃO et al., 2015); seguido da região norte (Rondônia e Pará) (QUIXABEIRA-SANTOS ET AL., 2011; ASSIS ET AL., 2013) e nordeste (Maranhão, Bahia e Pernambuco) (OLIVEIRA et al., 2013; ASSIS et al., 2015; LIMA et al., 2019).

Nos últimos anos, outros países da América do Sul relataram a ocorrência do VACV. Além do Brasil, na Argentina e no Uruguai foram demonstradas evidências da circulação viral em bovinos de leite, enquanto na Colômbia trabalhadores da cadeia leiteira foram afetados comos surtos de VB (FRANCO-LUIZ et al., 2014; 2016; USME-CIRO et al., 2017; STYCZYNSKI et al, 2019; LAITON-DONATO et al., 2020).

No contexto dos surtos de VB associados à circulação natural do VACV em ambiente rural no Brasil, a avaliação da patogênese em bovinos sugere que esses animais apresentam papel fundamental como amplificadores virais, sendo capazes de excretar uma significativa carga viral nas fezes, por tempo prolongado, que poderia favorecer a manutenção do VACV no ambiente e a infecção de outros hospedeiros (GUEDES et al., 2013).

Os surtos de VB afetam principalmente as propriedades de pequeno e médio porte, que realizam ordenha manual e apresentam precariedade na infraestrutura e na adoção de medidas sanitárias. Entretanto, a doença também foi descrita em propriedades de ordenha mecânica. Frequentemente, a produção leiteira é a principal fonte de subsistência para as famílias afetadas (LOBATO et al., 2005; REHFELD et al, 2017; MATOS et al., 2018).

Um estudo experimental em bovinos demonstrou o aparecimento de vesículas em tetos de animais cujo processo de cicatrização das lesões primárias havia sido concluído, indicando que pode ocorrer reinfecção em um mesmo surto, e o ordenhador pode atuar como disseminador do VACV nesses eventos (REHFELD et al., 2013). A movimentação de animais associada à comercialização de bovinos e o trânsito de trabalhadores leiteiros são fatores geopolíticos que poderiam favorecer a dispersão da circulação viral (QUIXABEIRA-SANTOS et al., 2011).

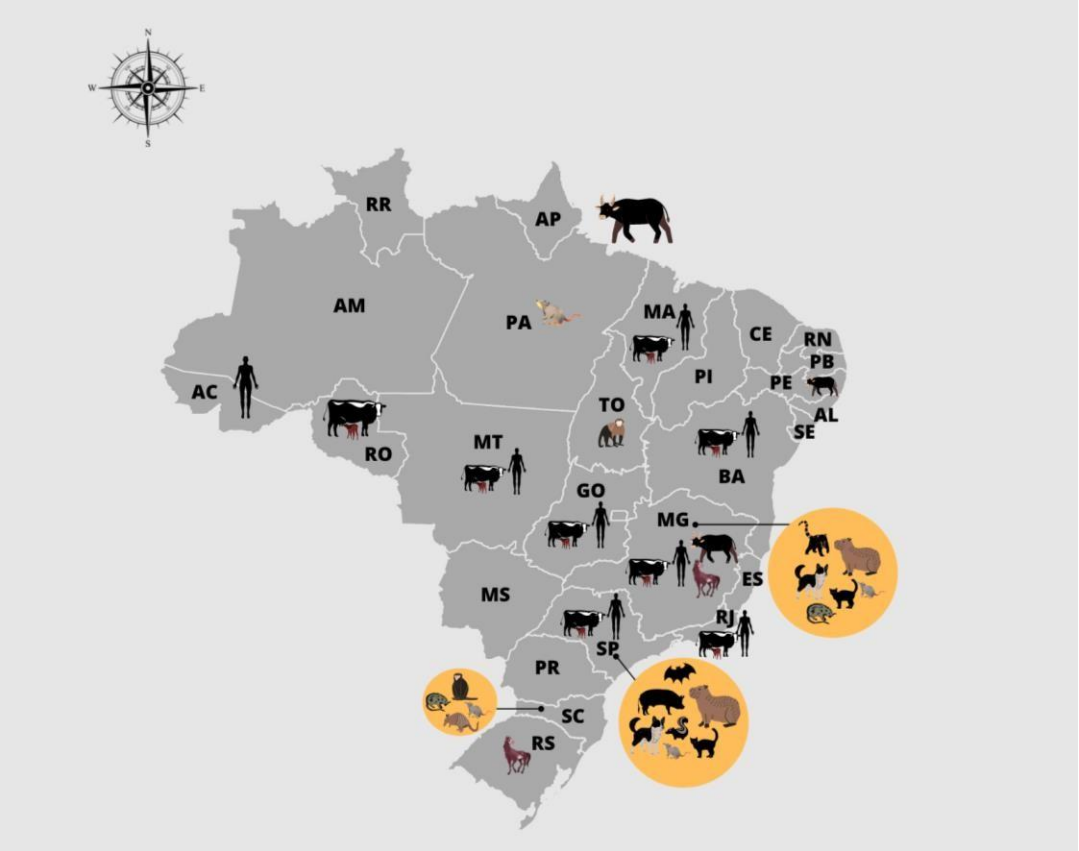


Figura 7. Detecção e distribuição do VACV no Brasil

Mapa do Brasil destacando os estados onde a distribuição do VACV foi relatada e os diversos hospedeiros onde o vírus foi detectado através de técnicas sorológicas, biologia molecular ou isolamento viral. Os estados que registraram a detecção do VACV são: Acre (AC), Rondônia (RO), Mato Grosso (MT), Goiás (GO), Tocantins (TO), Maranhão (MA), Pará (PA) – Ilha de Marajó, Pernambuco (PE), Bahia (BA), Minas Gerais (MG), Rio de Janeiro (RJ), São Paulo (SP), Santa Catarina (SC) e Rio Grande do Sul (RS). Nos estados de MG e SP foram registrados uma diversidade de hospedeiros expostos aos vírus, extrapolando o contexto dos surtos de VB e evidenciando a circulação do VACV em animais domésticos e silvestres. Fonte: Oliveira, et al., 2017 - modificado.

Outros hospedeiros zoonóticos foram relatados no ambiente rural, como os bubalinos e equídeos (BRUM et al., 2010; CAMPOS et al., 2011; ASSIS et al., 2012B; ABRAHÃO et al., 2016; FRANCO-LUIZ et al., 2016A; BORGES et al., 2018; LIMA et al., 2019). Nas propriedades rurais, os equídeos podem atuar nas atividades agrícolas auxiliando no transporte de alimentos e conduzindo os bovinos no percurso curral/pastagem. Dessa forma, em pequenas propriedades, os equídeos poderiam acessar livremente áreas de curral e compartilhar pastagens com bovinos. Eventualmente, isso poderia favorecer a exposição desses animais aos bovinos infectados (BORGES et al., 2018).

Apesar das frequentes descrições dos surtos e a evidente dispersão do VACV pelo território brasileiro, muito ainda é desconhecido sobre a circulação natural do VACV e seus possíveis hospedeiros reservatórios. Os primeiros VACV isolados no Brasil datam da década de 60, período que antecede a emergência dos surtos associados a bovinos. Esses isolados (Cotia

SPAn 232 e BeAn 58058) foram obtidos a partir de roedores sentinelas e silvestres, respectivamente, durante pesquisas de arbovírus conduzidas pelo Instituto Rockefeller (FONSECA et al., 1998; DA FONSECA et al., 2002).

Após emergência dos casos de VB, a detecção da mesma amostra viral circulando em humanos, bovinos e em roedor *Mus musculus* durante um surto, possibilitou o desenvolvimento de um modelo ecológico propondo a participação dos roedores como carreadores do vírus entre o ambiente silvestre e rural na cadeia de transmissão do VACV (ABRAHÃO et al., 2009A). Posteriormente, essa hipótese foi reforçada mediante a detecção da circulação do VACV em diversas espécies de roedores, dentre essas, espécies com hábitos generalistas, que podem se adaptar a ambientes perturbados (MIRANDA et al., 2017).

Além da possível atuação como pontes que favorecem a transmissão do VACV entre áreas conservadas e antropizadas; os roedores são apontados como potenciais fontes para a manutenção do VACV na natureza através da eliminação do vírus nas fezes e urina (FERREIRA et al., 2008; PERES et al., 2018). Embora a implicação epidemiológica desse achado ainda não seja clara, as partículas do VACV eliminadas em fezes de camundongos infectados experimentalmente foram detectadas por até 20 dias pós-infecção. Esse achado sugere que a eliminação de partículas virais viáveis nas excretas de roedores poderia favorecer a manutenção e/ou dispersão do vírus no ambiente (Figura 8) (ABRAHÃO et al., 2009b).

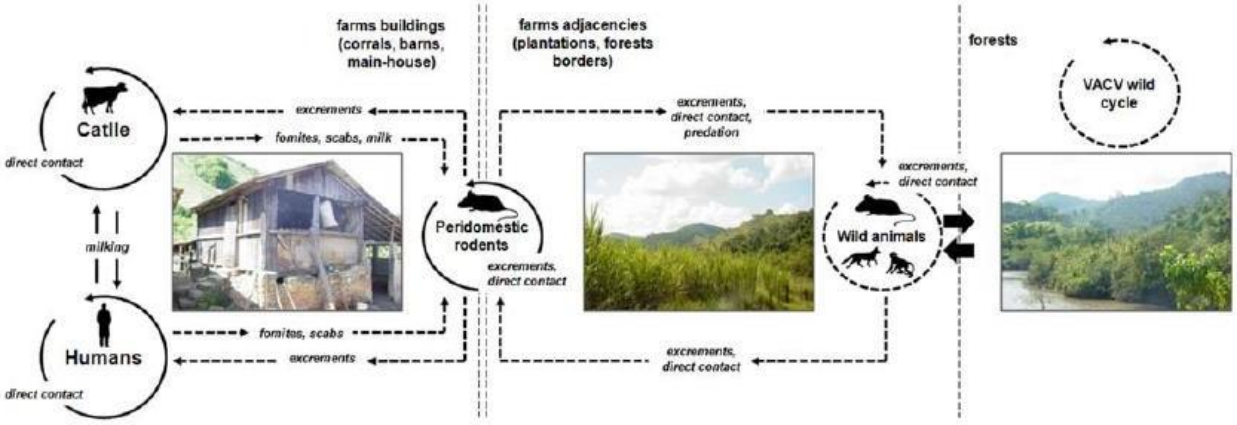


Figura 8. Modelo hipotético do ciclo de transmissão do VACV-Br

Roedores peridomésticos podem promover a transmissão do VACV entre animais silvestres, bovinos ou humanos, uma vez que circulam tanto nas propriedades rurais, quanto na mata ao redor. Este diagrama foi proposto por Abrahão e colaboradores (2009) com base em dados epidemiológicos e laboratoriais do VACV, levando em consideração as características comportamentais dos roedores e animais silvestres brasileiros. As linhas sólidas indicam dados determinados experimentalmente e as linhas tracejadas representam proposições hipotéticas ainda sob investigação. Fonte: Abrahão et al., 2009.

Dessa forma, o controle das populações de roedores em propriedades rurais seria uma medida preventiva relevante, visto que, além do potencial risco de trazer o patógeno à propriedade, existe também a possibilidade dos roedores se contaminarem no pasto e carrear o vírus para o ambiente silvestre. Um estudo experimental em camundongos expostos às fezes de bovinos contaminados com o VACV resultou em infecção dos roedores, com detecção molecular do vírus no sangue e nas fezes dos animais (D'ANUNCIAÇÃO et al., 2012).

O cenário da manutenção do VACV através de um ciclo silvestre tem sido consolidado através da detecção do vírus em diferentes biomas brasileiros assim como, em um amplo espectro de hospedeiros que envolvem espécies de animais silvestres (Tabela 2), como primatas não humanos, procionídeos, cingulados, marsupiais, morcegos e roedores silvestres, dentre eles o *Caluromys philander*, uma espécie arborícola que vive em ambiente de mata (ABRAHÃO et al., 2010B; PERES et al., 2013, 2016, 2018; DUTRA et al., 2016; MIRANDA et al., 2017; COSTA et al., 2017; 2018; MARTINS-COSTA et al., 2020).

Tabela 2. Espectro de hospedeiros susceptíveis.

Hospedeiros cuja associação com a circulação do VACV-Br foi detectada através da detecção do genoma ou de partículas virais.

	Família/Espécies de hospedeiros	Marcador laboratorial detectado	Referencial teórico
Associado a surtos e/ou infecções humanas Ł	Bovidae / búfalos domésticos (Bubalus bubalis) e bovinos (Bos taurus)	DNA e partícula viral	Damaso et al., 2000; Trindade et al., 2003; Nagasse-sugahara et al., 2004; Megid et al. 2008;2012; Schatzmayr et al., 2011; Quixabeira-Santos et al., 2011; Sant'ana et al., 2013; Oliveira et al., 2013; Assis et al., 2012a; 2013a; 2013b; Abrahão et al., 2015; 2016; Franco-luiz et al., 2016)
	Equidae / cavalos (Equus ferus caballus)	DNA e partícula viral	Brum et al., 2010; Campos et al., 2011; Abrahão et al., 2016
	Equidae / burro (Equus africanus sp.) e mula (Equus mulus)	DNA	Abrahão et al., 2016;
	Muridae / camundongo (Mus musculus)	DNA e partícula viral	Abrahão et al., 2009a
Não associado a surtos e/ou infecções humanas	Cebidae / macaco prego (Sapajus apella), macaco de cheiro (Saimiri sciureus)	DNA	Abrahão et al., 2010b; Martins-Costa et al., 2020
	Atelidae / bugio preto (Alouatta caraya)	DNA	Abrahão et al., 2010b

	Didelphidae / gambá de orelha preta/saruê (Didelphis aurita), gambá de orelha branca (Didelphis albiventris), cuíca (Caluromys philander), cuíca graciosa (Gracilinanus microtarsus), cuíca-de-cauda-grossa (Lutreolina crassicaudata)	DNA	Miranda et al., 2017; Peres et al., 2016; Martins-Costa et al., 2020
	Leporidae / Lebre (Lepus europaeus)	DNA	Martins-Costa et al., 2020
	Procyonidae / quati de cauda anelada (Nasua nasua)	DNA	Costa et al., 2018
	Felidae / gatos (Felis catus)	DNA	Costa et al., 2017
	Canidae / cachorro (Canis familiaris)	DNA	Peres et al., 2016; Costa et al., 2018
	Chlamyphoridae / tatu peba (Euphractus sexcintus)	DNA	Martins-Costa et al., 2020
	Molossidae / morcego (Molossus rufus e Eumops perotis)	DNA	Martins-Costa et al., 2020
	Cricetidae / Oryzomys spp., *Nectomys squamipes*	DNA e partícula viral	Martins-Costa et al., 2020
	Cricetidae / rato do mato (*Oligoryzomys nigripes*, *Oligoryzomys flavenscens*, *Sooretamys angouya*, *Calomys* spp., *Akodon* spp., *Cerradomys subflavus, Euryoryzomys russatus*), ratinho do cerrado (*Necromys Lasiurus*), rato pitoco (Thaptomys nigrita)	DNA	Miranda et al., 2017; Peres et al., 2018; Martins-Costa et al., 2020
	Echimyidae / rato de espinho (*Trinomys setosus*)	DNA	Miranda et al., 2017
	Erethizontidae / Sphiggurus spinosus	DNA	Martins-Costa et al., 2020
	Muridae / rato preto (*Rattus rattus*) camundongo (Mus musculus)	DNA	Miranda et al., 2017; Peres et al., 2018; Martins-Costa et al., 2020
	Sciuridae / caxinguelê (Guerlinguetus aestuans)	DNA	Martins-Costa et al., 2020
	Caviidae / capivaras (*Hydrochoerus hydrochaeris*)	DNA	Dutra et al., 2016; Martins-Costa et al., 2020

Ɨ Detecção de hospedeiro infectado no contexto de surto de vaccínia bovina e/ou relato de transmissão para humanos.

Atualmente, a circulação do VACV no Brasil tem transposto o ambiente silvestre e rural, tendo alcançado também o ambiente urbano. O VACV tem sido detectado em animais domésticos como canídeos e felinos (COSTA et al., 2017; 2018*)*. Embora não se saiba, ainda, o papel desses animais domésticos na cadeia epidemiológica do VACV, é importante destacar o potencial de transmissão do VACV desses animais domésticos para humanos, assim como é relatado para CPXV na Europa (CARLETTI et al., 2009; ZABA et al., 2017; TALAREK ET AL., 2018; KRANKOWSKA et al., 2020; HADDADEEN et al., 2020).

No contexto das infecções por CPXV, os felinos são os animais domésticos mais afetados, principalmente devido ao seu comportamento predatório em relação aos roedores, que são os principais reservatórios do vírus nos ambientes doméstico e peridomésticos (THOMSETT et al., 1978; PFEFFER et al., 2002; ESSBAUER et al., 2010; VOGEL et al., 2012; O'HALLORAN et al., 2016; BREHENY et al., 2017; JUNGWIRTH et al., 2018). Logo, a infecção de gatos domésticos por CPXV é associada a um maior risco de exposição a humanos no ambiente doméstico, o que nos faz questionar se o perfil das infecções naturais causadas pelo VACV-Br seria similar.

A exposição dos humanos ao VACV-Br fora do contexto da VB ainda é pouco explorada. O primeiro estudo soroepidemiológico em uma população de humanos foi realizado no estado do Acre, localizado na região amazônica. Esse trabalho envolveu 294 indivíduos pertencentes a assentamentos rurais, sendo encontrada uma soro prevalência de 27,89% (MOTA et al., 2010).

Outro estudo fez uma análise retrospectiva, avaliando soros amostrados nos anos de 1995-1996, período que antecede o primeiro surto de VB documentado. Neste trabalho, a população analisada era composta por indivíduos da região norte e do sudeste brasileiro. Dos 132 participantes, treze (9,8%) tiveram anticorpos neutralizantes detectáveis, sendo a idade e a vacinação as variáveis estatisticamente significativas associadas à presença de anti-OPV (FIGUEIREDO et al., 2015).

Por fim, um estudo de soro prevalência conduzido na região rural do Serro, importante bacia leiteira do estado de Minas Gerais, avaliou 241 indivíduos e detectou soro prevalência de 30,1% entre a população rural analisada. Dentre esses indivíduos com anticorpos anti-OPV, 9,5% enquadram-se como não vacinados para a varíola. Além disso, revelou a presença de DNA viral no sangue e altos títulos de anticorpos neutralizantes anti-OPV em indivíduos que não praticam qualquer atividade clássica de exposição ao VACV como, por exemplo, ordenha de bovinos, mas geralmente consome leite cru e queijo, sugerindo possível papel do consumo desses alimentos como rota alternativa de transmissão do VACV (COSTA, et al., 2015; 2016).

1.2.3. Detecção do VACV-Br no leite e em derivados lácteos

A emergência do VACV no Brasil no final da década de 90 ocorreu em um cenário rural. A VB é uma zoonose ocupacional que afeta principalmente o gado leiteiro e os ordenhadores. Nesse contexto, questiona-se o papel do leite e dos derivados na cadeia epidemiológica da doença. O isolamento de partículas virais e/ou detecção do genoma viral a partir de leite coletado de rebanhos de bovinos e bubalinos infectados durante surtos em Minas Gerais e Pernambuco, respectivamente, tem contribuído para a construção da epidemiologia do VACV

no Brasil no que tange esta possível rota de infecção (ABRAHÃO et al., 2009C; REHFELD et al., 2018; LIMA et al., 2019).

A eliminação de VACV foi detectada no leite de vacas infectadas experimentalmente a partir do 1° até o 81° dia pós-infecção (d.p.i). O vírus foi detectado, tanto no leite de animais submetidos à ordenha manual, quanto naqueles em que foi introduzido um cateter em suas glândulas mamárias (OLIVEIRA et al., 2015). Possivelmente, a eliminação do VACV no leite decorre de uma infecção viral sistêmica e, em alguns casos, subclínica, o que dificulta o monitoramento desses alimentos e consequentemente pode contribuir para a exposição dos consumidores (REHFELD et al., 2017A; MATOS et al., 2018).

A transmissão de VACV a partir de leite contaminado foi avaliada por Rehfeld e colaboradores, utilizando modelo murino. Embora tenha sido descrita ausência de sinais clínicos, foi demonstrada disseminação viral sistêmica e detecção do DNA viral nas fezes e na mucosa oral dos animais. Entretanto, o vírus não foi detectado no estômago dos animais, o qual, por se tratar de um meio extremamente ácido pode inativar patógenos ingeridos, incluindo vírus envelopados, como é o caso do VACV, e desnaturar proteínas antes mesmo da degradação por enzimas proteolíticas, características que reforçam a hipótese de que a região orofaríngea poderia ser um local primário de multiplicação viral após a exposição por via oral (REHFELD et al., 2015).

Outro estudo que sugere o consumo de leite contaminado como rota de transmissão foi conduzido a partir da investigação de um surto causado por uma amostra de VACV denominada buffalopox (BPXV) na Índia, e no qual foi descrita a infecção de humanos com desenvolvimento de lesões na boca em 10,2% dos afetados, (GURAV et al., 2011).

Outro achado relevante foi a demonstração de que as partículas virais no leite cru, contaminado experimentalmente, permanecem viáveis quando submetido ao processo de estocagem comumente utilizado, cujo alimento é mantido a -20°C ou a 4°C por 48 horas; e após pasteurização, que consiste em um tratamento térmico a 65°C por 30 minutos (OLIVEIRA et al., 2010).

Além disso, o VACV resiste ao processamento do leite durante a produção de queijo artesanal, permanecendo detectável no soro e no queijo (OLIVEIRA et al., 2010). Quando o tempo de maturação do queijo artesanal foi avaliado, conseguiu-se recuperar partículas virais de queijos maturados há 60 dias mantidos na temperatura de 25°C (REHFELD et al., 2017B).

1.2.4. Transmissão do VACV em infecções naturais

A via clássica de transmissão do VACV no contexto da VB ocorre através do contato direto entre humanos e bovinos infectados (Figura 9). Logo, a prática da ordenha é um importante fator de exposição associado ao VACV. Esse contato favorece a infecção do indivíduo e a propagação do vírus dentro do rebanho e até mesmo entre propriedades (TRINDADE et al., 2003; LEITE et al., 2005; LOBATO et al., 2005; KROON et al., 2011; OLIVEIRA et al., 2017).

Figura 9. Via clássica de transmissão do VACV

Contato direto entre humano e bovino infectado por VACV. Essa via clássica de transmissão caracteriza a vaccínia bovina como zoonose ocupacional. Fonte: Trindade et al., 2003; 2009; Lobato et al., 2005.

Além do contato direto com o animal infectado, o VACV pode ser transmitido por fômites. O vírus foi isolado a partir de utensílios domésticos no ambiente interno de uma propriedade afetada por surto de VB (ASSIS et al., 2013). Outra abordagem consiste na transmissão intrafamiliar de VACV. Nesta perspectiva, um caso foi descrito em 2002 no estado do Rio de Janeiro, onde o pai ordenhador foi infectado pelo VACV e transmitiu o vírus ao filho adolescente, que não realizou ordenha manual e não mantivera contato com bovinos

(PEREIRA-OLIVEIRA et al., 2014). Em outro relato, é descrita a transmissão após contato sexual, resultando em desenvolvimento de lesões na região genital feminina (BATISTA et al., 2009).

Como a VB é uma zoonose que afeta o gado leiteiro, questiona-se o papel do leite e de derivados no ciclo epidemiológico da doença. De fato, a infecção na ausência de contato direto com bovinos infectados através da ordenha foi relatada, apontando para possíveis novas vias de transmissão do VACV (MOTA et al., 2010; COSTA et al., 2015).

A circulação do VACV em um cluster familiar residente em área endêmica, no entanto, em indivíduos não praticantes de ordenha tem alertado para a possível existência de rotas alternativas de transmissão viral. Apesar de não se submeter à forma clássica de exposição ao vírus, esse cluster relatou consumo frequente de leite e queijo artesanal (COSTA et al., 2015).

Embora, até o momento, apenas um relato de exposição a leite durante um surto causado por BPXV na Ásia tenha sido associado à presença de manifestações clínicas (GURAV et al., 2011), diversos trabalhos têm evidenciado o potencial papel do leite e queijo artesanal como fontes de exposição ao VACV no Brasil (ABRAHÃO et al., 2009C; OLIVEIRA et al., 2010;2015;2017A; REHFELD et al., 2015;2017;2018; MATOS et al., 2018).

Além disso, a descrição do VACV em ambiente urbano no Brasil, infectando canídeos e felinos têm levantado a hipótese do possível papel desses animais no ciclo de transmissão da zoonose (COSTA et al., 2017; 2018). De fato, animais domésticos têm sido fundamentais na cadeia epidemiológica do CPXV na Europa. O CPXV é transmitido aos seres humanos principalmente por gatos domésticos que estão em contato com roedores, os reservatórios naturais do vírus (ESSBAUER et al*.,* 2009; CARLETTI et al., 2009; ZABA et al., 2017; TALAREK et al., 2018; KRANKOWSKA et al., 2020; HADDADEEN et al., 2020). No entanto, o CPXV também já foi descrito causando infecção com desenvolvimento de lesões em cães (SMITH et al., 1999; VON BOMHARD et al., 2010).

1.2.5. Diagnóstico, detecção e caracterização do VACV.

O diagnóstico das infecções causadas por OPV deve sempre basear-se na detecção laboratorial. Pode ser sorológico, através da pesquisa de anticorpos IgM, IgG ou neutralizantes; molecular, por meio da técnica de reação em cadeia da polimerase (PCR) e suas variações; ou por detecção direta do vírus, que consiste em microscopia eletrônica, onde é possível visualizar as partículas virais ou por isolamento viral em células de linhagem contínua, cultivo primário de fibroblastos de embrião de galinha ou em membrana corioalantóide de ovos embrionados (DAMON, 2013; KROON et al., 2016).

Através da pesquisa de anticorpos é possível detectar a infecção em fase aguda (IgM) ou estimar infecções passadas (IgG) (KAREM et al., 2005). Entretanto, devido à resposta imunológica cruzada entre as espécies do gênero OPV, essa metodologia não determina qual OPV foi responsável pelo estímulo (JACOBS et al., 2009; MILLER et al., 2011).

O diagnóstico molecular permite a detecção em nível de espécie, possibilitando a identificação das variabilidades através da detecção de mutações, deleções ou inserções a partir da análise dos principais genes pesquisados. O diagnóstico clínico das infecções naturais por VACV se mostra complexo. No Brasil, as lesões e os sinais clínicos presentes nos animais infectados sobrepõem os sintomas de outras doenças que afetam bovinos, como febre aftosa, estomatite vesicular, pseudovaríola e mamilite herpética. Além disso, em humanos, não raramente, o indivíduo acometido apresenta infecção bacteriana secundária que dificulta o diagnóstico clínico (RIET-CORREA et al., 1996; SANT'ANA et al., 2013; LAGUARDIA-NNASCIMENTO et al., 2016; ALVES et al., 2016).

No diagnóstico da VB, os espécimes clínicos utilizados para detecção viral podem ser crostas ou secreções obtidas diretamente das lesões (ricas em partículas virais), o sangue e soro dos pacientes e animais infectados (KROON et al., 2016). Os protocolos comumente utilizados para detecção do DNA do VACV no Brasil incluem como alvos C11R (*vgf/viral growth factor* – fator de crescimento viral) A26L (*ati/A type inclusion boddy protein* – proteína do corpúsculo de inclusão), e A56R (ha/gene da hemaglutinina), este último permite a caracterização da amostra viral entre os dois grupos circulantes (ROPP et al., 1995; MEYER et al., 1997; DRUMOND et al., 2008; TRINDADE et al., 2008; ABRAHÃO et al., 2010A; ASSIS et al., 2012A; KROON et al., 2016).

O isolamento viral, onde é possível recuperar partículas virais envolvidas na infecção, contribui para a caracterização das amostras virais circulantes (ABRAHÃO et al., 2009C; KROON et al., 2016). Após o isolamento é possível realizar estudos de patogênese para avaliar as características biológicas do vírus e sua relação com seus hospedeiros (FERREIRA et al., 2008; CAMPOS et al., 2011; TRINDADE et al., 2016; KROON et al., 2016).

No Brasil, o diagnóstico laboratorial da VB não faz parte do escopo de vigilância laboratorial dos Laboratórios Centrais de Saúde Pública (LACEN), sendo centralizado em uma rede de resposta laboratorial composta por alguns centros de pesquisa ou instituições governamentais, distribuídos em Minas Gerais, São Paulo e Rio de Janeiro (região Sudeste); e no Estado do Rio Grande do Sul (região Sul) (Figura 10). Esse cenário reflete como a circulação do VACV no país ainda é negligenciada e reforça a necessidade de formar uma rede cooperativa com ações estratégicas para o fortalecimento das políticas públicas de vigilância em saúde (DOMINGOS et al., 2021).

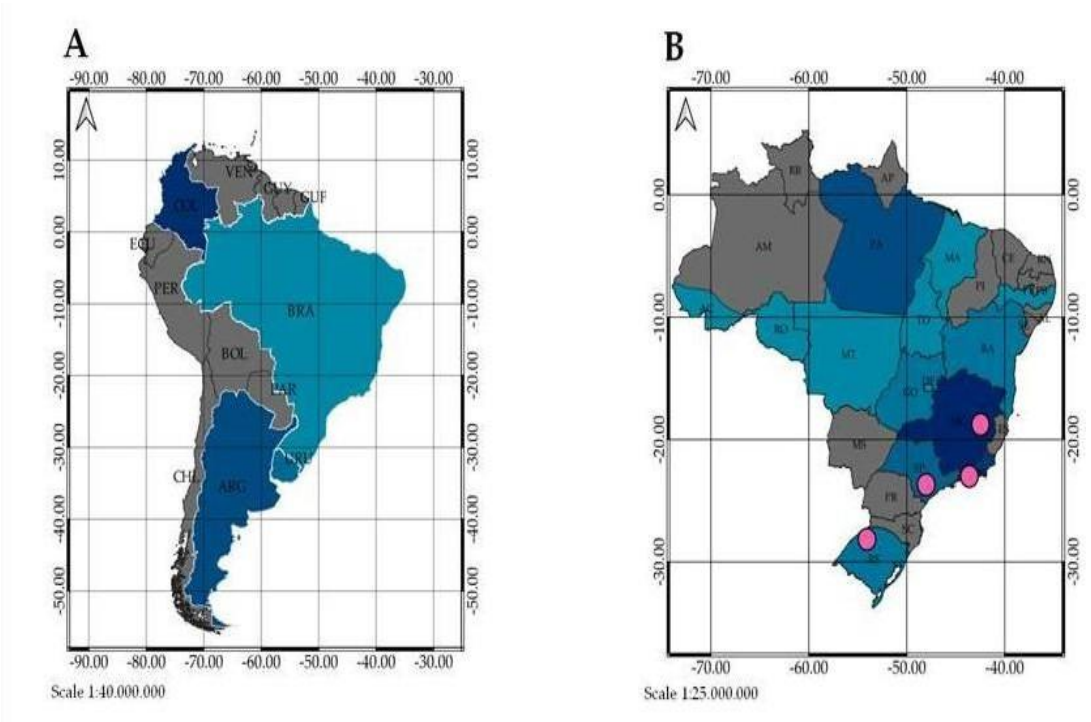


Figura 10. Detecção do VACV no Brasil e localização dos laboratórios de referência

Mapa do Brasil indicando os estados onde a circulação do VACV foi registrada. Os estados em cinza não registraram detecção do VACV até o momento. O gradiente da cor azul destaca os estados, considerando o índice mais baixo ao mais alto de registros relatados por publicações científicas. Acre (AC), Maranhão (MA), Mato Grosso (MT), Pernambuco (PE), Rondônia (RO), Tocantins (TO) e Distrito Federal (DF) apresentam apenas um registro cada. Por outro lado, Minas Gerais (MG) é o estado com maior número de casos de VACV. Os círculos rosas indicam os estados do Brasil onde existem laboratórios de referência, certificados para o diagnóstico de doenças por Poxvírus. Fonte: Domingos et al., 2021.

1.2.6. Impacto da VB na saúde pública

A VB é uma doença de alta morbidade. Em mais de 80% das propriedades afetadas por surtos de VB são detectados casos em seres humanos que além de apresentar lesões nos sítios mais comuns como nos membros superiores, frequentemente apresentam lesões secundárias derivadas do processo de autoinoculação (LOBATO et al., 2005; TRINDADE et al., 2007; LIMA et al., 2018). Apesar do impacto causado pelos surtos de VB, o monitoramento e controle ainda são ineficientes e, portanto, o número de casos é subestimado (OLIVEIRA et al*.,* 2017A; DOMINGOS et al., 2021).

Clinicamente, a infecção por VACV em humanos pode ser confundida com outras infecções vesico-pustulares como parapoxvirus, leishmaniose e estafilococos (SILVA et al., 2008; TRINDADE et al., 2009). Um inquérito epidemiológico realizado com profissionais de saúde que atuam em área endêmica para a VB no Brasil, revelou que 43% dos profissionais envolvidos no estudo desconhecem completamente a zoonose. De maneira geral, o estudo

demonstrou a existência de uma lacuna em termos de vigilância epidemiológica do VACV. Dessa forma, as equipes de saúde, mesmo em região endêmica, demonstram dificuldade em identificar e diagnosticar corretamente a doença. Além da subnotificação dos casos, isso pode resultar na aplicação de tratamento inadequado e agravamento do quadro clínico dos pacientes (OLIVEIRA et al., 2017B).

O VACV é transmitido por contato direto, portanto o potencial de transmissão pessoa a pessoa e a possibilidade de infecção nosocomial não devem ser negligenciados. No Paquistão, foi descrito um surto de BPXV, a amostra de VACV circulante no país, acometendo 19 indivíduos atendidos em unidades de tratamento de queimaduras. O atraso no reconhecimento e investigação do surto possibilitou a transferência de pacientes entre unidades de atendimento, o que ampliou o número de expostos (ZAFAR et al., 2007).

Além disso, atenção adicional deve ser dada a pacientes imunocomprometidos cuja infecção pode progredir de forma grave. Na Alemanha, um paciente infectado com o vírus da imunodeficiência humana (HIV), co-infectado com o Epstein-Barr e vírus da hepatite B e C, desenvolveu infecção generalizada por CPXV com evolução para o óbito (FASSBENDER et al., 2016). Em outro relato relacionado à CPXV, um paciente jovem, com imunossupressão pós-transplante renal foi infectado após contato com o gato doméstico e desenvolveu um quadro grave que resultou em falência múltipla de órgãos (GAZZANI et al*.,* 2016).

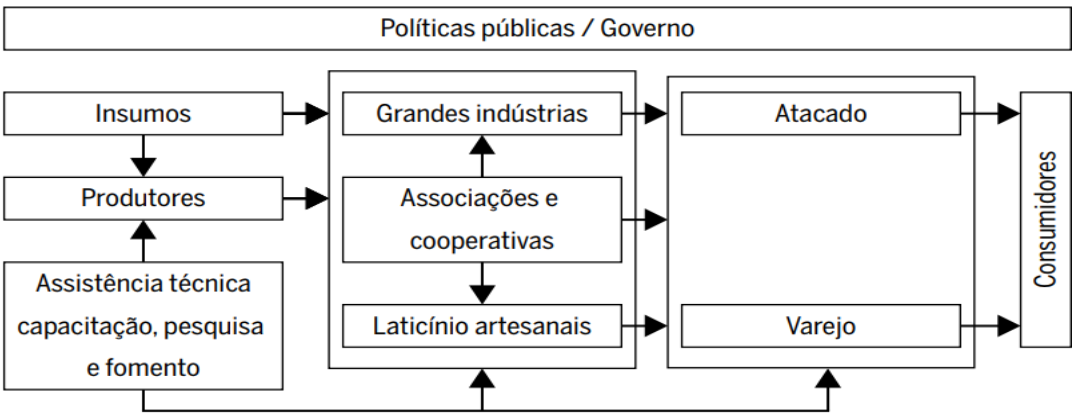
Nesse contexto, foi relatada uma condição grave e frequentemente letal, conhecida como vaccínia progressiva, em um paciente colombiano portador do HIV. O caso, que atuava como ordenhador, foi exposto ao VACV no ambiente de trabalho e não possuía histórico vacinal contra a varíola. O paciente apresentou um quadro persistente, que foi revertido apenas após transfusão sanguínea, sugerindo que a reversão da condição imunossupressora deve ser tratada em casos específicos e que são necessárias estratégias adicionais de prevenção, tratamento e controle de infecção (LAITON-DONATO et al., 2020).

1.2.7. Impacto econômico da VB na agropecuária brasileira

No Brasil, a agropecuária consiste em uma das atividades econômicas mais rentáveis. No âmbito da produção de leite, o país ocupa o quarto lugar no ranking mundial. Em 2020, o Brasil produziu cerca de 25,52 bilhões de litros de leite, registrando o equivalente a 76 milhões de dólares em exportações no mesmo período (CONAB, 2021). Embora as propriedades brasileiras produtoras de leite sejam menores quando comparadas aos outros países, no Brasil, as fazendas têm crescido quanto ao volume individual de produção (PEROBELLI *et al.,* 2018).

A cadeia produtiva do leite em Minas Gerais é fundamental na geração de empregos diretos e indiretos (Figura 11). O estado possui tradição na produção leiteira e lidera a

produtividade nacional, sendo responsável por 25,5% do volume total de leite produzido (CONAB, 2021). Devido a mudanças técnicas nas propriedades rurais de Minas Gerais, a produção leiteira teve um aumento de 87% no período de 1996 a 2014 (VILELA et al., 2017). No entanto, o desenvolvimento do estado é muito heterogêneo, aproximadamente 13% dos municípios mineiros apresentam melhor desenvolvimento, enquanto 60,5% são pouco ou nada desenvolvidos no contexto da produção de leite (OVIEDO-PASTRANA et al., 2014).



]Figura 11. Cadeia produtiva do leite no Brasil

A cadeia é iniciada a partir dos insumos, fatores para produção ou matérias primas. Esses insumos incluem aqueles utilizados no trato com o animal, bem como de maquinário para ordenha, resfriamento, armazenagem e distribuição do leite e são fornecidos aos produtores ou unidades de produção primária. A rede de distribuição e logística atuam como intermediários na distribuição do leite, ainda in natura, facilitando o acesso entre produtores e indústrias. Os laticínios artesanais e as grandes indústrias captam o leite e desenvolvem produtos a partir dele. A assistência técnica, capacitação e pesquisa no setor são desenvolvidas por instituições públicas e privadas como Embrapa, Emater, Instituições Financeiras, Cooperativas, a Secretaria Especial de Agricultura Familiar e Desenvolvimento Agrário (antigo MDA), Ministério da Agricultura, Pecuária e Abastecimento (MAPA), dentre outros. As redes de atacado e varejo atuam como centros de comercialização final, garantindo que o leite e seus derivados cheguem até os consumidores finais. Fonte: Perobelli et al., 2018.

Os bovinos de leite e os ordenhadores, elementos fundamentais na cadeia produtiva do leite em Minas Gerais, são os principais afetados pela VB. Os surtos são relatados em propriedades que realizam ordenha manual e/ou mecânica (OLIVEIRA et al., 2017A; BORGES et al., 2017). As pequenas propriedades que realizam a ordenha manual correspondem a cerca de 90% dos casos e a taxa de ataque nos rebanhos é muito alta, podendo atingir 100% (LOBATO et al., 2005; MATOS et al., 2018). Dessa forma, as pequenas propriedades, que dependem exclusivamente da produção de leite, são amplamente afetadas, assim como os municípios cuja principal atividade econômica baseia-se na produção leiteira (LOBATO et al., 2005; OLIVEIRA et al., 2017A).

As manifestações clínicas apresentadas no curso da infecção por VACV compreendem principalmente o desenvolvimento de lesões exantemáticas acompanhadas de processo

inflamatório e infecções bacterianas secundárias (OLIVEIRA et al., 2017A; MATOS et al., 2018). O desenvolvimento dessas lesões no epitélio do bovino associado à mastite pode resultar em perda econômica para o produtor através da diminuição da produção de leite e gasto com medicamentos e veterinários. Bovinos de leite, infectados experimentalmente, apresentaram uma redução média de 32,94% da produção de leite, a partir do 1º ao 32º dpi (Rehfeld et al., 2013). Além disso, pode levar ao aumento da contagem de células somáticas no leite produzido, alterando a qualidade da produção e consequentemente o rendimento industrial (FONSECA *et al.,* 2000; Rehfeld et al., 2013).

A infecção do ordenhador leva à perda temporária de recursos humanos nas propriedades afetadas, onde há o afastamento ou até mesmo a demissão do profissional. Isso causa prejuízo tanto para o empregado, que pode perder sua fonte de renda; quanto para o empregador diante da necessidade de contratação de um novo funcionário para suprir a falta do mesmo (TRINDADE et al., 2003; LEITE et al., 2005; LOBATO et al., 2005; TRINDADE et al., 2006; TRINDADE et al., 2007A; OLIVEIRA et al., 2017A).

Portanto, o estado de Minas Gerais é significativamente afetado pelos surtos de VB, uma vez que consiste no maior produtor de leite do Brasil, sendo responsável por aproximadamente 1/3 da produção de leite no país. (IBGE, 2018; CONAB 2021). Dados da Secretaria de Estado da Agricultura e do Abastecimento relatam que Minas Gerais possui tradição na produção leiteira, um grande rebanho de animais, condição climática favorável, o uso de tecnologias de produção e boa nutrição dos rebanhos, o que resulta no sucesso da produtividade (SEAB, 2015). Segundo o Banco Nacional de Desenvolvimento Econômico, a análise por estrato de produção revela a importância de estabelecimentos com escalas de produção pequena e média na produção nacional de leite (BNDES, 2016).

Outro gargalo associado à VB corresponde à precisão do diagnóstico clínico. Na área médica veterinária e na zootecnia, a VB frequentemente é confundida com outras doenças vesiculares de bovinos, como febre aftosa, estomatite vesicular, pseudovaríola e mamilite herpética, o que pode resultar em subnotificação de casos e mascarar a proporção dos surtos (ABRAHÃO et al., 2010; SANT'ANA et al., 2013). Diante do exposto, faz-se necessário o desenvolvimento de estudos que possam subsidiar o desenvolvimento de políticas públicas voltadas ao diagnóstico, controle e prevenção da ocorrência de VACV no Brasil.

2. JUSTIFICATIVA

A emergência massiva do VACV no Brasil ocorreu no final da década de 90 e tem causado doenças em bovinos e humanos. Apesar da evidente dispersão do vírus pelo território brasileiro, muito ainda é desconhecido sobre a circulação natural do VACV e seus hospedeiros reservatórios. A VB é uma zoonose emergente, cujo agente etiológico é o VACV.

No Brasil, em 2020 foram produzidos 25,52 bilhões de litros de leite e o estado de Minas Gerais foi responsável por 25,5% da produção. Os surtos de VB foram descritos em dez estados, dentre eles Minas Gerais, que devido à sua representatividade na produção de leite, acaba afetando diretamente a economia leiteira no Brasil, uma vez que bovinos de leite infectados podem apresentar uma redução média de 32,9% da produção de leite durante o período de infecção ativa.

Por ser uma zoonose ocupacional, a VB também representa um problema de saúde pública. O quadro clínico decorrente da infecção implica no afastamento do trabalhador e/ou na sua hospitalização. Geralmente, o tratamento médico aplicado é impróprio devido à carência ou ausência de informação sobre a doença por parte dos profissionais de saúde. É observada uma alta frequência de infecção por VACV em humanos, relatada em mais de 80% das propriedades rurais afetadas, mesmo entre indivíduos vacinados contra a varíola e que em tese deveriam estar protegidos contra qualquer infecção por OPV. Por se tratar de uma zoonose, a doença é difícil de combater devido à existência de reservatórios silvestres e animais amplificadores do vírus, fazendo-se necessária a aplicação de medidas de controle visando minimizar os surtos e/ou danos associados.

Quanto aos humanos, a exposição ao VACV fora do ambiente rural é pouco explorada, resumindo-se a três estudos de soro prevalência no Brasil. Embora conduzidos em populações rurais, a circulação do VACV em humanos não expostos à forma clássica de transmissão do vírus tem apontado a possível existência de rotas alternativas de transmissão. Dentre essas vias, a hipótese do consumo de leite e/ou queijo artesanal contaminado tem sido consolidada. Embora não tenha sido descrito nenhum caso de infecção humana claramente associada ao consumo de leite no Brasil, existem relatos sobre um surto de VACV-like na Índia. Portanto, essa via alternativa de transmissão não deve ser negligenciada visto que é comum que indivíduos de áreas rurais consumam o produto na sua forma *in natura* e o mesmo é utilizado na produção de queijo artesanal, o que pode levar a uma constante e silenciosa exposição ao vírus.

Outro fator é a circulação do VACV em animais domésticos inseridos em ambiente urbano. Apesar do papel desses animais no ciclo epidemiológico do VACV ainda ser desconhecido, esses achados se assemelham à cadeia epidemiológica do CPXV, demonstrando a importância dos animais domésticos como possível fonte de transmissão para humanos. Considerando os dados recentes associados a possíveis rotas alternativas de transmissão do VACV no Brasil, esse estudo visa contribuir para o mapeamento da circulação do VACV em ambiente urbano. Além disso, esse estudo também visa aplicar o conhecimento gerado, podendo subsidiar ações de vigilância em saúde e controle frente à emergência do VACV em áreas urbanas.

3. OBJETIVO GERAL

Avaliar o papel do consumo e manipulação do leite *in natura* e do queijo artesanal como uma rota alternativa de infecção pelo VACV em uma área urbana do estado de Minas Gerais e determinar a prevalência de anticorpos anti-OPV na população urbana estudada através de ensaios de soro neutralização e imuno enzimático.

4. OBJETIVOS ESPECÍFICOS

1.1. Verificar o potencial do queijo artesanal produzido e comercializado em Minas Gerais como fonte de exposição ao VACV;

1.2. Verificar o potencial de exposição de humanos ao VACV através do consumo de leite *in natura* e queijo artesanal;

1.3. Estimar a ocorrência de infecções recentes através da pesquisa de anticorpos do tipo IgM por ELISA de captura;

1.4. Avaliar os fatores de risco associados à circulação de OPV em população urbana a partir de um inquérito soroepidemiológico.

1. FLUXOGRAMA DE TRABALHO

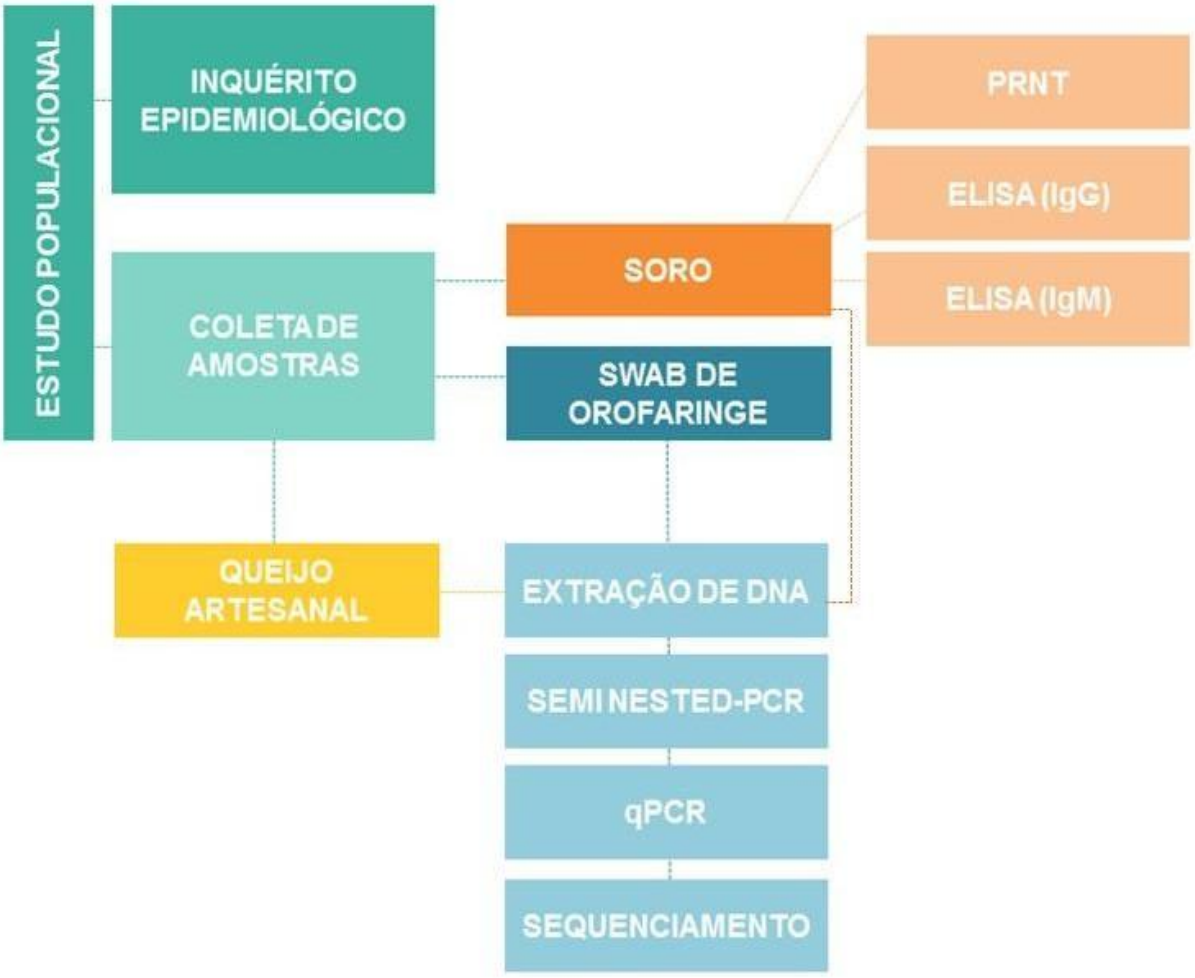


Figura 12. Fluxograma do estudo.

5. METODOLOGIA

5.1.1. Coleta do queijo artesanal produzido e comercializado em Minas Gerais

Em Minas Gerais, o queijo artesanal é reconhecido como um item de patrimônio imaterial e é tradicionalmente feito utilizando o leite cru. Para compreender o papel do queijo artesanal como possível fonte de transmissão do VACV, amostras de queijo artesanal comercial produzido no estado de Minas Gerais foram analisadas. Em Belo Horizonte, capital do estado, e na bacia leiteira do Serro, amostras de queijo artesanal foram coletadas no período de junho de 2015 a setembro de 2018. As amostras de queijo foram fornecidas por produtores da bacia

leiteira do Serro ou adquiridas em supermercados da capital, tendo sido produzidos nas principais bacias leiteiras, correspondendo ao Serro, Araxá, Canastra e Cerrado (Alto Paranaíba e Serra do Salitre).

5.1.2. Estudo epidemiológico de base populacional

Foi realizado um estudo transversal de base populacional para caracterização demográfica de indivíduos residentes em área urbana do estado de Minas Gerais, no período de 2015 a 2018. O inquérito epidemiológico foi realizado utilizando um questionário semiestruturado e pré-codificado (anexo 1). Foram avaliados fatores de risco relacionados à infecção assintomática ou doença clínica, histórico de infecções, antecedentes vacinais e aspectos comportamentais dos participantes envolvendo manipulação e consumo de leite *in natura* e/ou queijo artesanal.

5.1.3. Local do Estudo

O estudo foi desenvolvido na área urbana do município do Serro, localizado na macrorregião Jequitinhonha, estado de Minas Gerais (Figura 13). O município possui uma população de 20.993 habitantes, sendo 11.060 residentes em área urbana (IBGE, 2018). A região é endêmica para VACV (TRINDADE *et al.,* 2009; 2016; ASSIS *et al.,* 2012; COSTA, *et al.,* 2015; 2016; OLIVEIRA et al., 2017) e a principal atividade econômica consiste na produção de leite e queijos, dentre eles, o queijo artesanal do Serro, Patrimônio Imaterial segundo o Instituto Estadual do Patrimônio Histórico e Artístico de Minas Gerais (IEPHA/MG). O estudo foi conduzido entre os anos de 2015 e 2018 em uma amostragem aleatória de indivíduos dessa população.

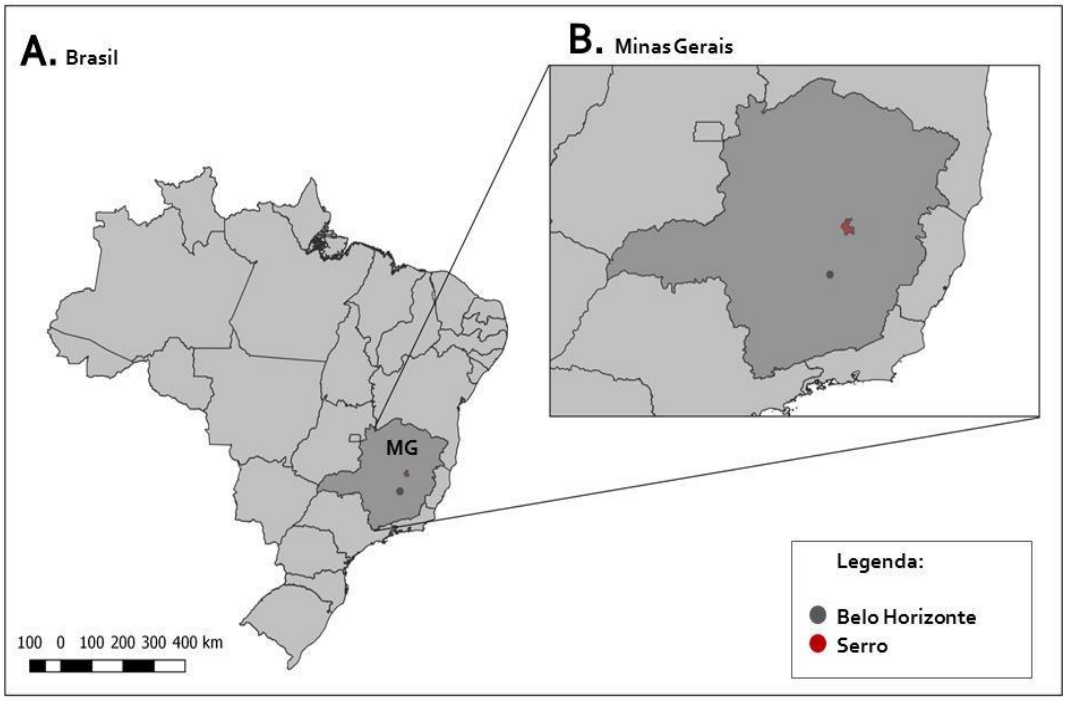


Figura 13. Localização geográfica do Serro-MG

O município do Serro (em vermelho) está inserido em uma importante bacia leiteira. O serro fica localizado a 312 km de distância da capital Belo Horizonte, capital do estado de Minas Gerais. Oliveira, JS, dados não publicados.

5.1.4. Definição do tamanho da amostra

No Brasil, os estudos epidemiológicos existentes até o momento são relacionados à investigação de surtos. Portanto, não há dados suficientes para estimar a prevalência das infecções por VACV em áreas urbanas. Utilizando a versão 3.0 do Programa Open-Epi (*Open Source Epidemiologic Statistics for Public Health*) foi calculado o número amostral apontando-se uma prevalência esperada de 50%, erro alfa de 5%, população urbana do município, (precisão de 10% em torno da estimativa e efeito de desenho de 1,0). Dessa forma, foi determinada uma amostra mínima de 372 indivíduos para a realização de um estudo com intervalo de confiança de 95% no Serro.

5.1.5. Seleção dos Participantes

Durante o período de 2015, 2017 e 2018 foram realizadas visitas ao município do Serro, com o apoio da secretaria municipal de saúde, sendo percorridos os bairros que compreendem a área urbana do município. Para coleta nos bairros, foi realizado um cálculo para distribuição da amostra de acordo com o percentual populacional residente em cada bairro do município. Os bairros foram percorridos e as casas foram visitadas alternadamente, fazendo-se o convite aos residentes para participação no estudo. Para a realização das coletas, foram montados pontos de referência em cada bairro, onde os participantes foram concentrados. Indivíduos de todas as faixas etárias foram contatados e aqueles que concordaram em participar voluntariamente, responderam a uma entrevista e tiveram amostras de sangue e swab oral coletadas. Foram excluídos do estudo, participantes com idade menor que 18 anos, cuja autorização expressa de um responsável legal não tenha sido disponibilizada.

5.1.6. Considerações Éticas

A participação no estudo foi voluntária e a todos os indivíduos contatados foi apresentado um termo de consentimento pós-esclarecimento, oral e por escrito, com informações sobre o estudo, seus propósitos, procedimentos e riscos envolvidos. Para participar do estudo, cada voluntário assinou o termo de consentimento livre e esclarecido (TCLE – anexo 2). Nos casos em que o participante tinha idade inferior a 18 anos, o TCLE foi assinado pelo representante legal.

O documento assinado permaneceu em poder da equipe de pesquisadores envolvida no projeto, enquanto uma cópia foi entregue ao voluntário. Caso o participante não soubesse assinar, poderia optar por ter o termo de consentimento assinado por algum familiar ou ter a impressão digital coletada. Os participantes receberam os resultados dos exames individuais realizados, encaminhados via secretaria municipal de saúde, acompanhados da interpretação dos resultados e seu significado. Esta proposta foi aprovada pelo Comitê de Ética em Pesquisa da UFMG (COEP) sob registro de parecer 1.974.249.

5.1.7. Instrumentos para Coleta de Informações

Foram utilizados questionários semiestruturados, pré-codificados, contendo perguntas sobre cada um dos participantes (Anexo 1). Foram investigadas as características demográficas, como sexo, idade, ocupação, escolaridade; e características comportamentais, como hábitos pessoais, prática da ordenha, consumo e manipulação de leite *in natura*, contato com animais domésticos e silvestres, histórico vacinal, ocorrência prévia da doença etc. para avaliação de fatores de exposição ao VACV. As informações coletadas foram digitalizadas e foram estruturados bancos de dados no *Microsoft Office Excel*. Foi realizada dupla digitação para garantir a confiabilidade dos dados.

5.1.8. Plano de Análise

A análise dos dados foi realizada utilizando a versão 3.0 do Programa Open-Epi *(Open Source Epidemiologic Statistics for Public Health)*. Foram realizadas as análises descritivas de todos os dados coletados e cálculo da prevalência de anticorpos anti-OPV.

As variáveis foram analisadas através de tabelas de contingência, usadas para avaliar a associação entre um possível fator de risco ('Exposição') e um desfecho ('Doença'). Neste estudo, o desfecho não consistiu em “doença”, mas em detecção de material genético viral ou desenvolvimento de anticorpos, indicadores de contato com o VACV, mas não necessariamente associadas à manifestação clínica na população estudada. Foi realizada a análise uni variada com comparação das frequências de cada variável nos diferentes grupos, utilizando o teste de qui-quadrado, teste exato de Fisher e cálculo da estimativa de risco relativo (*Odds Ratio*), para avaliação dos fatores de risco para infecção por OPV na população estudada.

5.1.9. Amostras Clínicas

A coleta foi realizada, após a assinatura do TCLE de cada participante ou responsável legal. Foram coletadas amostras de swab orofaríngeo para prospecção molecular do VACV na população urbana do Serro-MG. Os swabs foram mantidos “a seco” e armazenados a -20°C até o processamento no Laboratório de Vírus, ICB/UFMG. Também foram coletadas amostras de sangue venoso, as quais foram processadas por centrifugação para obtenção de soro, em um

laboratório de apoio oferecido pela secretaria municipal de saúde. As amostras processadas foram armazenadas a -20°C e transportadas ao Laboratório de Vírus, ICB/UFMG, onde foram analisadas.

5.1.10. Avaliação Clínica

Durante a coleta das amostras, uma inspeção visual foi realizada para observar a presença de lesões na mucosa oral dos indivíduos. Além disso, foi feita uma inspeção do braço esquerdo daqueles participantes que poderiam ter sido vacinados de acordo com a idade. Considerando a obrigatoriedade da vacinação contra a varíola no Brasil até o ano de 1975 e a erradicação mundial declarada em 1980, estimou-se nesse estudo que indivíduos com idade superior a 40 anos poderiam ter sido vacinados (HOCHMAN *et al.,* 2011).

5.1.11. Metodologia laboratorial

Para avaliação do consumo e manipulação de queijo artesanal e/ou outro derivado lácteo frente à exposição ao VACV em área urbana, as amostras de queijo comercializadas e os swabs de orofaringe da população amostrada foram processados e submetidos à prospecção molecular através da reação em cadeia da polimerase (PCR). A detecção do DNA viral nas amostras foi confirmada por sequenciamento. Algumas das amostras com material genético viral "detectável" na prospecção molecular foram submetidas à isolamento viral em cultivo celular.

Para determinação da soro prevalência e avaliação de exposição prévia aos OPV, o soro dos participantes foi analisado através de duas metodologias laboratoriais distintas: o ensaio de soro neutralização por redução de placas, considerado o padrão ouro para o diagnóstico de infecção por OPV, sendo possível avaliar o grau de proteção do indivíduo (STORCH & WANG, 2013); e ensaio imuno enzimático (ELISA) para detecção de imunoglobulina do tipo G (IgG).

Para detecção de exposição recente a OPV, foi realizado ELISA de captura visando detectar imunoglobulina do tipo M (IgM). Os ensaios imuno enzimáticos foram realizados através de colaboração com o grupo de pesquisa em poxvírus e raiva do *Centers for Disease Control and Prevention* (CDC/Atlanta, USA) (KAREM et al., 2005). Para isso, os soros foram

enviados ao CDC e processados pelo pesquisador Galileu Barbosa Costa, egresso do grupo de pesquisa em Ecologia de Viroses Emergentes (ECOVIR) e coorientador deste estudo.

5.1.12. Processamento do queijo artesanal

As amostras de queijo coletadas foram armazenadas em tubos cônicos estéreis e mantidas a -20 °C. Para extração do DNA, foi realizada uma adaptação dos volumes utilizados no protocolo descrito por Rehfeld et al., 2017 e Oliveira et al., 2017. A maceração foi realizada em beadbeater a partir de 0.1g do queijo em 0.9 mL de solução salina tamponada com fosfato (PBS) 1x. Do produto obtido, 200uL foi submetido à extração de DNA e o remanescente foi armazenado a -20°C.

5.1.13. Processamento do swab orofaríngeo

As amostras de swab foram inseridas em um tubo de 0,6 mL contendo um furo na cavidade inferior, o qual foi acoplado a outro microtubo de 1,5 mL. No microtubo de 0,6 mL contendo o swab foi adicionado 500 uL de PBS 1x para hidratação por 5 minutos. Após esse tempo os microtubos acoplados foram centrifugados. O filtrado agora localizado no tubo de 1,5 mL foi submetido a três ciclos de congelamento e descongelamento, o que favoreceu a lise das células presentes na amostra clínica.

Em seguida, o material foi sonicado sendo submetido a três ciclos de 30 segundos no ultrassom alternados com 30 segundos no gelo. Essa etapa é fundamental no manuseio de amostras para detecção de OPV, pois favorece a homogeneização da amostra desfazendo os agregados de partículas virais frequentemente formados.

5.1.14. Extração de DNA viral

A extração de DNA viral foi realizada utilizando o kit *High Pure Viral Nucleic Acid Kit*, da Roche. Esse kit permite a extração de RNA e DNA viral. Em cada tubo de 1,5 mL foi adicionado 200 uL da amostra, 200 uL da *working solution* e 50 uL de proteinase K, esse material foi homogeneizado em vortex e incubado a 72°C por 10 minutos em thermoblock. Posteriormente, foi adicionado 100 uL de *binding buffer* e homogeneizado. O produto foi transferido para uma coluna com filtro e centrifugado por 1 minuto a 8.000 g. O líquido filtrado foi descartado e foi adicionado 500 uL de inibidor removal buffer e novamente foi centrifugado por 1 minuto a 8.000 g. O líquido foi descartado e, em seguida, foi adicionado 450 uL de wash

buffer, esse material foi centrifugado por 1 minuto a 8.000 g, o líquido descartado e, foi repetida essa etapa de lavagem. O tampão de lavagem foi descartado novamente e o tubo coletor foi submetido a spin em velocidade máxima por 10 segundos. O filtro foi inserido em um novo tubo de 1,5 mL e foi adicionado 50 uL de elution buffer, por fim, foi centrifugado por 1 minuto a 8.000g. O produto obtido foi quantificado, e analisado quanto à pureza da extração, em nanodrop.

5.1.15. Reação em cadeia da polimerase (PCR)

O DNA extraído do swab de orofaringe e de queijo artesanal foi submetido à triagem para a detecção de OPV por biologia molecular. Para isso, foram utilizadas as reações de qPCR e semi-nested PCR para amplificação de um fragmento do gene C11R que codifica o fator de crescimento viral – vgf. Foi utilizado o protocolo descrito por Kroon e colaboradores (2016). Com relação à semi-nested PCR, nas duas etapas de amplificação foram utilizadas as mesmas condições salinas e enzimáticas, descritas a seguir: 2,0 mM de $MgCl_2$, 10 mM de cada nucleotídeo (dNTP), 2U de Taq DNA polimerase (Promega), 2,0 μL de Tampão 10X de Taq polimerase, 4 ρmol dos iniciadores específicos, 1 μL de amostra e 500 ng de albumina sérica bovina (BSA), em um total de 20 μL de reação. As condições térmicas utilizadas para a amplificação na primeira reação foram: um ciclo inicial de 94ºC por 9 minutos, seguido por 30 ciclos de 94ºC por 1 minuto, 45ºC por 1 minuto, 72ºC por 2 minutos, e uma extensão final de 72ºC por 10 minutos. O volume de 1 μL desta 1ª reação foi utilizado como "template" na 2ª reação, cujas condições de amplificação foram: 94ºC por 5 minutos; 30 ciclos de 94ºC por 1 minuto, 45ºC por 1 minuto, 72ºC por 1 minuto, seguido de uma extensão final de 72ºC por 10 minutos.

O DNA extraído foi submetido à técnica de PCR em tempo real para pesquisa de outro alvo viral, capaz de caracterizar o vírus em nível de espécie. O protocolo utilizado na prospecção de VACV em queijo artesanal foi padronizado por Oliveira, DB (dados não publicados). A reação foi desenhada para amplificar um fragmento de 120pb do gene A56R que codifica a hemaglutinina viral (ha). Foi utilizado o seguinte par de primers HA-HRM/F (5'- AAC CAC CGA TGA TGC GGA T -3') e HA-HRM/R (5'-TGC CAC GGC CGA CAA TAT AA -3') capaz de amplificar amostras de VACV-BR do grupo 1 e 2.

As amplificações foram avaliadas e as amostras que apresentaram amplificações detectáveis na curva de amplificação, apresentando a temperatura de dissociação (*tm*) semelhante ao controle positivo (VACV-WR) e com *Ct (Threshold cycle)* abaixo de 38 foram consideradas positivas, naquelas amplificações em que o *tm f*oi divergente, abaixo ou acima de 2ºC do controle positivo, e/ou, o *Ct* foi acima de 38, foram consideradas amplificações

inespecíficas.

5.1.16. Caracterização molecular

Para a caracterização de VACV nesse estudo, a reação utilizada amplifica um fragmento nesse mesmo gene, no entanto permite a caracterização molecular das amostras, sendo possível diferenciar VACV do grupo 1 e do grupo 2 (TRINDADE ET AL., 2008). O protocolo é composto por três reações diferentes que podem ser realizadas sob mesma condição em uma só placa de 48 poços. A combinação das três reações propostas pelo protocolo é capaz de amplificar DNA proveniente de material clínico e distinguir amostras diferentes da espécie VACV. As reações foram preparadas utilizando-se o SYBR green e conduzidas em placas de 48 poços acoplada em termociclador do tipo ABI STEP ONE seguindo os seguintes ciclos: 95°C por 10 minutos; seguido por 40 ciclos de 95°C por 10 segundos e 58°C por 40 segundos. Os swabs testados que apresentaram amplificações com *tm* variável abaixo ou acima de 1ºC, em relação ao controle positivo, ou que apresentaram amplificação acima do *Ct (Threshold cycle)* 38, foi considerado como amplificação inespecífica.

Naqueles participantes do estudo que apresentaram evidência de infecção aguda, ou seja, DNA do VACV detectável em amostras de swab orofaríngeo ou que apresentaram anticorpos do tipo IgM, também foi realizada a PCR em amostras de soro, com o objetivo de verificar a potencial infecção.

5.1.17. Sequenciamento genético

Pela viabilização do sequenciamento genético, agradecemos aos colaboradores do Instituto René Rachou – Fiocruz Minas, que disponibilizaram a realização do sequenciamento em ambos os sentidos com iniciadores específicos utilizados em cada reação de PCR descrita através do método didesoxi descrito por Sanger (SANGER, 1977).

5.1.18. Alinhamento de sequências genéticas

A qualidade das sequências obtidas foi analisada através do SeqTrace 0.9.0. (BRIAN STUCKY *et al.,* 2014). As sequências foram analisadas manualmente utilizando o programa MEGA 7.0 (TAMURA, 2011) e comparadas através do programa BLAST N com sequências depositadas no banco de dados do "*National Center of Biothecnology Information*" (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (ALTSCHUL, 1990).

5.1.19. Isolamento viral

O protocolo de isolamento viral descrito por KROON et al., 2016 foi realizado utilizando monocamadas de células Vero, cultivadas em meio de cultura simples (meio mínimo de Eagle – MEM) (Cultilab, Brasil) e acrescido de soro fetal bovino (SFB) a 5% (Cultilab, Brasil) para o crescimento celular, antimicrobianos estreptomicinas (100 ug/mL), penicilina (100 U/mL) e anfotericina B (2 ug/mL) para inibir o crescimento de bactérias e fungos, em estufa a 37°C suplementada com 5% de CO_2. Também foi utilizado cultivo primário de fibroblastos de embrião de galinha (CEF), cultivado em meio Dulbecco MEM (DMEM) (Cultilab, Brasil), acrescido soro fetal bovino (SFB) a 10% (Cultilab, Brasil) e os mesmos antimicrobianos utilizados no cultivo das células Vero. Para o isolamento do VACV, o remanescente do material processado anteriormente usado na prospecção molecular foi diluído. Do queijo, foram feitas diluições 1:5, 1:10, 1:50, 1:100 e 1:500; Em relação aos swabs, o material obtido após o processamento foi diluído 1:1 em PBS contendo o dobro da quantidade usual de antibióticos. O volume de 800 µL (400 µL por poço) ou 600 µL (330 µL por poço) foi inoculado em sistema celular, submetido à adsorção por 1 hora e mantido com MEM 2% de SFB acrescido do dobro da concentração usual de antibiótico. Cada amostra foi incubada em atmosfera úmida a 5% de CO_2 até o aparecimento do efeito citopático. Caso o efeito citopático não fosse detectado até sete dias, foram feitas passagens subsequentes. Não havendo efeito citopático até a terceira passagem, a amostra foi descartada.

5.1.20. Cultivo celular

Células de linhagem contínua BSC-40 são células epiteliais derivadas do rim de macaco verde africano *(Cercopithecus aethiops*), obtidas da *American Type Culture Collection* (ATCC), Maryland, EUA. As células foram cultivadas em meio de cultura simples (meio mínimo de Eagle – MEM) (Cultilab, Brasil), e acrescido soro fetal bovino (SFB) a 5% (Cultilab, Brasil) para o crescimento celular, antimicrobianos: estreptomicina (100 ug/mL), penicilina (100 U/mL) e anfotericina B (2 ug/mL) para inibir o crescimento de bactérias e fungos, em estufa a 37°C suplementada com 5% de CO_2. O cultivo foi realizado em garrafas apropriadas e, posteriormente, as células foram transferidas para placas de seis poços para a realização do teste de soro neutralização.

5.1.21. Amostra de VACV

Para os testes de soro neutralização foi utilizado o VACV amostra Western Reserve. A amostra VACV-WR foi gentilmente cedida pelo Dr. C. Jungwirth (Universitat Wurzburg, Alemanha) e faz parte da coleção de vírus do Laboratório de Vírus, ICB, UFMG. Para os ensaios imunoenzimáticos foi utilizada a amostra viral VACV-ACAM2000 purificada e amplamente utilizada pelo CDC.

5.1.22. Soros Controles

Como soro controle positivo e negativo foi utilizado um pool de soros pertencentes à soroteca do Laboratório de Vírus da UFMG, previamente testados por ensaio de soro neutralização. Para os ensaios imuno enzimáticos, os soros controles fazem parte da coleção do CDC e foram cedidos por profissionais de saúde, militares e pesquisadores.

5.1.23. Ensaio de soro neutralização por redução de placa (plaque reduction neutralization test – PRNT)

O teste foi desenvolvido segundo o protocolo de Newman (2003), com adaptações. Os soros foram triados em duplicata na diluição de 1:40. Essa diluição foi realizada em meio MEM sem adição de SFB e adicionado volume igual de MEM contendo entre 30 e 300 unidades formadoras de placas (UFP) de VACV-WR. Antes da diluição, as proteínas do sistema do complemento foram desnaturadas através da incubação do soro a 56ºC por 30 minutos em banho-maria. Para o controle de vírus, fundamental para a revelação da técnica, o mesmo processo foi adotado exceto pelo soro diluído, o qual foi substituído por um soro negativo ou SFB. As soluções de soro e vírus foram homogeneizadas e incubadas por aproximadamente 16 horas a 37°C em atmosfera suplementada com 5% CO_2.

Essas soluções foram então inoculadas em placas de seis poços com monocamadas de BSC-40 entre 90 e 100% de confluência para adsorção. Durante esse processo, a placa foi incubada a 37°C em atmosfera de 5% de CO_2 e homogeneizada a cada quinze minutos durante uma hora. Após a adsorção, 2 ml de MEM com 2% de SFB foi adicionado a cada um dos poços, um volume suficiente para que a placa permanecesse por 48 horas a 37°C e suplementada 5% de CO_2 sem que a monocamada desidratasse. Decorridas 48 horas de incubação, as placas foram fixadas com solução de formalina a 10% (formaldeído 37%) e coradas com solução de cristal violeta a 1%. As placas de lise viral foram visualizadas e contadas. A partir da média do número de placas de cada duplicata, foram consideradas positivas as amostras que apresentarem média de redução maior ou igual a 50% (PRNT50), comparadas àquelas encontradas no controle de vírus.

Para a titulação, as amostras de soros positivas foram diluídas serialmente na razão de dois iniciando-se pela diluição de 1:40 até a diluição de 1:5120 e a execução do teste ocorreu conforme mencionado anteriormente. Para calcular o título de anticorpos neutralizantes por mL (unidades neutralizantes por mL – UN/mL), foi dividido 1 mL do volume da solução vírus/soro pelo volume inoculado na monocamada celular e multiplicado esse valor pela última diluição onde foi encontrada redução maior ou igual a 50% do número de placas de lise em relação ao controle de vírus.

5.1.24. Ensaio imunoenzimático (Enzyme Linked Immunosorbent Assay – ELISA-IgG)

As amostras de soro coletadas foram submetidas à triagem para presença de anticorpos IgG anti-OPV pelo método de ELISA, cuja sensibilidade é de 100% e especificidade 88,5% (KAREM *et al.,* 2005). Para isso, foram testados em duplicata, junto a dois controles positivos e cinco controles negativos. Cada placa de 96 poços [Placas de micro titulação de poliestireno de alta ligação (*Thermo Fisher Scientific, USA*)] foi sensibilizada com VACV-ACAM2000 purificado, diluído em tampão carbonato 0.01M (*Sigma-Aldrich®, USA*), e inativado por formaldeído a $1.2x10^5$ UFP/poço. Em cada poço foi adicionado 100 µl da diluição. Posteriormente, a placa permaneceu overnight à temperatura de 4°C, tempo este necessário para adesão das partículas virais ao fundo da placa.

Em seguida, a placa foi lavada novamente com PBS-Tween 0,05%. Após esse tempo, a placa foi bloqueada através da adição de tampão fosfato-salino (PBS) mais 0,05% de Tween 20 (PBS-Tween 0,05%), 5% de leite em pó desnatado, 2% de albumina de soro bovino (BSA) e 2% de soro de cabra. Para cessar a adesão, a placa foi incubada por 30 minutos a temperatura ambiente. Em seguida, a placa foi lavada novamente com PBS-Tween 0,05%. Foram adicionados 100µL do soro teste e soros controles diluídos na razão de 1:100 (2.5 µL de soro a 247.5 µL de solução de bloqueio) a cada poço, seguido de incubação em estufa a 37ºC por 1 hora.

Posteriormente, a placa foi lavada e adicionados 100µL de imunoglobulina caprina anti-IgG humano conjugado a peroxidase (Laboratórios KPL – Gaithersburg, MD) diluído na razão 1:2000. A placa foi incubada por 1 hora a 37°C, depois lavada e foi acrescentado 100µL do substrato TMB (3,3',5,5'-Tetramethylbenzidine) (Laboratórios KPL – Gaithersburg, MD) sob incubação de 4 minutos à temperatura ambiente. A reação será interrompida pela adição de 100µL de solução de parada. A leitura da placa foi realizada no Enspire (Perkim-Elmer, USA) a 450 nm. O ponto de corte (PC) foi determinado pela média dos cinco controles negativos, mais três vezes o desvio padrão. Foi realizada uma normalização, sendo: valor da densidade óptica (DO – PC). Logo, após normalização, qualquer resultado acima de zero foi considerado

positivo. Vale lembrar que a determinação do ponto de corte foi realizada em cada placa.

5.1.25. Ensaio imunoenzimático (Enzyme Linked Immunosorbent Assay – ELISA-IgM)

Para detecção de anticorpos IgM, foi realizado um ELISA de captura (KAREM *et al.,* 2005). Placas de 96 poços (Immulon II) foram incubadas com 100 µl de imunoglobulina caprina anti-IgM humano (*Laboratórios KPL – Gaithersburg, MD*) na diluída 1:800 em PBS (pH 7,4) por 1 hora a 37°C. As placas foram lavadas cinco vezes com PBS-Tween 0,1% e bloqueadas por 30 minutos a 3 horas em temperatura ambiente com a solução de bloqueio composta por PBS-Tween, 0,5% de gelatina, 2% de BSA, 5% de leite em pó desnatado e 2% de soro de cabra. As placas foram lavadas, os soros foram diluídos na razão de 1:50 (5 µL de soro a 245 µL solução de bloqueio) e adicionados.

Prosseguiu-se com incubação por 1 hora a 37°C, seguida de lavagem. O antígeno, VACV-ACAM2000 purificado foi então adicionado a uma concentração de $6.2x10^5$UFP/poço e a placa foi incubada por 1 hora a 37°C. As placas foram lavadas e uma diluição de 1:250 de um fluido ascítico murino hiper imune com anticorpos poli clonais anti-varíola (HMAF, Bangladesh) foi adicionado. As placas foram lavadas e uma diluição de 1:6000 de imunoglobulina caprina anti-IgG murino conjugado com peroxidase (BIO-RAD) foi adicionado e incubado por 30 minutos a 37°C.

A placa foi lavada e foi acrescentado 100 µL do substrato TMB (3,3',5,5'-Tetramethylbenzidine) (*Laboratórios KPL – Gaithersburg, MD*) sob incubação de 8 minutos à temperatura ambiente. A reação será interrompida pela adição de 100 µL de solução de parada. A leitura da placa foi realizada no Enspire (Perkim-Elmer, USA) a 450 nm. O ponto de corte (PC) foi determinado pela média dos cinco controles negativos, mais três vezes o desvio padrão. Foi realizada uma normalização, sendo: valor da densidade óptica (DO – PC). Os resultados > 0,04 foram considerados positivos. Esse teste apresenta 92% de sensibilidade e 100% de especificidade.

6. RESULTADOS E DISCUSSÃO

6.1.1. Detecção do VACV associada ao consumo de leite e derivados em Minas Gerais: Prospecção molecular de VACV em queijo artesanal comercializados

Durante o período de 2015 a 2018, foi amostrado um total de 71 queijos artesanais produzidos em diferentes regiões do estado de Minas Gerais. Um total de 32 amostras (45%) foi proveniente da mesorregião do Jequitinhonha, representando a bacia leiteira do Serro; a mesorregião do triângulo representa 43,6% da origem dos queijos analisados, sendo 31 amostras de queijos produzidos no Alto Paranaíba (5), Araxá (24), Cerrado (1) e Serra do Salitre (1); entre o Triângulo Mineiro e a região oeste do estado têm-se a Serra da Canastra, de onde 3 amostras (4,22%) foram analisadas; representando o noroeste tem-se uma amostra de Carmo do Paranaíba (1,4%), assim como uma amostra de queijo de Itabira (1,4%), região central e uma amostra da Serra da Mantiqueira (1,4%), localizada no Sul de Minas.

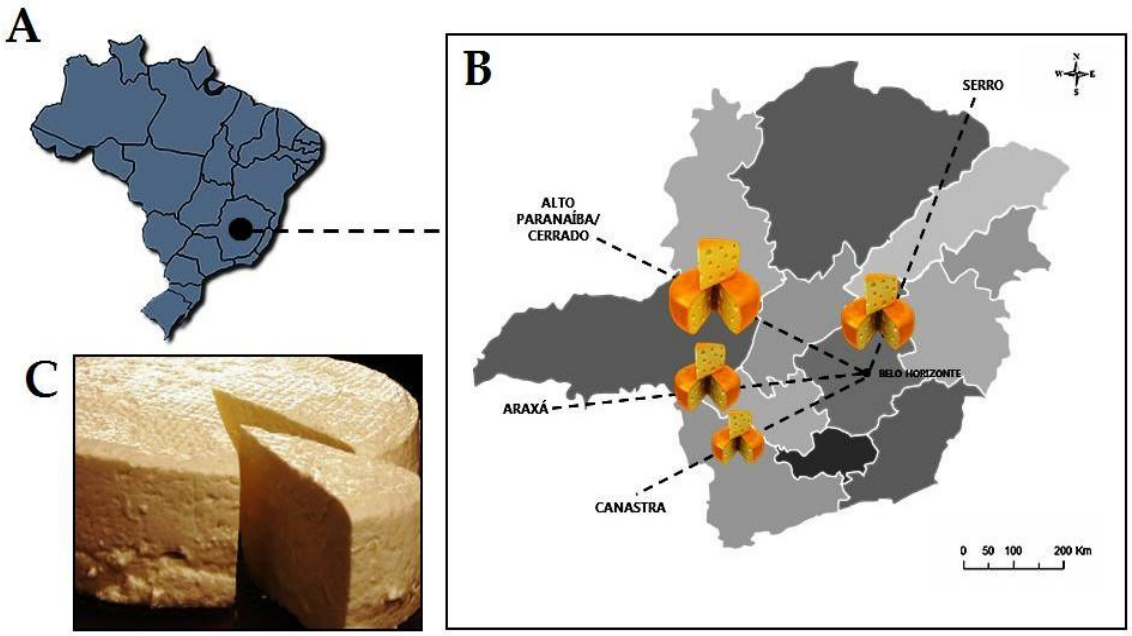


Figura 14. Localização geográfica das bacias leiteiras de Minas Gerais

(A) Mapa do Brasil destacando Minas Gerais e a capital Belo Horizonte. (B) Localização das bacias leiteiras no estado. (C) Queijo artesanal produzido em Minas Gerais. Fonte: Oliveira et al., 2017.

Dentre os 71 queijos amostrados, onze (15,5%) apresentaram o DNA do VACV, quando o gene C11R foi pesquisado. Foram obtidas três sequências de nucleotídeos equivalentes a 157 pb, 119 pb e 171 pb respectivamente, as quais foram alinhadas a sequências homólogas de VACV brasileiros ou outros OPV. Duas dessas sequências foram provenientes de amostras de queijo coletadas no município do Serro, no ano de 2015, durante uma visita para realização do

inquérito populacional. Embora as amostras tenham apresentado alguns SNPs (*single nucleotide polymorphism*) na região analisada, quando comparadas à amostra de VACV-WR utilizada como referência, a análise e interpretação desse dado é limitada pela baixa cobertura obtida no sequenciamento (Figura 15).

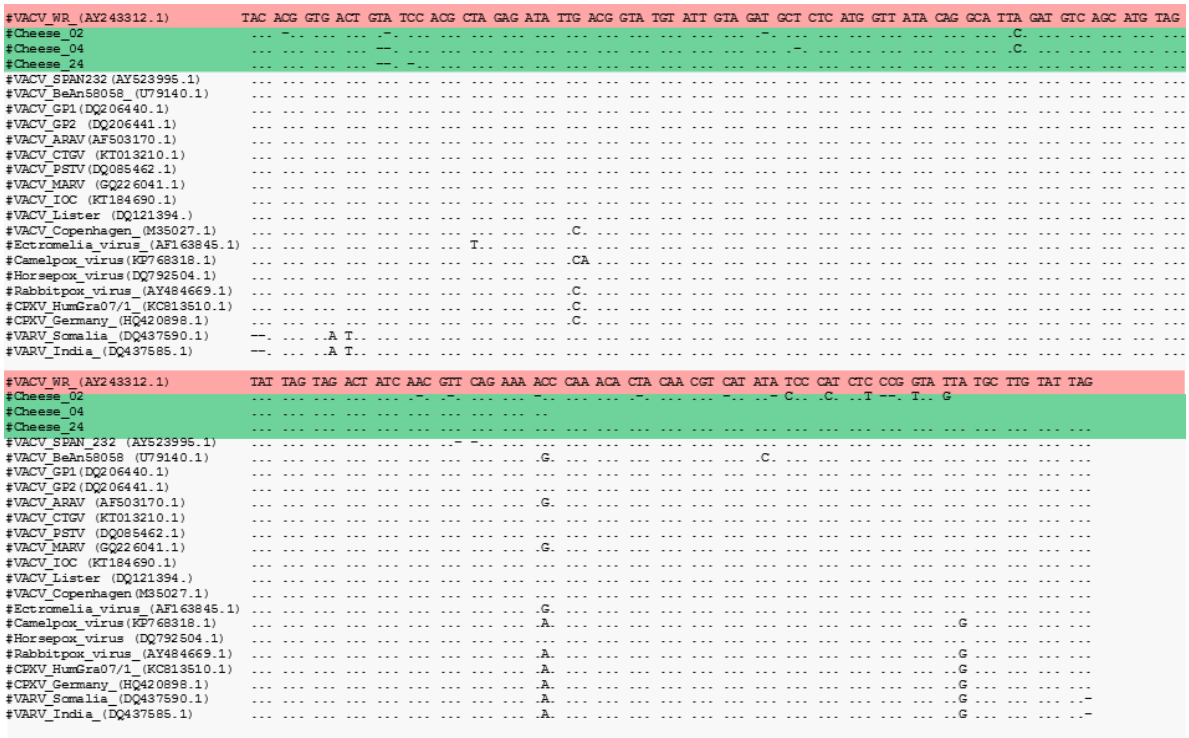


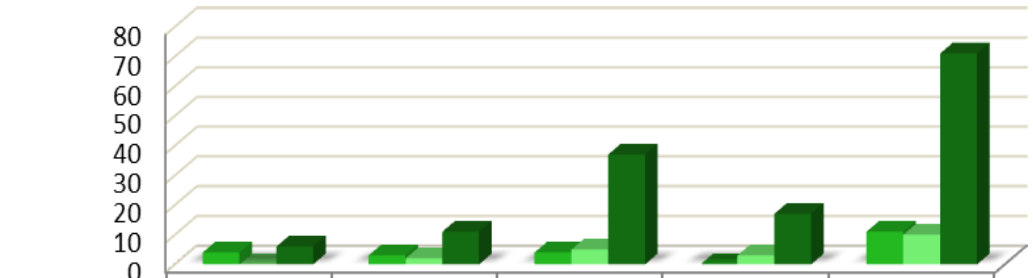
Figura 15. Alinhamento de sequências do gene C11R de três amostras de queijo artesanal comercial (em verde) com sequências homólogas de VACV brasileiros e outros orthopoxvírus

É possível identificar um SNP na mesma posição, compartilhado entre as amostras Cheese 02 e 04, ambas coletadas no Serro em 2015. Os pontos representam bases conservadas, eliminação de traços e letras nas bases divergentes. Os fragmentos foram sequenciados em ambas as orientações pelo método didesoxi na plataforma ABI 3130 (Applied Biosystems, Foster City, CA, USA), a qualidade da sequência foi avaliada utilizando o SeqTrace. Sequências foram alinhadas por ClustalW (http://www.genome.jp/tools/clustalw). Legenda: Cheese 02 (157 pb), 04 (119 pb) e 24 (171 pb) (verde) corresponde às sequências obtidas de queijo artesanal.

Através da prospecção do gene A56R o material genético do VACV foi detectado em dez (14%) queijos que apresentaram amplificações no *tm* esperado para o alvo do gene testado. Alguns queijos testados apresentaram amplificações detectáveis na curva de amplificação, no entanto a temperatura de dissociação foi divergente do controle positivo. Quando essa temperatura variou abaixo ou acima de 2ºC, a amplificação foi considerada inespecífica.

O DNA do VACV foi detectado em queijos artesanais amostrados em todos os anos de coleta e em todas as bacias leiteiras, embora em diferentes períodos. Um dos queijos, produzido no Alto do Paranaíba, apresentou DNA amplificado em ambos os alvos moleculares

pesquisados. Os dados referentes à prospecção do VACV em queijo artesanal são descritos na figura 16.



	2015	2016	2017	2018	Global
C11R+	4	3	4	0	11
A56R+	0	2	5	3	10
N amostral	6	11	37	17	71

Figura 16. Prospecção Do VACV em queijo artesanal comercializado

Um total de 71 amostras de queijo artesanal foi analisado por biologia molecular para prospecção do VACV utilizando dois alvos genéticos distintos. Em 28,1% dos queijos analisados neste estudo foi possível detectar o DNA do VACV. O gráfico descreve o número de amostras coletadas (n) e positivas para os respectivos alvos moleculares testados, de acordo com o ano de coleta da amostra. As amostras de queijo referente ao ano de 2015 foram coletadas durante a visita ao Serro, para amostragem da população deste estudo. Pode-se observar que o VACV foi detectado em 66,6% das amostras coletadas nesse período e local, representando a maior taxa de detecção por período.

O DNA do VACV foi detectado em 28,1% das amostras de queijo coletadas durante o período deste estudo. No ano de 2015 foram amostrados seis queijos, todos provenientes do Serro-MG, sendo amplificado DNA do VACV em 4/6 amostras analisadas (66,6%). As amostras de queijo coletadas no ano de 2015 foram todas provenientes da bacia leiteira do Serro, coletadas durante o estudo epidemiológico, tratando-se, portanto, de queijos frescos, o que poderia ter favorecido a detecção do vírus. Além disso, esse achado chama a atenção para a possível existência de surtos na região, entretanto sem registro de notificação às autoridades sanitárias.

Em 2016 o VACV foi detectado em ¼ queijos do Serro (25%) e 3/6 queijos do triângulo mineiro (50%). Em 2017 obteve-se a maior amostra em relação ao ano de produção do queijo e, além disso, foi amostrada uma maior diversidade de queijos produzidos em regiões distintas de Minas Gerais, assim como em diferentes períodos de maturação, neste ano foi possível detectar o VACV em 4/19 queijos do Serro (21%), 3/12 do triângulo (23%) e 2/3 da Serra da Canastra (66,6%). Em 2018 o DNA do VACV foi detectado em 3/13 queijos do triângulo mineiro (23%) (Figura 17).

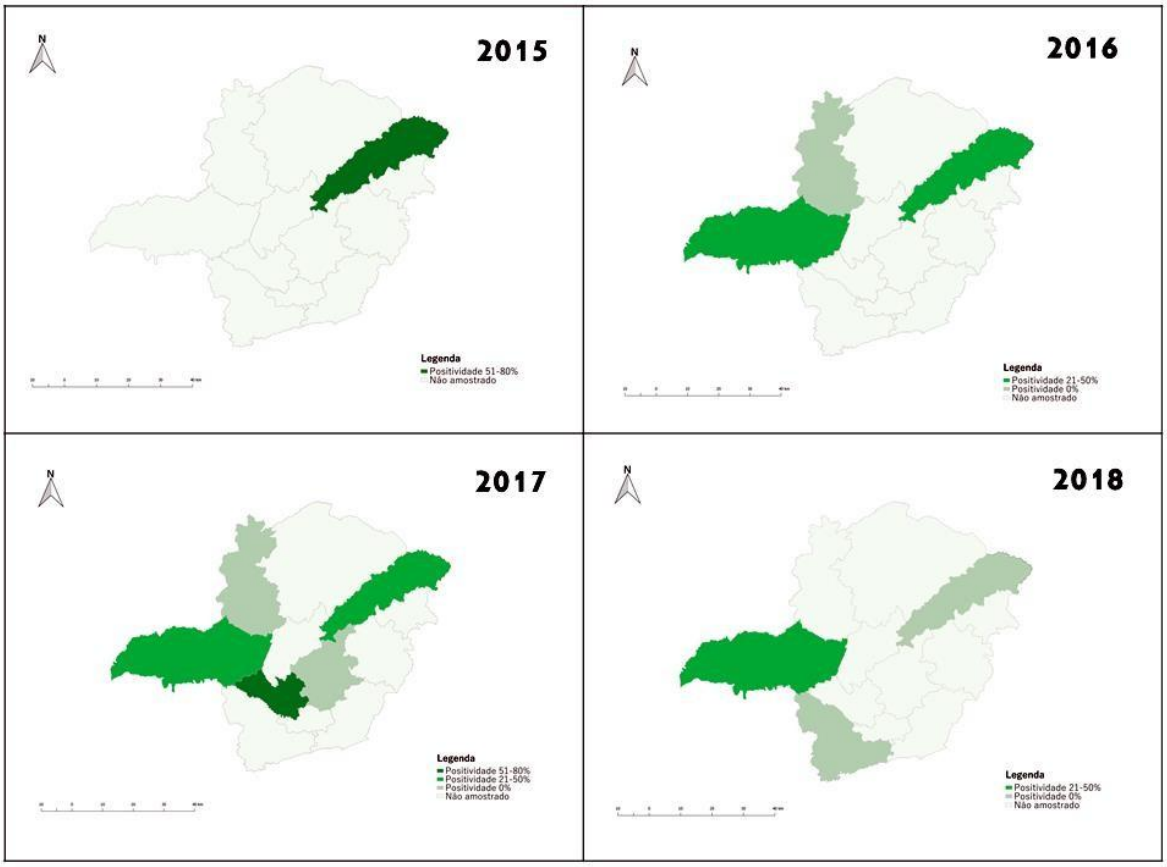


Figura 17. Série histórica da taxa de detecção do DNA do VACV em queijo artesanal produzido em diferentes regiões de Minas Gerais

Quando analisamos o percentual de detecção em relação ao ano de coleta da amostra, é possível identificar uma taxa de detecção molecular decrescente ao longo do período: 66,6% em 2015, 45% em 2016, 24,3% em 2017 e 17,6% em 2018. Entretanto, não há uma normalização do número de amostras analisadas ao longo dos anos e do número de amostras analisadas por região, limitando as inferências. Embora o número de amostras analisadas no ano de 2015 tenha sido o menor em relação aos anos seguintes, neste ano foi identificada a maior taxa de detecção molecular do VACV no queijo artesanal deste estudo. Adicionalmente, todas as amostras coletadas no ano de 2015 foram provenientes da bacia leiteira do Serro, tratando-se de queijos frescos, amostradas no momento de visita ao local para realização do estudo populacional.

A circulação natural do VACV no Brasil influencia diretamente na cadeia produtiva do leite. O estado de Minas Gerais, referência na produção de leite, é inserido na região sudeste, epicentro da circulação do vírus no país. A partir do primeiro relato da contaminação do leite com VACV durante os surtos ocorridos entre 2005 e 2008 em Minas Gerais, uma possível fonte de exposição alternativa ao vírus passou a ser considerada (ABRAHÃO *et al*., 2009C).

Essa hipótese foi reforçada por estudos subsequentes que relataram a eliminação do VACV mesmo na ausência de manifestação clínica no bovino (OLIVEIRA *et al*., 2015). A eliminação do VACV no leite de animais com infecção subclínica dificulta o monitoramento desses alimentos, podendo contribuir para a exposição dos consumidores (REHFELD *et al*., 2017; MATOS *et al*., 2018).

Além do leite, o queijo artesanal produzido com leite *in natura*, também possui representatividade na economia do estado. Minas Gerais tem 7 regiões produtoras de QMA: Araxá, Canastra, Campos das Vertentes, Cerrado, Serra do Salitre, Serro e Triângulo Mineiro, que produzem o queijo com leite cru, pingo (fermento lácteo natural) e sal. A produção de QMA foi registrada como patrimônio imaterial do estado de Minas Gerais. Dessa forma, além da importância econômica, o QMA possui valor cultural e social reconhecido pelo Instituto Estadual do Patrimônio Histórico e Artístico do Estado de Minas Gerais - IEPHA-MG. Outro fator que deve ser considerado é o tradicional uso do pingo no processo de produção de alguns queijos artesanais, que pode vir a favorecer a contaminação dos queijos produzidos.

Sabe-se que a partícula do VACV resiste ao processo de produção de queijos artesanais e, embora o processo de maturação possa dificultar a detecção do vírus, geralmente presente em títulos menores, as partículas do VACV podem ser recuperadas em QMA mesmo após um período extenso de maturação, em torno de 60 dias (OLIVEIRA *et al*., 2010; REHFELD *et al*., 2017). A detecção do DNA do VACV em QMA comercial reforça esses achados experimentais e sinaliza para a possibilidade de introdução do VACV em grandes centros urbanos mediante a comercialização de QMA.

Alguns dos queijos coletados nesse estudo foram adquiridos em diferentes supermercados de Belo Horizonte. Esses supermercados atuam como fontes de distribuição local, na cidade e na região metropolitana. Além disso, esses queijos também são comercializados no Mercado Central de Belo Horizonte, onde as variedades de QMA "Canastra" e "Serro" são os itens laticínios mais procurados (NETTO, 2009).

No entanto, vale destacar que o material genético do VACV foi detectado em queijo produzido em todas as bacias leiteiras de Minas Gerais. Há nove décadas o Mercado Central possui papel significativo no turismo da capital do estado. Portanto, o QMA comercializado nesse centro de referência cultural pode ser encaminhado a inúmeros destinos, seja regional, estadual, nacional e até mesmo internacional (Mercado Central de Belo Horizonte, 2021 -

http://mercadocentral.com.br/sobre/).

No âmbito da saúde pública, a contaminação de QMA por VACV aponta para um risco de exposição aos consumidores. Embora os relatos de infecção natural através do consumo de alimentos contaminados por VACV sejam inexistentes, a exposição de seres humanos ao VACV por essa via deve ser prevenida. Esse é um princípio básico da vigilância em saúde, que visa à prevenção e promoção da saúde. A transmissão do VACV através da ingestão de leite contaminado foi demonstrada em modelo murino, sendo desenvolvida uma infecção assintomática nos animais que integraram o estudo (REHFELD *et al*., 2015).

A amostra viral utilizada nesse estudo experimental corresponde a um VACV brasileiro do grupo 1, cujo perfil de virulência anteriormente demonstrado foi sutil nesses animais (FERREIRA *et al*., 2008; CALIXTO *et al*., 2018). Portanto, a dinâmica da exposição via oral ao VACV ainda é incerta e, sendo assim, a investigação dessa via como possível forma alternativa de transmissão do VACV para humanos faz-se relevante.

A partir da emergência do VACV no Brasil na década de 90, a via clássica de transmissão foi caracterizada como o contato direto entre o ordenhador e o bovino infectado (LOBATO *et al*., 2005; LEITE *et al*., 2005; OLIVEIRA *et al*., 2017A). No entanto, o perfil das propriedades rurais produtoras de leite tem sido padronizado mediante a introdução da ordenha mecanizada. Dentre as práticas da cadeia produtiva do leite, a ordenha mecânica e a inserção de sanitizantes na rotina da ordenha através de dispositivos de imersão foram apontadas como importantes fatores de exposição ao VACV (BORGES *et al*., 2017).

Considerando a ordenha mecânica, dois fatores poderiam contribuir para o risco de exposição ao VACV: a ausência ou inadequação da limpeza aplicada nos extratores mecânicos e /ou a pressão utilizada na sucção dos tetos, que eventualmente poderia lesionar os animais (BORGES *et al*, 2017).

Além dessas características, o compartilhamento de agulhas na administração de hormônios ou outros eventuais fômites poderiam contribuir para a propagação do VACV nos rebanhos, além do próprio trabalhador que atua como um facilitador da transmissão viral (BORGES *et al*., 2017; OLIVEIRA *et al*., 2017A).

Diante do exposto, além de configurar um problema de saúde pública, a demonstração de QMA possivelmente contaminado com VACV pode ser um gargalo para o setor econômico.

O controle de qualidade de alimentos lácteos preconizado pela portaria Nº1305, de 30 de abril de 2013, do Instituto Mineiro de Agropecuária não inclui a pesquisa laboratorial do VACV. Essa alternativa poderia ser inviável na prática devido ao custo agregado.

No entanto, a implementação de estratégias educacionais via intervenção comunitária é uma interessante alternativa visando à prevenção da emergência do VACV nesse contexto. Essa estratégia tem sido aplicada na África, visando o controle de MPXV, pelo Centro de Controle e Prevenção de Doenças (*Centers for Disease Control and Prevention* – CDC), referência mundial em doenças infecciosas emergentes (ROESS *et al*., 2011; REYNOLDS *et al*., 2013; SHIFERAW *et al*., 2017).

Dessa forma, através do mapeamento da cadeia produtiva do leite e do queijo artesanal, podem-se desenvolver medidas educativas que resultarão em mudanças práticas objetivando a prevenção dos surtos de VB nas propriedades e garantindo a inocuidade dos produtos lácteos gerados. A intervenção nas propriedades rurais pode contribuir para a contenção dos casos de VB e prevenir a introdução do VACV em centros urbanos.

Nesse sentido, Costa e colaboradores (2021) propuseram materiais educacionais voltados para indivíduos com residência em áreas rurais (principalmente fazendeiros e leiteiros), fornecendo uma visão geral e informações básicas sobre medidas preventivas contra infecções por VACV que podem melhorar o controle e a prevenção da VB, especialmente para populações vulneráveis localizadas em áreas endêmicas (COSTA et al., 2021).

Neste estudo, o DNA do VACV foi detectado em queijos produzidos na bacia leiteira do Serro. Os produtos da região têm destaque no mercado nacional e internacional e fomentam a economia local. Adicionalmente, a VB é uma doença endêmica nesta região e neste contexto dados importantes sobre a epidemiologia da doença foram descritos anteriormente pelo grupo de pesquisa Ecovir e subsidiaram a escolha desta região produtora de QMA para o desenvolvimento do estudo populacional.

6.1.2. Estudo epidemiológico de base populacional: Perfil demográfico

O estudo epidemiológico de base populacional realizado no município do Serro-MG incluiu 372 participantes amostrados na área urbana. A caracterização demográfica revela que a população estudada é composta por 201 mulheres (54%) e 171 homens (46%). Em relação à

idade, a mediana da idade de todos os participantes foi equivalente a 38,5 anos. Considerando a descontinuidade da vacinação contra a varíola no Brasil a partir do ano de 1978 e a erradicação mundial declarada em 1980, estimou-se nesse estudo que indivíduos com idade superior a 40 anos poderiam ter sido vacinados contra a varíola.

Segundo a etnia, a maioria da população se classifica como pardo (64,2%), seguido de branco (18,6%) e negro (17,2%). Ao analisarmos a escolaridade, a maioria dos indivíduos amostrados possui ensino primário (51%) ou nível médio (43,5%). A população é composta por estudantes (16%), aposentados (12,3%), desempregados (7,8%), prestadores de serviço (48,6%), profissionais de saúde (4,8%) e trabalhadores rurais (7,8%). Pouco mais da metade da população (55,1%) possui renda familiar mensal superior a 1 salário-mínimo (Figura 18).

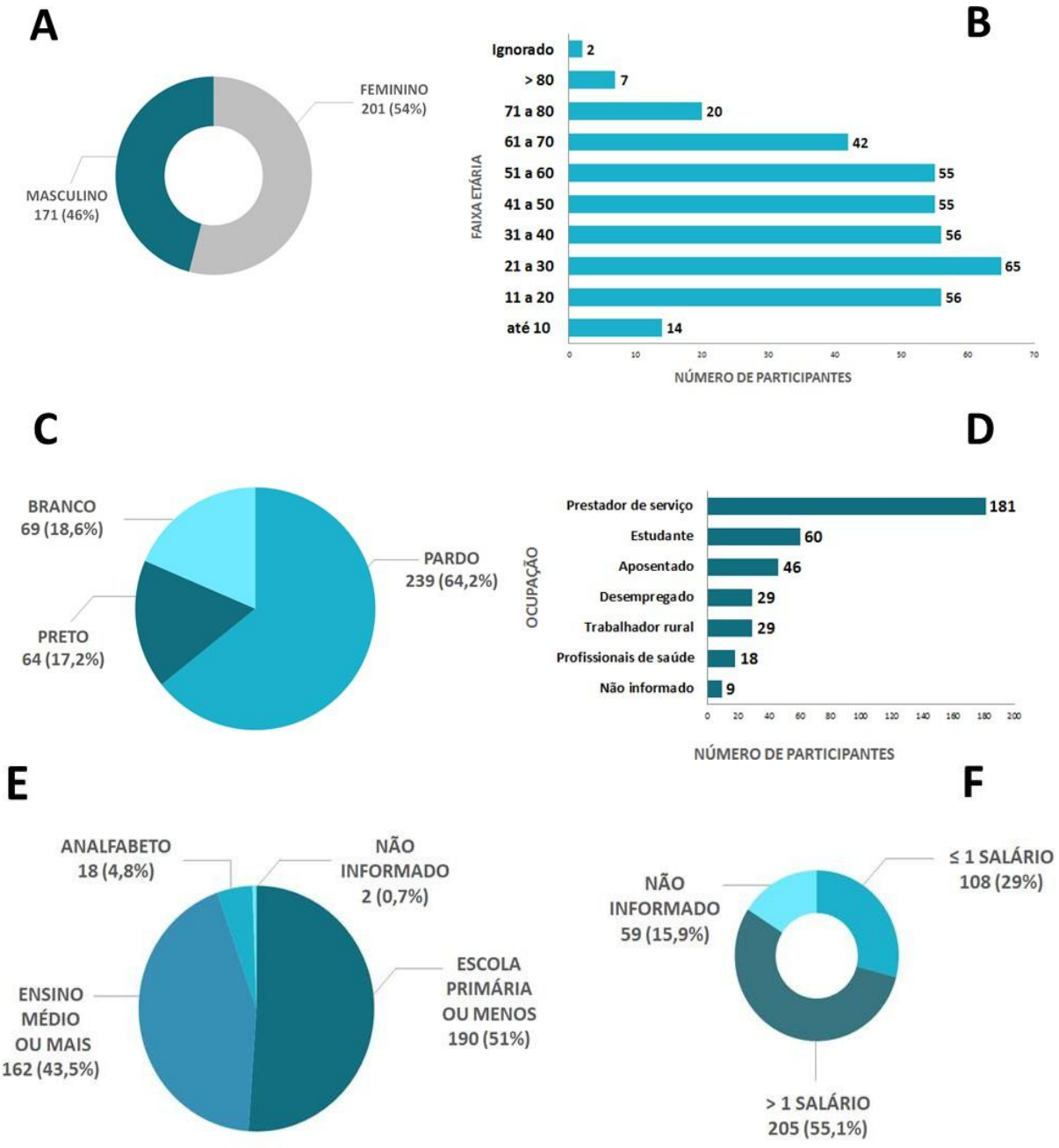


Figura 18. Caracterização demográfica da população participante deste estudo.

A) Distribuição percentual dos participantes de acordo com o gênero; B) Distribuição da população por faixa etária (número absoluto): A média de idade dos participantes é de 40 anos, variando entre 5 e 94 anos de idade; 48,1% do grupo populacional possui idade > 40 anos e foram considerados como possíveis vacinados; C) Classificação da população de acordo com a autodeclaração étnica; D) Ocupação dos participantes do estudo; E) Nível de escolaridade; F) Renda familiar média mensal.

6.1.3. Identificação de exposição humana ao VACV tendo como potencial fonte de exposição o consumo de derivados do leite in natura em Minas Gerais

Nesse estudo, um total de 372 swabs de orofaringe foram coletados durante os anos de 2015, 2017 e 2018; e submetidos à PCR para a detecção do VACV. Para ampliar a detecção, foram utilizados dois alvos na pesquisa por VACV, o gene C11R (pesquisado por qPCR e nested-PCR) e o gene A56R (qPCR), o que possibilitou a detecção do DNA do VACV em 25 amostras de swab orofaríngeo, obtendo-se uma taxa de detecção molecular equivalente a 6,7%. Dentre as amostras com DNA de VACV detectável, 21/25 (84%) foram positivas na prospecção quando o gene C11R foi analisado e quando o alvo analisado foi o gene A56R 10/25 (40%) foram positivas, apresentando amplificações no *tm* esperado para o alvo do gene testado. Em seis amostras com DNA do VACV detectável, foi possível identificar ambos os alvos moleculares pesquisados (6/25 = 24%) (Figura 19).

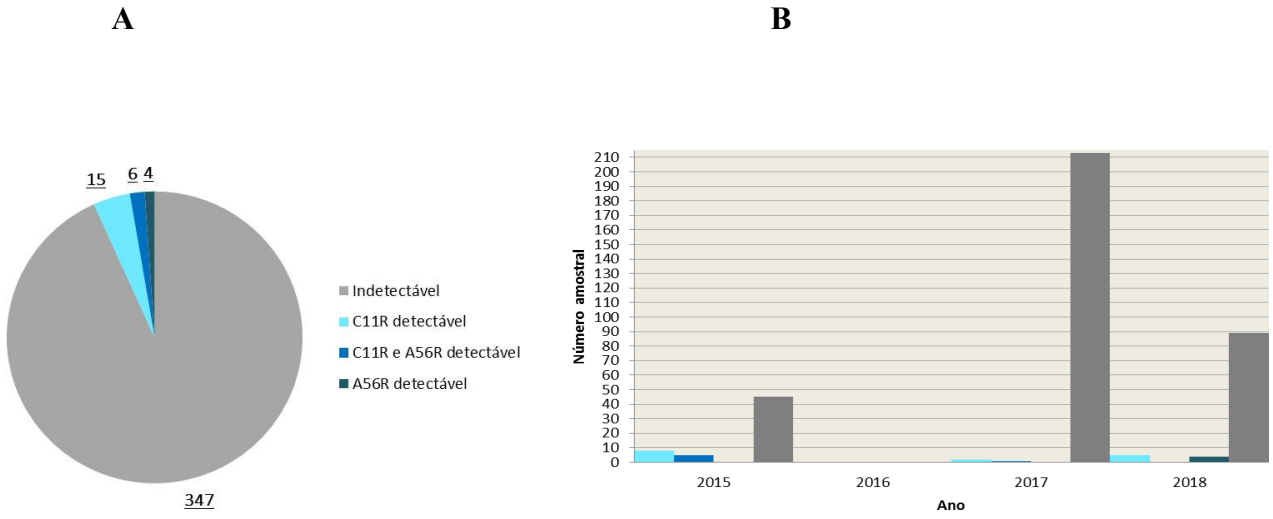


Figura 19. Detecção do DNA do VACV em swab orofaríngeo na população do Serro-MG

A população participante neste estudo corresponde a 372 indivíduos assintomáticos. A) O gráfico demonstra o número absoluto de amostras referente à detecção de cada alvo molecular testado (C11R – azul claro; A56R – verde escuro; ou em ambos os alvos - azul); B) Distribuição do número de amostras e seus respectivos resultados de acordo com o ano de coleta da amostra foram analisados as amostras de swab de 58 participantes amostrados no ano de 2015; 216 amostras referentes a participantes amostrados no ano de 2017; e 98 participantes amostrados em 2018.

Conforme supracitado, a amplificação do DNA do VACV foi identificada neste estudo em 6,7% das amostras coletadas. Entretanto a coleta das amostras ocorreu em três momentos

distintos e foi possível identificar flutuação na taxa de detecção de acordo com o ano de coleta da amostra. Em 2015, o DNA do VACV foi detectado em 22,4% (13/58) das amostras coletadas; em 2017 a taxa foi 1,38% (3/216) e em 2018 identificou-se 9,18% (9/98).

Ao ser analisado separadamente, o ano de 2015 se destaca por apresentar a maior proporção de swabs orofaríngeo (13/58 = 22,4%) e também de queijos artesanais (4/6 = 66,6%); amostrados na região do Serro/Jequitinhonha com DNA do VACV detectável. Esse achado poderia ser associado à ocorrência de surto na região, entretanto não foram encontrados registros publicados que subsidiem tal associação. O detalhamento dos resultados obtidos na prospecção molecular é visualizado na Tabela 3.

Tabela 3 Descritivo das amostras positivas e os respectivos alvos identificados durante a amplificação do DNA do VACV em swab orofaríngeo na população estudada no Serro, Minas Gerais.

Identificação da amostra	Nested-PCR gene C11R	qPCR gene C11R	qPCR gene A56R
SO_05_2015	+	+	+
SO_14_2015	+	-	-
SO_15_2015	+	+	-
SO_19_2015	+	+	-
SO_20_2015	+	-	-
SO_24_2015	+	+	+
SO_26_2015	+	+	-
SO_28_2015	+	+	+
SO_41_2015	+	+	+
SO_50_2015	+	+	-
SO_51_2015	+	+	-
SO_52_2015	+	NT	+
SO_57_2015	+	+	-
SO_113_2017	+	-	-
SO_115_2017	+	+	-
SO_212_2017	+	+	+
SO_223_2018	-	NT	+
SO_224_2018	-	NT	+

SO_225_2018	-	NT	+
SO_251_2018	+	-	-
SO_252_2018	+	-	-
SO_254_2018	+	-	-
SO_286_2018	-	NT	+
SO_307_2018	+	-	-
SO_312_2018	+	-	-

+: positivo; -: negativo; NT: não testado.

Conforme observado na tabela acima, neste estudo, 21/25 amostras de swab oral humano tiveram DNA viral detectável na abordagem nested-PCR para a pesquisa do fragmento do gene C11R. Este gene encontra-se duplicado no genoma dos OPV, o que aumenta a sua chance de detecção como marcador molecular. Outra vantagem deste protocolo consiste na realização de duas reações de amplificação, onde o produto da primeira reação é utilizado como amostra na reação seguinte, o que favorece o aumento da sensibilidade do teste. Além disso, o protocolo padronizado por Abrahão e colaboradores dispensa a prévia extração de material genético, tornando-o um protocolo indicado para a prospecção molecular de OPV. Essas reações foram escolhidas para triagem, pois permitem a rápida detecção de OPV diretamente em espécimes clínicos e, além disso, o alvo genético pesquisado encontra-se duplicado no genoma dos OPV, o que aumenta a sensibilidade de detecção do teste (ABRAHÃO et al., 2010A).

Adicionalmente, a técnica de qPCR foi empregada neste estudo, utilizando a alternativa da pesquisa do gene C11R, que neste caso, foi detectável em 12/25 amostras; e a pesquisa do gene A56R, que detectou o VACV em 10/25 amostras. Esses achados demonstram que a abordagem multialvo, assim como a diversidade de protocolos visando à pesquisa de marcador molecular pode ampliar a possibilidade de prospecção do VACV.

A detecção do VACV em amostras de swab de orofaringe foi confirmada por sequenciamento de nucleotídeos. No alinhamento a seguir, são apresentadas sequências nucleotídicas referente a três amostras que foram positivas em qPCR para o gene A56R, sendo duas de queijo e uma amostra de swab orofaríngeo (Figura 20).

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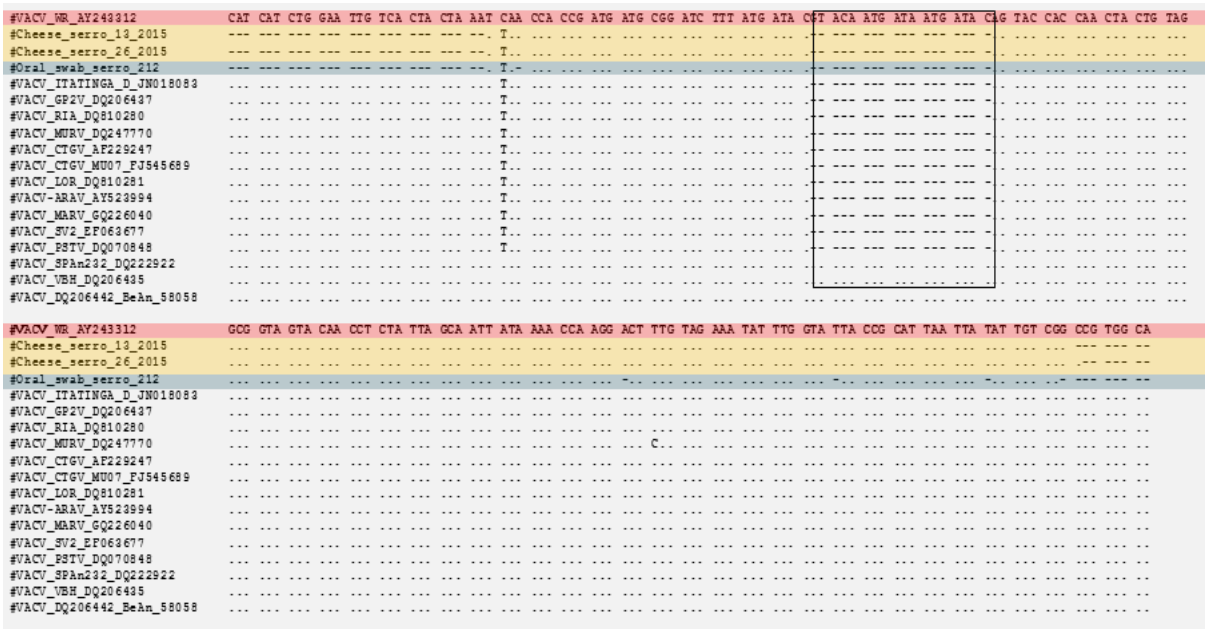


Figura 20. Alinhamento de sequências do gene A56R de três amostras de queijo artesanal comercial (em amarelo) e swab orofanríngeo humano (em azul) com sequências homólogas de VACV brasileiro

Os pontos representam bases conservadas, eliminação de traços e letras nas bases divergentes. Os fragmentos foram sequenciados em ambas as orientações pelo método dideoxi em plataforma ABI3130 (Applied Biosystems, Foster City, CA, USA), a qualidade da sequência foi avaliada utilizando o SeqTrace. Sequências foram alinhadas por ClustalW (http://www.genome.jp/tools/clustalw).

O alinhamento das sequências revelou similaridade com outras sequências de VACV depositadas no GenBank. O alinhamento das sequências obtidas com sequências de referência revela a deleção de 18nt no gene A56R, agrupando as amostras de VACV detectadas em swab de orofaringe e queijo às amostras de VACV do grupo 1 circulantes no Brasil

Sabe-se da co-circulação dos VACV brasileiros. Nesse contexto, foi reportada anteriormente a identificação dos dois diferentes grupos genéticos do VACV-Br na região do Serro. A maioria dos isolados de VACV-Br apresentam a deleção de 18 nt no gene A56R e baixa virulência em modelo murino, características do grupo 1 de VACV-Br. Este grupo de vírus, cuja detecção foi relatada neste estudo, em amostras de swab orofaríngeo de humano assintomático e queijos da bacia leiteira do Serro, foi, anteriormente isolado e/ou detectado em momentos distintos na região, ora associado com a infecção de humanos e bovinos durante um surto em 2005, ora prospectado em animais silvestres amostrados entre 2012 e 2013 (TRINDADE et al., 2009; 2016; MIRANDA et al., 2017); Adicionalmente, o grupo 2 de VACV-Br também foi isolado a partir de humanos e bovinos infectados no Serro, durante um

surto registrado em 2011 (ASSIS et al., 2012).

A caracterização dos participantes analisados nesse estudo e a correlação entre os dados demográficos e a detecção molecular do VACV em orofaringe são descritas na Tabela 4. Nenhuma variável demográfica analisada demonstrou associação estatisticamente significativa com a detecção do DNA do VACV.

Tabela 4 Variáveis demográficas analisadas frente à detecção de DNA do VACV em amostras de orofaringe

Variável demográfica	N (%)	PCR positivo (%)	PCR negativo (%)	Valor de P	ODDS IC*
Gênero					
Feminino	201 (54,0)	13 (6,5)	188 (93,5)	NS	
Masculino	171 (46,0)	12 (7,0)	171 (93,0)		
Idade (anos)					
Até 10	14 (3,8)	1 (7,1)	13 (92,9)		
11 a 20	56 (15)	3 (5,3)	53 (94,7)		
21 a 30*	65 (17,5)	6 (9,2)	59 (90,8)		
31 a 40	56 (15)	2 (3,6)	54 (96,4)	NS	
41 a 50	55 (14,8)	7 (12,7)	48 (87,3)		
51 a 60	55 (14,8)	2 (3,6)	53 (96,4)		
61 a 70	42 (11,3)	1 (2,4)	41(97,6)		
71 a 80	20 (5,3)	0 (0)	20 (100)		
> 80	7 (1,9)	2 (28,5)	5 (71,5)		
Ignorado	2 (0,5)	1 (50)	1 (50)		
Etnia					
Pardo*	239 (64,2)	19 (7,9)	220 (92,1)		
Preto	64 (17,2)	3 (4,7)	61 (95,3)	NS	
Branco	69 (18,6)	3 (4,3)	66 (95,7)		
Nível educacional[a]					
Escola primária ou	190 (51,0)	11 (5,8)	179 (94,2)		
menos*	162 (43,5)	12 (7,2)	150 (92,8)	NS	
Ensino médio ou mais	18 (4,8)	2 (11,1)	16 (88,9)		
Analfabeto	2 (0,7)	0 (0,0)	2 (100,0)		
Não informado					
Ocupação					
Estudante	60 (16,1)	4 (6,7)	56 (93,3)		

Aposentado	45 (12,0)	2 (4,4)	44 (95,6)	
Desempregado	29 (7,8)	1 (3,4)	28 (96,6)	NS
Profissionais de saúde	19 (5,1)	3 (15,8)	15 (84,2)	
Prestador de	179 (48,1)	12 (6,7)	169 (93,3)	
serviço[b]*	32 (8,6)	1 (3,1)	28 (96,9)	
Trabalhador rural	8 (2,1)	2 (25)	7 (75)	
Não informado				
Renda[c]				
≤ 1 salário mínimo	108 (29,0)	12 (11,1)	96 (88,9)	
> 1 salário mínimo*	205 (55,1)	12 (5,8)	193 (94,2)	NS
Não informado	59 (15,9)	1 (1,7)	58 (98,3)	
Total	**372 (100,0)**	**25 (6,7%)**	**347 (93,3%)**	

a: Escola primária ou menos (≤ 8 anos de estudo), Ensino médio ou mais (> 8 anos de estudo); b: Esse grupo inclui profissionais como professor, comerciantes, eletricista, cozinheiro etc.; c: O salário- mínimo no Brasil em 2021 = R$ 1.100,00; * categoria utilizada como referência; NS: não significativo.

Os relatos de prospecção molecular do VACV em orofaringe, associados à infecção natural são escassos. Entretanto, estudos relacionados à prática da vacinação relataram a detecção do VACV em indivíduos submetidos à vacinação nos Estados Unidos (KLOTE *et al.,* 2005; SAVONA *et al.,* 2006; COHEN *et al.,* 2007).

Para avaliar o risco de transmissão do VACV por meio de indivíduo vacinados, Klote e colaboradores (2005) avaliaram 801 amostras de esfregaços de garganta coletadas nas duas semanas após a vacinação de 144 vacinados com Dryvax e não encontraram evidências do VACV por meio da PCR, cultura ou detecção de antígeno.

Cohen e colaboradores (2007) analisaram indivíduos também vacinados com a Dryvax e encontraram o DNA viral detectáveis no sangue desses indivíduos vacinados, mas indetectável em esfregaços orofaríngeos, não sendo possível isolar a partícula viral em nenhuma das amostras analisadas.

Em contraste, Savona e colaboradores (2006) descreveram indivíduos vacinados com Dryvax na Califórnia, cujo DNA do VACV na orofaringe, no curativo da lesão formada no local de aplicação da vacina e no sangue foram avaliados em diferentes momentos. Entre 6 e 8 dias decorridos da vacinação, 16% (12/74) dos indivíduos tiveram DNA detectável em algum tipo de amostra avaliada: em 6/12 participantes, o DNA foi detectável apenas na orofaringe, em

2/12 apenas no sangue; em 1/12 na orofaringe e no sangue; em 1/12 na orofaringe e no curativo; 1/12 no sangue e o curativo aplicado sobre a lesão; e em 1/12 apenas no curativo aplicado no local da vacinação.

Após 20 dias decorridos da vacinação, o vírus permaneceu detectável em 22% dos vacinados (11/51) e a orofaringe permaneceu representando o segundo local amostrado com maior positividade (4/51 = 8%), ficando atrás apenas da detecção no curativo utilizado no local da vacinação (6/51 = 12%) (SAVONA *et al.,* 2006).

Recentemente, o mesmo grupo relatou que em indivíduos vacinados com a Dryvax, que apresentaram o curso clínico esperado resultando no desenvolvimento de vesículas no local da vacinação, o DNA do VACV foi detectável no sangue (5%), na orofaringe (11%) e no curativo em contato com a lesão vacinal (4%); entretanto, o grupo de vacinados que não desenvolveu vesículas, também apresentou o DNA viral detectável no sangue (33%) e na orofaringe (17%), sugerindo a disseminação sistêmica e multiplicação viral na ausência do desenvolvimento de lesões características no local de vacinação (DELACRUZ et al, 2019).

Essa premissa vem sendo discutida no meio científico, onde novas descobertas sugerem que a ausência de lesões, poderia ser indicativa do comprometimento de resposta imune mediada por células T em vacinados, mas não necessariamente relacionada à ausência de multiplicação viral ou produção de anticorpos (KELSO et al., 2005; TREANOR et al., 2006). Nesse contexto, a detecção de DNA do VACV em sangue e na orofaringe de vacinados nos Estados Unidos, na ausência de lesões características da multiplicação viral no local de aplicação da vacina, poderia apoiar esta hipótese (DELACRUZ et al, 2019).

A prospecção molecular do VACV em orofaringe na população urbana do Serro foi realizada com o objetivo de compreender a dinâmica da possível transmissão do VACV através da ingestão de leite *in natura* ou queijos artesanais possivelmente contaminados (Tabela 5). No entanto, para a população analisada não houve correlação estatística significativa entre os fatores de exposição analisados e a detecção do VACV em orofaringe.

Tabela 5. Fatores de exposição analisados frente à detecção de DNA do VACV em amostras de orofaringe

Fator de exposição	N (%)	PCR positivo (%)	PCR negativo (%)	Valor de P ODDS IC
Consumo de leite				
Sim	333 (89,5)	22 (6,6)	311(93,4)	NS
Não	39 (10,5)	3 (7,7)	36 (92,3)	
Consumo de leite *in natura*				
Sim	70 (18,8)	6 (8,5)	64 (91,5)	NS
Não	302 (81,2)	19 (6,3)	283 (93,7)	
Consumo de queijo artesanal				
Sim	333 (89,5)	23 (6,9)	310 (93,1)	NS
Não	39 (10,5)	2 (5,1)	37 (94,9)	
Produção de derivados lácteos				
Sim	39 (10,5)	4 (10,2)	35 (89,8)	NS
Não	333 (89,5)	21 (6,3)	312 (93,7)	
Total	**372 (100,0)**	**25 (6,7%)**	**347 (93,3%)**	

NS: Não significativo.

Embora não tenha sido demonstrada significância estatística entre os fatores de exposição analisados neste estudo e a detecção de DNA do VACV em amostras de orofaringe da população avaliada no Serro, a possibilidade de exposição através do consumo de alimentos lácteos possivelmente contaminados não deve ser descartada. A detecção de DNA do VACV em amostras de swab oral foi descrita por Rehfeld e colaboradores (2015) ao avaliar a infecção experimental em modelo murino através da ingestão de leite contaminado. Esta detecção foi acompanhada de soroconversão nos animais infectados, embora nenhum sinal clínico tenha sido

observado.

Neste estudo populacional conduzido no Serro-MG, a detecção de DNA do VACV em amostras de orofaringe pode ser associada a duas hipóteses principais: (1) Os indivíduos poderiam ser infectados através da ingestão do leite/queijo contaminado, ocorrendo à multiplicação viral na mucosa oral e possibilitando a detecção do VACV na orofaringe. (2) Os indivíduos podem ter sido expostos ao VACV através do consumo do queijo, mas não necessariamente teriam sido infectados. No entanto, cabe esclarecer que outras potenciais formas de exposição não identificadas nesse estudo podem existir.

Para testar as hipóteses deste trabalho, foi realizada a inoculação do material coletado, em monocamada celular, l para a prospecção da partícula infecciosa a partir de algumas amostras de queijo artesanal e lavado do swab orofaríngeo que apresentaram DNA do VACV detectável neste estudo. Para isso, foram selecionadas algumas amostras que apresentaram menores valores de *ct* nos ensaios de qPCR. No entanto não foi possível visualizar o aparecimento de placas de lise, indicativo da multiplicação viral do VACV. Desta forma, após a realização de três passagens cegas em cultivo de célula Vero e CEF, na ausência de confirmação visual do aparecimento do efeito citopático, o material foi descartado.

Neste estudo, a ausência de isolamento da partícula infecciosa do VACV em amostras de orofaringe humana ou de queijo, limita as inferências acerca da hipótese de infecção dos indivíduos. Entretanto, cabe destacar que neste estudo o número de amostras submetidas à isolamento viral foi um limitante. A seleção das amostras foi atrelada aos menores valores de *ct* observados nos ensaios de qPCR, o que seria indicativo de uma maior carga viral e possivelmente aumentaria a chance de isolamento.

A população avaliada no Serro foi assintomática no momento da amostragem. No entanto, a dinâmica associada à via oral de exposição ao VACV ainda é desconhecida e o desfecho clínico em humanos não foi relatado no Brasil até o momento. O único relato de lesão oral possivelmente associada ao consumo de leite contaminado foi descrito na Índia. As lesões foram detectadas na região oral em 17 indivíduos (10,2%) e foram acompanhadas de sintomas clássicos da infecção por VACV-like. No entanto, é importante ressaltar que, esses casos foram descritos em um contexto de um surto de BPXV, quando geralmente têm-se uma alta carga viral, diferente do contexto no qual a população do Serro foi avaliada (GURAV *et al.,* 2011).

Sabe-se que o vírus é eliminado no leite de vacas infectadas, mesmo em casos de

infecção subclínica, fato que dificulta o monitoramento sanitário desses alimentos, sendo a estratégia de educação em saúde, uma alternativa aplicável para promoção da redução da circulação viral através da conscientização dos produtores rurais (ABRAHÃO *et al.,* 2009C; REHFELD *et al.,* 2017; 2018; OLIVEIRA *et al.,* 2017; MATOS *et al.,* 2018).

Para análise da exposição oral ao VACV, deve-se considerar a carga viral possivelmente contida no alimento contaminado, a estabilidade da partícula nesses alimentos, a resistência da partícula viral quando entra em contato com as enzimas do sistema digestivo e o sítio primário de multiplicação viral. Entender a interação entre esses fatores e o VACV será fundamental para compreender o desfecho associado à exposição ao vírus por essa via alternativa.

Dentre os fatores citados, a presença e estabilidade da partícula do VACV em leite e queijo artesanal foram exploradas em outros estudos. No leite, foi demonstrado através de contaminação laboratorial que, embora haja diminuição na carga viral, o VACV pode resistir à pasteurização (OLIVEIRA *et al.,* 2010), processo comumente realizado, principalmente no interior de Minas Gerais, como no Serro, onde o consumo de leite *in natura* ou pasteurizado ainda é amplamente difundido, enquanto o consumo de leite UHT (*ultra high temperature*) é menos frequente. Dessa forma, o risco de exposição ao VACV através da ingestão de leite torna-se notável.

A produção laboratorial de queijo artesanal utilizando leite contaminado demonstrou a resistência das partículas do VACV ao processamento e manutenção a 4°C (OLIVEIRA *et al.,* 2010). É importante destacar que, partículas virais foram detectadas tanto no queijo produzido, quanto no soro, popularmente conhecido como *"pingo"*. No queijo, as proteínas e células somáticas poderiam atuar como fatores mecânicos que facilitariam a manutenção do vírus, dificultando a degradação das partículas virais (OLIVEIRA *et al.,* 2010; 2015).

Outro achado interessante é a manutenção da viabilidade do VACV considerando diferentes intervalos de maturação dos queijos artesanais. Foi demonstrado que o vírus pode resistir no queijo submetido à maturação por até 45 e 60 dias a 25°C (REHFELD *et al.,* 2017). Esse extenso intervalo no qual o VACV permanece viável nos queijos artesanais aumenta a oportunidade de exposição dos consumidores e torna inviável a revisão do período de maturação dos queijos como possível medida de intervenção frente ao risco de exposição oral. Diante disso, poderia ser considerado o papel do leite e derivados possivelmente contaminados como fonte de exposição ao VACV. A iniciativa de investigar a presença do vírus

na mucosa oral é fundamental para a composição da cadeia epidemiológica envolvendo alimentos possivelmente contaminados. A detecção molecular do VACV na orofaringe pode subsidiar a hipótese de rotas alternativas de transmissão do vírus. Para tanto, faz-se interessante conhecer o curso clínico decorrente da exposição oral ao VACV em humanos.

A ingestão do VACV poderia expor o vírus, inicialmente a enzimas e ao pH ácido da mucosa oral, geralmente inferior a 4,5. Não se tem dados referentes à resistência do VACV nessas condições; no entanto, Essbauer e colaboradores (2007) descreveram a manutenção do título viral em pH levemente ácido (pH 4,5 a 5,7) em águas pluviais não estéreis armazenadas em 4ºC durante 56 dias, quando a amostra de água levemente ácida foi suplementada com proteínas (soro fetal bovino) e mantidas na faixa de temperatura, o OPV foi detectável por até 166 dias. Em superfícies de alimentos, como salada, pão e salsichas, o VACV permaneceu viável por 14 dias.

Considerando os dados descritos nessa tese, surgem inúmeras perspectivas a fim de entender a dinâmica da exposição e possível infecção via oral pelo VACV. Para auxiliar no esclarecimento a cerca destes achados, a pesquisa por marcadores sorológicos de infecção recente (IgM e anticorpos neutralizantes) ou mesmo a detecção do DNA viral no soro são fundamentais para subsidiar a discussão referente a estes dados. Isso poderá esclarecer o papel que a ingestão de alimentos contaminados pode vir a representar no ciclo epidemiológico do VACV no Brasil.

6.1.4. Prevalência de anticorpos anti-OPV na população urbana do Serro-MG

Para detectar a presença de anticorpos anti-OPV foram realizadas diferentes técnicas sorológicas com o objetivo de realizar uma análise mais robusta em relação à estimativa da prevalência de diferentes tipos/classes de anticorpos na população analisada. Dentre os 372 participantes, 63 apresentaram anticorpos neutralizantes anti-OPV detectáveis em PRNT50, o que representa uma prevalência global de 16,9% (IC 95% = 13.45 – 21.09). O título de anticorpos neutralizantes variou de 100 a 800 UN/mL.

A prevalência entre os indivíduos classificados como “vacinados”, de acordo com o critério da idade superior a 40 anos, foi 28,5% (IC 95% = 22.37 – 35.52). Entre os “não vacinados”, ou seja, com idade inferior a 40 anos, essa taxa foi ligeiramente menor, equivalente

a 6,3% (IC 95% = 3.52 – 10.76) de indivíduos com anticorpos neutralizantes anti-OPV detectáveis (p<0.0001).

Quando a idade desses indivíduos é analisada, tem-se para os indivíduos com anticorpos neutralizantes detectáveis, a mediana da idade corresponde a 57 anos, variando entre 15 e 88 anos, enquanto, os indivíduos sem anticorpos neutralizantes apresentam a idade mediana equivalente há 36 anos, variando entre 5 e 94 anos, sendo identificada significância estatística associada à detecção de anticorpos em indivíduos pertencentes a uma maior faixa etária. Através da análise *Odds ratio*, identificamos que os participantes com faixa etária acima de 40 anos de idade apresentaram aproximadamente seis vezes mais chance de apresentar anti-OPV detectável, em relação aos participantes com idade inferior a 40 anos (Tabela 6).

Tabela 6. Variáveis demográficas associadas à presença de anticorpos neutralizantes anti-OPV na população urbana do Serro-MG.

Variável demográfica	N (%)	PRNT positivo (%)	PRNT negativo (%)	Valor de P	Odds IC*
Gênero					
Feminino	201 (54,0)	32 (15,9)	169 (84,1)	NS	
Masculino	171 (46,0)	31 (18,1)	140 (81,9)		
Idade (anos)					
≤ 40 anos	191 (51,3)	12 (6,3)	179 (93,7)		5.94
> 40 anos	179 (48,1)	51 (28,5)	128 (71,5)	<0.0001	(2.96 - 12.7)
Não informado	2 (0,6)	0 (0,0)	2 (100,0)		
Etnia					
Pardo*	239 (64,2)	39 (16,3)	200 (83,7)		
Preto	64 (17,2)	10 (15,6)	54 (84,4)	NS	
Branco	69 (18,6)	14 (20,3)	55 (79,7)		
Nível educacional[a]					
Escola primária ou	190 (51,0)	32 (16,8)	158 (83,2)		
menos*	162 (43,5)	28 (17,3)	134 (82,7)	NS	
Ensino médio ou mais	18 (4,8)	3 (16,6)	15 (83,4)		
Analfabeto	2 (0,7)	0 (0,0)	2 (100,0)		
Não informado					
Ocupação					

Aposentado[I]	46 (12,3)	16 (34,8)	30 (65,2)		3.32
Trabalhador rural[I]	29 (7,8)	10 (34,5)	19 (65,5)		(1.46 – 7.36)
Prestador de serviço[b]*	181 (48,6)	25 (13,8)	156 (86,2)	<0.05	
Desempregado	29 (7,8)	4 (13,8)	25 (86,2)		3.28
Estudante	60 (16,1)	6 (10,0)	54 (90,0)		(1.20 - 8.43)
Profissionais de saúde	18 (4,8)	1 (5,5)	17 (94,5)		
Não informado	9 (2,6)	1 (11,1)	8 (88,9)		
Renda[c]					
≤ 1 salário mínimo	108 (29,0)	24 (22,2)	84 (77,8)		
> 1 salário mínimo	205 (55,1)	37 (18,0)	168 (82,0)	NS	
Não informado	59 (15,9)	2 (3,4)	57 (96,6)		
Total	**372 (100,0)**	**63 (16,9)**	**309 (83,1)**		

a: Escola primária ou menos (≤ 8 anos de estudo), Ensino médio ou mais (> 8 anos de estudo); b:Esse grupo inclui profissionais como professor, comerciantes, eletricista, cozinheiro etc.; c: O salário-mínimo no Brasil em 2021 =R$ 1.100,00; * categoria utilizada como referência; Ɨ fator de exposição associado à presença de anticorpos anti-OPV; NS: não significativo.

Adicionalmente à idade, a ocupação como "aposentado" e "trabalhador rural" também demonstrou uma associação estatisticamente significativa ao desfecho de apresentação de anticorpos neutralizantes detectáveis. Esse resultado é esperado, visto que a categoria de "aposentados" agrupa os indivíduos com a maior faixa etária e inclui indivíduos possivelmente vacinados contra a varíola. Considerando que a vacinação contra a varíola no Brasil ocorreu até o ano de 1975 e a erradicação mundial da doença foi declarada em 1980, estimando-se, portanto, nesse estudo que indivíduos com idade superior a 40 anos poderiam ter sido vacinados. Na categoria de trabalhador rural, os indivíduos com anticorpos neutralizantes também possuem idade superior a 40 anos (Tabela 7) e, por atuarem em ambiente rural onde os surtos de VACV são descritos, podem ser mais expostos ao vírus.

Tabela 7. Distribuição dos participantes de acordo com a ocupação e detecção de anticorpos neutralizantes anti-OPV; e descritivo de outras variáveis demográfica/fatores de exposição de interesse.

Ocupação		N	PRNT +	PRNT -	IgM/IgG	PCR	Idade	Take	Bairro	Aninais	Leite cru	Queijo	Produção	Ordenha
Trabalhador da Saúde	Acompanhante hospitalar	1	0	1										
	Agente de combate a endemias (ACE)	1	0	1										
	Agente de saúde	4	0	4										
	Assistente social	1	0	1										

	Enfermeiro	3	0	3										
	Farmacêutico	1	0	1										
	Fisioterapeuta	1	0	1										
	Técnico de enfermagem	**7**	**1**	**6**	Negativo	Negativo	22	Não	Morro de areia	Sim	Não	Sim	Não	Não
	Açougueiro	1	0	1										
	Auxiliar de cozinha	1	0	1										
	Cozinheira	**4**	**1**	**3**	Negativo	Negativo	53	Sim	Nossa Senhora Aparecida	Sim	Não	Sim	Não	Não
	Padeiro	1	0	1										
	Advogada	2	0	2										
	Artista/comunicadora	3	0	3										
	Atendente / Recepcionista / Secretária	**5**	**1**	**4**	Negativo	Negativo	26	Não	Leiteiro	Sim	Sim	Sim	Não	Não
	Autônomo	9	0	9										
	Babá	3	0	3										
	Balconista	**8**	**1**	**7**	Negativo	Negativo	24	Não	Leiteiro	Não	Sim	Sim	Não	Não
	Bancária	1	0	1										
	Barbeiro/Cabelereira	**3**	**1**	**2**	IgG positivo	Negativo	73	Sim	Praia	Sim	Não	Sim	Não	Não
	Caixa	1	0	1										
	Carpinteiro	**2**	**1**	**1**	Negativo	Negativo	49	Sim	Morro da páscoa	Sim	Sim	Sim	Não	Não
	Comerciante	5	0	5										
	Cuidador de idoso	3	0	3										
	Eletricista	**2**	**1**	**1**	Negativo	Positivo	30	Não	Gambá	Sim	Sim	Sim	Sim	Não
	Empacotadora	1	0	1										
	Engenheiro	1	0	1										
	Estofador	1	0	1										
	Gerente Comercial	1	1	0										
Prestador de Serviço	Funcionário Público	2	0	2										
	Mecânico	**5**	**1**	**4**	Negativo	Negativo	50	Sim	Rosário	Sim	Sim	Sim	Não	Não
	Motorista	**6**	**2**	**4**	Negativo	Negativo	62	Não	Morro do Vigário	Sim	Sim	Sim	Não	Não
					IgM e IgG positivo	Negativo	63	Sim	Milho Verde	Sim	Não	Sim	Não	Não
	Operador de equipamento de mineração	1	0	1										
	Professor	**5**	**2**	**3**	IgG positivo	Negativo	59	Não	Centro	Sim	Não	Não	Não	Não
					Negativo	Negativo	67	Sim	Gambá	Sim	Sim	Sim	Sim	Não
	Vendedor	4	0	4										
	Ajudante	2	0	2										
	Auxiliar (cabelereiro, pedreiro, serviços gerais)	**23**	**1**	**22**	Negativo	Negativo	52	Não	Lazaredo	Não	Não	Sim	Não	Não
	Descarregador de caminhão	2	0	2										
	Doméstica	**46**	**8**	**38**	Negativo	Positivo	45	Sim	Centro	Sim	Não	Não	Não	Não
					Negativo	Negativo	38	Não	Morro da páscoa	Não	Não	Sim	Não	Não
					Negativo	Negativo	51	Não	Morro do Vigário	Não	Não	Sim	Não	Não
					Negativo	Negativo	63	Não	Morro do Vigário	Não	Não	Sim	Não	Não
					Negativo	Negativo	58	Sim	Morro do Vigário	Sim	Sim	Sim	Não	Não
					Negativo	Negativo	53	Sim	Morro do centenário	Sim	Não	Sim	Não	Não
					IgG positivo	Negativo	56	Sim	Morro do vento	Não	Não	Sim	Não	Não
					IgG positivo	Negativo	63	Sim	Machadinho	Não	Não	Sim	Não	Não

	Frentista	1	0	1											
	Pedreiro/Pintor	**20**	**4**	**16**	Negativo	Negativo	52	Sim	Morro da páscoa	Sim	Não	Sim	Não	Não
					Negativo	Negativo	61	Não	Bela Vista	Sim	Não	Sim	Não	Não
					Negativo	Negativo	64	Sim	Morro da páscoa	Não	Sim	Sim	Não	Não
					Negativo	Negativo	57	Sim	Morro da páscoa	Não	Não	Sim	Não	Não
	Técnico de manutenção	3	0	3										
	Vigilante	1	0	1										
Aposentado		**45**	**16**	**29**	Negativo	Negativo	64	Não	Centro	Não	Não	Sim	Não	Não
					IgG positivo	Negativo	83	Não	Centro	Não	Não	Não	Não	Não
					Negativo	Negativo	79	Sim	Leiteiro	Sim	Não	Sim	Não	Não
					Negativo	Positivo	83	Não	Gambá	Não	Não	Sim	Sim	Não
					IgG positivo	Negativo	78	Não	Gambá	Sim	Sim	Sim	Sim	Não
					Negativo	Negativo	88	Não	Praia	Sim	Não	Sim	Não	Não
					Negativo	Negativo	67	Não	Praia	Sim	Não	Sim	Não	Não
					Negativo	Negativo	87	Não	Morro do vigário	Sim	Não	Sim	Sim	Sim
					IgG positivo	Negativo	66	Não	Morro de areia	Sim	Não	Sim	Não	Não
					Negativo	Negativo	63	Não	Machadinho	Sim	Não	Sim	Não	Sim
					Negativo	Negativo	60	Sim	Boa Vista Lajes	Sim	Não	Sim	Não	Não
					Negativo	Negativo	72	Não	Centro	Não	Não	Sim	Não	Não
					Negativo	Negativo	73	Não	Praia	Não	Não	Sim	Não	Não
					IgM Positivo	Negativo	62	Não	Lucas	Sim	Não	Sim	Não	Não
					Negativo	Negativo	42	Sim	Machadinho	Não	Não	Não	Não	Não
					IgG positivo	Negativo	78	Não	Arraial de baixo	Sim	Não	Sim	Não	Não
Desempregado	Desempregado	7	0	7										
	Do lar	**22**	**4**	**18**	Negativo	Negativo	34	Não	Leiteiro	Sim	Não	Sim	Sim	Sim
					Negativo	Negativo	45	Não	Leiteiro	Sim	Sim	Sim	Não	Não
					IgG positivo	Negativo	75	Não	Morro da páscoa	Não	Não	Sim	Não	Não
					IgG positivo	Negativo	71	Não	Centro	Não	Não	Sim	Não	Não
Estudante		**60**	**6**	**54**	Negativo	Negativo	17	Não	Centro	Sim	Não	Não	Não	Não
					Negativo	Positivo	15	Não	Leiteiro	Sim	Não	Sim	Não	Não
					Negativo	Positivo	17	Não	Gambá	Sim	Sim	Sim	Não	Não
					Negativo	Negativo	17	Não	Cidade Nova	Sim	Sim	Não	Não	Não
					Negativo	Negativo	15	Não	Centro	Sim	Sim	Não	Sim	Sim
					Negativo	Negativo	21	Não	Cidade Nova	Não	Não	Não	Não	Não
Trabalhador Rural	Engenheiro agrônomo	1	0	1										
	Produtor rural / Pecuarista	**3**	**2**	**1**	IgG positivo	Negativo	63	Não	Centro	Sim	Não	Sim	Não	Não
					Negativo	Negativo	69	Não	Rancho St Antônio	Sim	Sim	Sim	Sim	Sim
	Lavrador	**18**	**5**	**13**	IgG positivo	Negativo	62	Não	Morro do vigário	Sim	Não	Sim	Sim	Não
					Negativo	Negativo	49	Sim	Machadinho	Sim	Sim	Sim	Não	Sim
					Negativo	Negativo	48	Sim	Arraial de baixo	Não	Não	Sim	Não	Não
					Negativo	Negativo	54	Sim	Lajeado	Sim	Não	Sim	Não	Não
					IgG positivo	Negativo	56	Não	Fazenda	Não	Não	Sim	Não	Sim
	Trabalhador rural	**10**	**4**	**6**	IgG positivo	Negativo	52	Sim	Nova Vista	Não	Não	Sim	Sim	Não
					Negativo	Negativo	46	Não	Morro do vento	Não	Não	Sim	Não	Não
					IgG positivo	Negativo	54	Sim	Córrego da Prata	Sim	Não	Sim	Sim	Sim

				Negativo	Negativo	64	Não	Morro de areia	Sim	Sim	Sim	Não	Sim
Não informado	8	0	8										
Total	372	63	309										

Cinza: indivíduos com idade inferior a 40 anos (não vacinados), mas que apresentaram anticorpos neutralizantes anti-OPV detectável em PRNT50.

A prevalência de anticorpos neutralizantes anti-OPV detectada na população residente em área urbana do Serro é menor do que a prevalência descrita para a população rural. O estudo transversal conduzido na zona rural do município amostrou 240 participantes nos anos de 2012 e 2013 e revelou uma soro prevalência de 30,8% (COSTA *et al*., 2016). De fato, é esperada uma soro prevalência menor na população urbana, visto que os surtos causados pelo VACV até o momento têm sido descritos em populações rurais (TRINDADE *et al*., 2009; 2016; ASSIS *et al*., 2012; COSTA *et al*., 2015; 2016).

Sabe-se que, alguns fatores são fundamentais na dinâmica da circulação do VACV no Brasil, dentre eles, o papel dos bovinos como amplificadores virais. A presença dos bovinos na propriedade rural, sendo infectados e eliminando uma alta carga viral nas fezes, pode favorecer a infecção de outros hospedeiros (GUEDES *et al*., 2013; MATOS *et al*., 2018). Nesse contexto, os animais silvestres também são peças importantes, pois poderiam colaborar na manutenção de um ciclo silvestre da circulação do vírus, que, eventualmente, poderia ser carreado entre esses dois ambientes através de roedores peridomiciliares (ABRAHÃO *et al*., 2009A; 2009B; MIRANDA *et al*., 2017).

Além disso, a soro prevalência detectada em nosso estudo é menor do que a relatada em residentes de um assentamento rural na região amazônica (27,9%). Assim como para a população rural do Serro, os indivíduos amostrados nesse estudo conduzido na Amazônia são residentes de assentamento rural localizado próximo ao ambiente silvestre, fatores que poderiam aumentar a chance de exposição dessa população ao VACV (MOTA *et al*., 2010).

Quando se compara a soro prevalência dos residentes em área urbana do Serro com um estudo retrospectivo conduzido por Figueiredo e colaboradores (9,8%), a prevalência de anticorpos neutralizantes no Serro é maior. O estudo de Figueiredo e colaboradores avaliou duas populações distintas no Brasil e foi amostrado antes da emergência dos surtos de VB no país. Portanto, essa população apresenta um perfil temporal diferente da população urbana do Serro, que, embora seja composta de residentes em área urbana, estão inseridos em uma área

endêmica para a circulação do VACV (FIGUEIREDO *et al*., 2015).

Em relação a anticorpos do tipo IgG, estes foram detectados em 26 indivíduos, resultando em uma prevalência global de 7% (IC 95% = 4.817 - 10.16) (Tabela 8). Dentre os "vacinados" segundo o *cut off* da idade, a prevalência corresponde a 12,3% (IC 95% = 8.195 - 17.97) e entre "não vacinados" a 2,1% (IC 95% = 0.639 - 5.533).

Tabela 8. Variáveis demográficas associadas à presença de anticorpos do tipo IgG anti-OPV.

Variável demográfica	N (%)	IgG positivo (%)	IgG negativo (%)	Valor de P	ODDS IC*
Gênero					
Feminino	199 (53,9)	17 (8,5)	182 (91,5)	NS	
Masculino	170 (46,1)	9 (5,3)	161 (94,7)		
Idade (anos)					
≤ 40 anos	188 (50,9)	4 (2,1)	184 (97,9)	<0.001	6.46
> 40 anos	179 (48,5)	22 (12,3)	157 (87,7)		2.11 - 26.16)
Não informado	2 (0,6)	0 (0,0)	2 (100,0)		
Etnia					
Pardo*	237 (64,2)	17 (7,2)	220 (92,8)		
Preto	64 (17,3)	4 (6,2)	60 (93,8)	NS	
Branco	68 (18,5)	5 (7,3)	63 (92,7)		
Nível educacional					
Escola primária ou	187 (50,7)	12 (6,4)	175 (93,6)		
menos*	162 (43,9)	11 (6,8)	151 (93,2)	NS	
Ensino médio ou	18 (4,9)	3 (16,6)	15 (83,4)		
mais	2 (0,5)	0 (0,0)	2 (100,0)		
Analfabeto					
Não informado					
Ocupação					
Aposentado	46 (12,5)	5 (10,8)	41 (89,2)		
Trabalhador	29 (7,8)	6 (20,7)	23 (79,3)		
rural[I]	180 (48,8)	8 (4,4)	172 (95,6)	<0.01	5.60

Prestador de	18 (4,9)	3 (16,6)	15 (83,4)		1.44 - 20.14)
serviço[b*]	29 (7,8)	3 (10,3)	26 (89,7)		
Profissionais de	58 (15,7)	1 (1,7)	57 (98,3)		
saúde	9 (2,5)	0 (0,0)	9 (100,0)		
Desempregado					
Estudante					
Não informado					
Renda[c]					
≤ 1 salário	106 (28,7)	8 (7,5)	98 (92,5)		
mínimo	204 (55,3)	18 (8,8)	186 (91,2)	NS	
> 1 salário	59 (16,0)	0 (0,0)	59 (100,0)		
mínimo					
Não informado					
Total	**369 (100.0**	**26 (7,0)**	**343 (93,0)**		

a: Escola primária ou menos (≤ 8 anos de estudo), Ensino médio ou mais (> 8 anos de estudo); b: Esse grupo inclui profissionais como professor, comerciantes, eletricista, cozinheiro etc.; c: O salário- mínimo no Brasil em 2019 = R$ 1.100,00; * categoria utilizada como referência; Ł fator de exposição associado à presença de anticorpos anti-OPV; NS: não significativo.

Sobre as variáveis demográficas, tanto a presença de IgG (OR = 6.46; IC 95% = 2.11 - 26.16), quanto a presença dos anticorpos neutralizantes (OR = 5.94; IC 95% = 2.96 - 12.7), foram associados estatisticamente à idade superior a 40 anos (TABELA 9). Além disso, as ocupações de “aposentado” (OR = 3.32; IC 95% = 1.46 – 7.36) e “trabalhador rural” (OR = 3.28; IC 95% = 1.20 - 8.43) foram associadas à presença de anticorpos neutralizantes, enquanto, para a presença de IgG apenas “trabalhador rural” (OR = 5.60; IC 95% = 1.44 - 20.14) mostrou associação (Tabela 9 e Figura 21).

Tabela 9. Variáveis demográficas associadas estatisticamente à presença de anticorpos anti-OPV

	Anticorpos neutralizantes N (%)	**Anticorpos IgG N (%)**
Idade		
≤ 40 anos	12 (19%)	4 (15,4%)
> 40 anos	**51 (81%)**	**22 (84,6%)**
Ocupação		
Aposentado	**16 (25,4%)**	5 (19,2%)
Trabalhador rural	**10 (15,9%)**	**6 (23,0%)**
Prestador de serviçob*	25 (39,7%)	8 (30,8%)
Profissionais de saúde	4 (6,3%)	3 (11,6%)
Desempregado	6 (9,5%)	3 (11,6%)
Estudante	1 (1,6%)	1 (3,8%)
Não informado	1 (1,6%)	0 (0,0%)
Total	63	26

Embora a detecção de anticorpos neutralizantes e do tipo IgG possam revelar uma exposição prévia à OPV, seja por infecção natural ou por vacinação, a soro prevalência de ambos foi divergente nesse estudo. Essa divergência pode ser justificada pelas características das metodologias empregadas, assim como a diferença entre as amostras virais utilizadas em ambos os testes. Além disso, sabe-se que os anticorpos neutralizantes, além do IgG, abrangem diferentes classes de imunoglobulinas e são fundamentais na resposta protetora contra OPV (KAREM *et al.,* 2005; HATAKEYAMA *et al.,* 2005; KWANCHUM *et al.,* 2017).

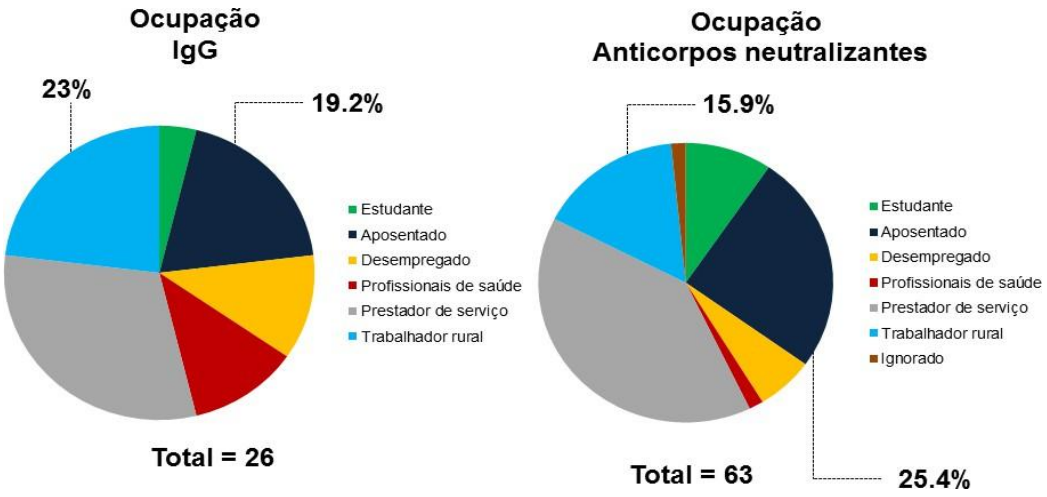


Figura 21. Variáveis demográficas associadas significativamente à presença de anticorpos anti-OPV na população urbana do Serro-MG.

Estratificação das amostras positivas para IgG (esquerda) e anticorpos neutralizantes (direita) baseada na "ocupação".

Estudos de soro prevalência em diferentes populações no mundo tem sido realizados com o objetivo de determinar o *status* imune dos indivíduos em resposta à vacinação antivariólica hipotetizando uma possível reexposição dos indivíduos ao VARV (HAMMARLUND *et al.,* 2003; HATAKEYAMA *et al.,* 2005; PUTZ *et al.,* 2005; LIU *et al.,* 2012; KWANCHUM *et al.,* 2017). De fato, a vacinação contra a varíola foi um importante fator de exposição na população analisada nesse estudo. No entanto, a população brasileira está inserida em um contexto distinto, pois no Brasil tem-se a possibilidade de exposição ao VACV-Br que circula naturalmente no território nacional causando infecções zoonóticas. Logo, anticorpos anti-OPV, tanto do tipo neutralizantes (12/189 = 6.3%), quando do tipo IgG (4/188 = 2.1%) foram detectados em indivíduos considerados "não vacinados", ou seja, àqueles com idade inferior a 40 anos.

É importante destacar que todos os indivíduos soropositivos "não vacinados" não apresentavam nenhum sinal de infecção por VACV e relataram ausência de infecção prévia. Esses dados reforçam a hipótese da existência de alguma rota alternativa de transmissão do VACV que pode colaborar para a circulação do vírus em ambiente urbano (COSTA *et al.,* 2015; OLIVEIRA *et al.,* 2017).

Ao analisarmos a presença de IgM, a prevalência global corresponde a 2,7% (10/369) (IC 95% = 1.412 - 4.983) (Tabela 10). Entre os indivíduos IgM positivos, 7 dos 10 eram "vacinados" de acordo com o critério da idade. No entanto, destes, cinco não possuíam anticorpos IgG e/ou neutralizantes detectáveis. Além disso, nenhuma variável demográfica analisada teve associação estatística significativa com a presença de IgM.

Tabela 10. Variáveis demográficas associadas à presença de anticorpos do tipo IgM anti-OPV na população urbana do Serro-MG.

Variável demográfica	N (%)	IgM positivo (%)	IgM negativo (%)	Valor de P	Odds IC*
Gênero					
Feminino	199 (53,9)	3 (1,5)	196 (98,5)		
Masculino	170 (46,1)	7 (4,1)	163 (95,9)	NS	
Idade (anos)					
≤ 40 anos	188 (50,9)	3 (1,6)	185 (98,4)		
> 40 anos	179 (48,5)	7 (3,9)	172 (96,1)	NS	
Não informado	2 (0,6)	0 (0,0)	2 (100,0)		
Etnia					
Pardo*	237 (64,2)	4 (1,7)	233 (98,3)		
Preto	64 (17,3)	2 (3,1)	62 (96,9)	NS	
Branco	68 (18,5)	4 (5,9)	64 (94,1)		
Nível educacional[a]					
Escola primária ou menos*	187 (50,7)	6 (3,2)	181 (96,8)		
Ensino médio ou mais	162 (43,9)	3 (1,8)	159 (98,2)		
Analfabeto	18 (4,9)	0 (0,0)	18 (100,0)	NS	
Não informado	2 (0,5)	1 (50,0)	1 (50,0)		
Profissão					
Aposentado	46 (12,5)	3 (6,5)	43 (93,5)		
Prestador de serviço[b]*	180 (48,8)	7 (3,9)	173 (96,1)		
Estudante	58 (15,7)	0 (0,0)	58 (100,0)		
Desempregado	29 (7,8)	0 (0,0)	29 (100,0)	NS	
Profissionais de saúde	18 (4,9)	0 (0,0)	18 (100,0)		
Trabalhador rural	29 (7,8)	0 (0,0)	29 (100,0)		
Não informado	9 (2,5)	0 (0,0)	9 (100,0)		
Renda[c]					
≤ 1 salário mínimo	106 (28,7)	1 (0,9)	105 (99,1)		
> 1 salário mínimo	204 (55,3)	9 (4,4)	195 (95,6)	NS	
Não informado	59 (16,0)	0 (0,0)	59 (100,0)		

Total	**369 (100.0)**	**10 (2,7)**	**359 (97,3)**

a: Escola primária ou menos (≤ 8 anos de estudo), Ensino médio ou mais (> 8 anos de estudo); b: Esse grupo inclui profissionais como professor, comerciantes, eletricista, cozinheiro etc.; c: O salário- mínimo no Brasil em 2019 = R$ 1.100,00; * categoria utilizada como referência; Ɨ fator de exposição associado à presença de anticorpos anti-OPV; NS: não significativo.

A sobreposição de anticorpos neutralizantes, IgG e IgM em um indivíduo com marca vacinal revela a indução da resposta humoral de fase aguda contra infecção por OPV, ou seja, evidência de exposição recente, mesmo em indivíduo vacinado. Esse dado corrobora com o estudo de Karem e colaboradores, onde indivíduos previamente vacinados tiveram níveis de IgM detectáveis quando expostos ao MPXV durante o surto descrito na América do Norte em 2003 (KAREM *et al*., 2005).

De fato, tem sido observado no Brasil que indivíduos vacinados, quando expostos ao VACV no contexto dos surtos de VB, são re-infectados, desenvolvem o quadro clínico característico e, embora não tenha sido descrita identificação de anticorpos do tipo IgM, têm-se altos títulos de anticorpos neutralizantes, que também atuam como indicativo de uma resposta humoral frente à exposição recente (SILVA-FERNANDES *et al*., 2009; OLIVEIRA *et al*, 2014; COSTA *et al*., 2015).

Apesar de não ter sido demonstrada associação entre a presença de anticorpos IgM e as características demográficas analisadas, vale destacar a detecção desse importante marcador de infecção aguda associada à ausência de sinais clínicos de infecção em indivíduos avaliados fora do contexto da VB. Para esclarecimento desses achados, dentre outros supracitados, que fortalecem o pressuposto de que a população analisada não têm sido exposta ao VACV pela via comumente associada a forma clássica de transmissão, que consiste no contato direto com o bovino infectado, mas através de uma forma alternativa, foram avaliados potenciais fatores de exposição.

6.1.5. Potenciais fatores de exposição ao VACV na população urbana do Serro-MG

Os potenciais fatores de exposição ao VACV são apresentados na Tabela 11. Entre eles, o contato com animais domésticos como gato e cachorro relatado por 302 (81,2%) indivíduos. No geral, 20,4% (n=76) dos participantes tiveram contato com bovinos e equídeos, enquanto

24,2% (n-=90) tiveram contato com animais silvestres. Aproximadamente 70% da população avaliada têm visto roedor próximo à propriedade onde mora. A ordenha, sabidamente fator de risco associada à circulação de VACV no Brasil, foi realizada por apenas 64 (17,2%) participantes em algum momento no passado, no entanto o consumo de leite é uma prática relatada por 333 (89,5%) indivíduos. O consumo do leite cru é realizado por 18,8% (n=70) dos entrevistados, o consumo de queijo artesanal por 89,5% (n=333) e a manipulação de leite na produção de derivados por apenas 10,5% (n=39).

Tabela 11. Fatores de exposição associados à presença de anticorpos anti-OPV em população urbana

Fator de exposição	N (%)	PRNT Positivo (%)	IgM Positivo (%)	IgG Positivo (%)	Valor de P ODDS IC
Marca vacinal[a]					<0,05
Sim	64 (17,2)	23 (36,5)	5 (50,0)	9 (34,6)	3,75*
Não	308 (82,8)	40 (63,5)	5 (50,0)	17 (65,4)	2,04-6,91
Contato com bovinos ou equídeos					
Sim	76 (20,4)	16 (25,4)	2 (20,0)	7 (26,9)	
Não	296 (79,6)	47 (74,6)	8 (80,0)	19 (73,1)	
Contato com animais domésticos[b]					<0,001
Sim	302 (81,2)	40 (63,5)	8 (80,0)	18 (69,3)	0,31
Não	70 (18,8)	23 (36,5)	2 (20,0)	8 (30,7)	0,17-0,56
Contato com animais silvestres					
Sim	90 (24,2)	17 (27,0)	4 (40,0)	8 (30,7)	
Não	282 (75,8)	46 (73,0)	6 (60,0)	18 (69,3)	
Presença de roedores					
Sim	259 (69,6)	42 (66,6)	8 (80,0)	20 (76,9)	
Não	113 (30,4)	21 (33,4)	2 (20,0)	6 (23,1)	
Consumo de leite					
Sim	333 (89,5)	59 (93,7)	10 (100,0)	26 (100,0)	
Não	39 (10,5)	4 (6,3)	0 (0,0)	0 (0,0)	
Consumo de leite cru					0,052
Sim	70 (18,8)	18 (28,6)	3 (30,0)	3 (11,5)	1,97
Não	302 (81,2)	45 (71,4)	7 (70,0)	23 (88,5)	1,06-3,68
Consumo de queijo artesanal					
Sim	333 (89,5)	54 (85,7)	9 (90,0)	23 (88,5)	

Não	39 (10,5)	9 (14,3)	1 (10,0)	3 (11,5)	
Produção de derivados					<0,05
Sim	39 (10,5)	12 (19,0)	0 (0,0)	7 (26,9)	2,45*
Não	333 (89,5)	51 (81,0)	10 (100,0)	19 (73,1)	1,17-5,16
Ordenha					
Sim	64 (17,2)	10 (15,9)	1 (10,0)	3 (11,5)	
Não	308 (82,8)	53 (84,1)	9 (90,0)	23 (88,5)	
Total	**372 (100,0)**	**63 (100,0)**	**10 (100,0)**	**26 (100,0)**	

a: Presença da marca vacinal no braço esquerdo; b: Esse grupo inclui cachorro, gato, galinha, passarinho, porco, dentre outros; *Os valores de OR e IC para a presença de anticorpos neutralizantes.

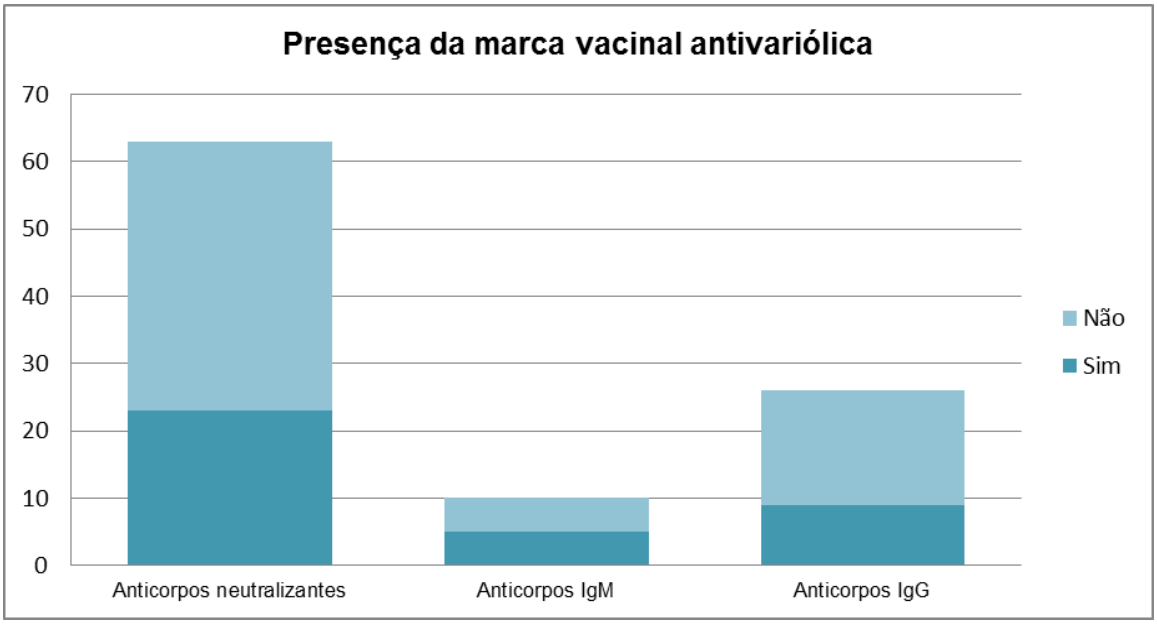
O ponto de corte na idade superior a 40 anos foi determinado para caracterizar os indivíduos como "vacinados". Avaliando a vacinação contra a varíola como fator de exposição, 48,1% (n=179) dos entrevistados foram vacinados. No entanto, apenas 17,2% (n=64) foram considerados de fato vacinados, devido à presença da marca vacinal no braço esquerdo.

6.1.6. Fatores de exposição estatisticamente associados à soro prevalência

A análise dos fatores de exposição revelou que indivíduos vacinados contra varíola tiveram aproximadamente 4 vezes mais chances de ter anticorpos neutralizantes em relação àqueles não vacinados (OR=3.7; IC 95% = 1.9-7.1), IgG (OR=2.8; IC 95% = 1.0-7.0) e IgM (OR=5.1; IC 95% = 1.1-22.9).

Outro fator de exposição associado foi à manipulação de leite cru durante a produção de derivados do leite, sendo demonstrado que, indivíduos que realizam essa prática têm duas vezes mais chances de ter anticorpos neutralizantes detectáveis (OR=2.45; IC 95% = 1.17-5.16) e foram quatro vezes mais propensos a ter anticorpos do tipo IgG (OR=3.6; IC 95% = 1.18-9.8) do que os participantes que não realizam essa prática (Figura 22).

Por outro lado, o contato entre o indivíduo e animais domésticos foi significativamente associado à diminuição da chance desse indivíduo ter anticorpos neutralizantes (OR= 0.31; IC 95% = 0.17-0.56).



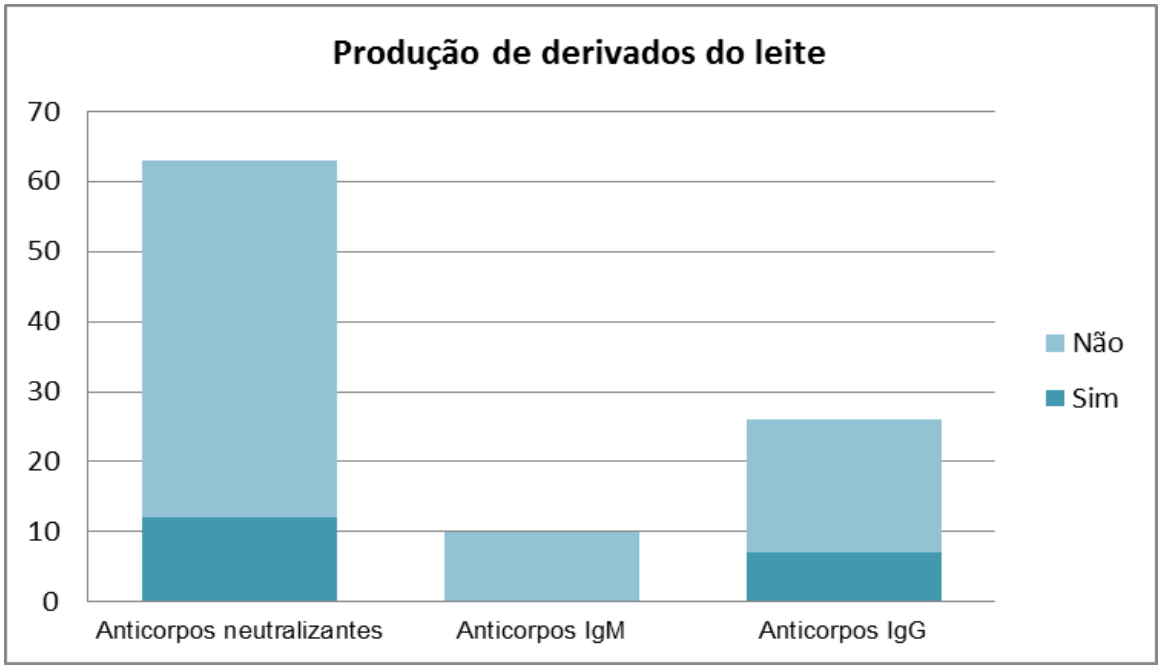


Figura 22. Fatores de exposição significativamente associados à presença de anticorpos anti-OPV.

Estratificação baseada na "presença da marca vacinal" e "produção de derivados do leite" para amostras positivas para IgM, IgG e anticorpos neutralizantes.

Considerando os indivíduos que possuem anticorpos neutralizantes detectáveis, cuja exposição laboral através da produção de derivados do leite foi relatada, têm-se um título de

anticorpos neutralizante variável entre 100-800 UN/mL (Tabela 12). Dentre esses indivíduos, quatro (33.3%) possuem a marca vacinal característica da vacinação antivariólica; e seis (50%) relataram a prática da ordenha de bovinos, exposição laboral sabidamente associada à circulação do VACV no Brasil.

Tabela 12. Título de anticorpos neutralizantes dos participantes envolvidos na produção de derivados do leite

Indivíduo Amostrado	**Marca vacinal**	**Título de Anticorpos neutralizantes**	Resultado PCR orofaringe	**Prática Da ordenha**
18/2015	Não	100 UN/mL	-	Sim
40/2015	Sim	100 UN/mL	-	Sim
42/2015	Não	100 UN/mL	-	Sim
51/2015	Não	NT	+	Não
52/2015	Não	100 UN/mL	+	Não
53/2015	Não	100 UN/mL	-	Não
55/2015	Sim	100 UN/mL	-	Não
60/2017	Não	200 UN/mL	-	Sim
65/2017	Não	400 UN/mL	-	Não
126/2017	Sim	800 UN/mL	-	Não
191/2017	Sim	200 UN/mL	-	Sim
210/2017	Não	100 UN/mL	-	Sim

NT: Não testado devido a volume insuficiente de amostra; +: positivo; -: negativo.

A comparação entre os tipos de anticorpos detectados nessa população revela uma sobreposição entre IgM, IgG e anticorpos neutralizantes em um indivíduo. Embora apresente anticorpos do tipo IgM, o participante não apresentou DNA viral detectável em swab orofaríngeo, nem DNA viral detectável no soro (Tabela 13 – linha 55).

Esse indivíduo é do sexo masculino, trabalha como taxista e relatou conhecer previamente a VB devido à existência de surtos na região. Este participante possui marca vacinal indicativa da vacinação antivariólica, e alto título de anticorpos neutralizantes equivalente a 800UN/ mL. Dentre os possíveis fatores de exposição associados à presença de OPV, foi relatado consumo de leite, entretanto industrializado, consumo de queijo artesanal,

presença de roedores na propriedade, contato com animais silvestres e contato com felinos domésticos. O participante negou a existência de lesões no felino doméstico, o quando questionado a respeito.

Além disso, foi detectada a sobreposição de anticorpos IgM e anticorpos neutralizantes (100 UN/mL) em uma mulher, com 62 anos de idade, no entanto sem marca vacinal (Tabela 13 – linha 68). A participante relatou residir em uma comunidade próxima à área rural e ter contato com canídeos, felinos, equinos e bovinos, embora a ordenha não consista em uma prática realizada pela mesma. Além disso, o consumo de leite (não industrializado) e queijo artesanal foi relatado. Entretanto, o DNA do VACV nas amostras de swab orofaríngeo e de soro foi indetectável, o que sugere que possivelmente não houve exposição via oral neste caso.

Em relação à presença de IgG e anticorpos neutralizantes, teve-se a sobreposição desses anticorpos em 15 participantes (Tabela 13 – linhas 4, 5, 20, 30, 36, 44, 48, 50, 52, 57, 58, 62, 73, 74 e 75). Todos possuem idade superior a 40 anos, no entanto apenas seis possuem a marca vacinal no braço esquerdo. Dois participantes relataram contato com paciente diagnosticado com VB; oito (53,3%) relataram contato com animais (canídeos, felinos, equinos e bovinos); três (20%) relatam proximidade a animais silvestres (primatas não humanos) e onze (73,3%) descreveram a presença de roedores próximos ao domicílio; todos os indivíduos (100%) consomem leite, sendo o consumo de leite não industrializado relatado por 10/16 participantes; e 14/16 (86,6%) consome queijo artesanal, enquanto 26,6% (n=4) participam da produção de derivados do leite e 13,3% (n=2) relataram a realização de ordenha.

Neste estudo, 98 indivíduos listados na tabela acima tiveram alguma evidência laboratorial de exposição ao VACV através da pesquisa sorológica (PRNT50, ELISA IgM e IgG) e da pesquisa molecular realizada no swab orofaríngeo. Nos participantes que apresentaram o DNA do VACV detectável nas amostras de swab orofaríngeo coletadas OU àqueles que apresentaram anticorpos do tipo IgM detectados por ELISA foi realizada a pesquisa de DNA do VACV também na amostra de soro com o objetivo de verificar a potencial infecção.

Nestes, foi possível detectar o DNA viral através de qPCR para o gene C11R no soro em dois participantes. Um deles é um homem, 30 anos de idade, logo, trata-se de um participante não vacinado contra a varíola. Este indivíduo trabalha no campo, como engenheiro agrônomo, e apresentou anticorpos IgM e IgG detectáveis, assim como o DNA viral no soro (Tabela 13 – linha 1). Dentre os possíveis fatores de exposição associados, o participante relatou

contato com animais domésticos e silvestres e consumo de leite cru. Entretanto, não foi possível detectar DNA viral em swab orofaríngeo, o que sugere que a exposição pode ter ocorrido por meio de outros fatores, como o contato com animais e ambiente rural/silvestre em decorrência da sua atividade ocupacional.

O segundo participante que apresentou DNA viral detectável no soro também corresponde ao sexo masculino, idade 52 anos, vacinado (marca vacinal presente no braço esquerdo). Embora vacinado, este indivíduo não apresentou anticorpos neutralizantes e IgG detectáveis nos testes realizados, o que pode ser indicativo da diminuição destes níveis de anticorpos ao longo dos anos ou mesmo pode estar associado a deficiência na produção dos anticorpos em resposta à vacinação, ambos cenários já relatados em outros estudos envolvendo vacinados (HAMMARLUND et al., 2005; KELSO et al., 2005; PUTZ et al., 2005; AMANNA et al., 2006; KENNEDY et al., 2016).

Neste participante, além do DNA viral detectável no soro, foi identificada a sobreposição de outros marcadores de infecção aguda (DNA viral em swab orofaríngeo e anticorpo do tipo IgM). Quando questionado sobre os possíveis fatores de exposição, foi informado que o contato com animais domésticos, e o consumo de leite cru e queijo artesanal são práticas frequentemente realizadas. Por outro lado, o participante descartou a prática de ordenha de bovinos e o histórico de contato com casos de vaccínia bovina.

O estudo de Costa e colaboradores (2015) descreveu a detecção molecular do VACV em sangue e altos títulos de anticorpos neutralizantes no soro de indivíduos em um núcleo familiar. Nesse cluster, apenas um integrante realizou ordenha de bovinos e foi exposto ao VACV por essa rota de transmissão. No entanto, a transmissão direta entre humanos ou transmissão ambiental devido à contaminação de fômites foi apontada como formas alternativas de exposição ao vírus nesse cluster. Vale ressaltar que, esses indivíduos relataram consumo de leite não pasteurizado e queijo artesanal. Esse relato contribui para fortalecer a perspectiva da investigação do consumo de derivados lácteos como possíveis fontes de transmissão do VACV (COSTA *et al.,* 2015).

Nesse contexto, embora o consumo de leite e derivados não tenha sido associado significativamente à soro prevalência de anti-OPV na população urbana analisada, essa associação não deve ser descartada, dado que o desfecho clínico decorrente da exposição oral ao VACV ainda é desconhecido. Sabe-se que, durante um surto causado pelo BPXV na Índia,

alguns indivíduos desenvolveram lesões na mucosa oral associadas ao consumo de leite contaminado (GURAV *et al*., 2011). Além disso, estudos epidemiológicos desenvolvidos com outro OPV, o MPXV, têm sugerido que a manipulação de carne de primatas não humanos (PNH) infectados e consumo de carnes de caça entre as populações urbanizadas poderia atuar como uma fonte alternativa de transmissão do MPXV na África (REYNOLDS *et al*., 2019).

A manipulação de leite durante a produção de queijo artesanal e outros derivados foi apontada como um importante fator de risco associado à circulação do VACV na população urbana analisada. Esse é um achado epidemiológico relevante, visto que a zoonose causada pelo VACV é caracterizada como uma doença ocupacional devido à exposição de ordenhadores ao gado leiteiro infectado (OLIVEIRA *et al*., 2017A). A descrição de uma nova fonte de exposição ocupacional acaba advertindo a vigilância em saúde (epidemiológica e sanitária), quanto à elaboração de estratégias de prevenção multissetoriais.

Em relação aos possíveis fatores de exposição ao VACV na população urbana do Serro, as análises revelam que a exposição a animais domésticos está significativamente associada à diminuição da chance do indivíduo ter anticorpos neutralizantes anti-OPV. De fato, Borges e colaboradores relataram algo semelhante; em um estudo conduzido em propriedades rurais em diferentes municípios de Minas Gerais, dentre eles o Serro, onde a presença de gatos domésticos nas propriedades amostradas foi inversamente proporcional à presença de anticorpos neutralizantes anti-OPV em bovinos (BORGES *et al*., 2017).

Animais domésticos como cães e gatos fazem parte da cadeia epidemiológica do CPXV, OPV zoonótico circulante na Europa. Os gatos domésticos, especificamente, desempenham um importante papel na transmissão do CPXV para humanos (SMITH *et al*., 1999; ESSBAUER *et al*., 2009; VON BOMHARD *et al*., 2010; CHOMEL *et al*., 2014; SWITAJ *et al*., 2015; ZABA *et al*., 2017; GAZZANI *et al*., 2016). Apesar da circulação do VACV ter sido descrita em animais domésticos como cães e gatos no Brasil, o perfil da infecção parece ser divergente em relação às descrições de CPXV (PERES *et al*., 2013; 2016; COSTA *et al*., 2017; 2018).

Um estudo de infecção experimental demonstrou que a infecção de felinos por VACV de fato resulta em um desenvolvimento de lesões discretas, quando comparada à infecção por CPXV (BENNETT *et al*., 1989). Isso corrobora com os dados de Costa e colaboradores, que descreve a circulação do VACV em uma amostragem de felinos recrutada em clínicas veterinárias; no entanto os animais não apresentavam sinais clínicos indicativos de infecção por

OPV (COSTA *et al*, 2017). Isso pode indicar que, assim como o perfil clínico da infecção por CPXV e VACV é divergente em felinos, possivelmente o papel desses animais no ciclo epidemiológico desses vírus também pode ser distinto.

A presença de felinos em residências pode controlar a população de pequenos roedores através da predação ou devido à influência de substâncias químicas presentes na urina desses predadores sobre a reprodução dos roedores (VOZNESSENSKAYA *et al*., 2013). Os pequenos roedores, por sua vez, são apontados como reservatórios do VACV no Brasil (ABRAHÃO *et al*., 2009A; MIRANDA *et al*., 2017). Logo, pode-se inferir que a presença de animais domésticos na residência, mais especificamente de gatos, atenuaria a introdução do VACV em determinado ambiente por meio de roedores (BORGES *et al*., 2017).

Diante dos dados apresentados nesta tese, foi proposto um novo modelo hipotético destacando a dinâmica da circulação do VACV e possíveis formas de transmissão em diferentes hospedeiros e ambientes (Figura 23) e um modelo hipotético das rotas de infecção dos OPV em humanos (Figura 24). Além disso, este estudo amplia as perspectivas sobre a investigação da circulação do VACV em ambiente urbano e propõe a intensificação das ações de vigilância em saúde, por meio daspráticas de educação em saúde como forma de prevenção frente à introdução do VACV em ambiente urbano.

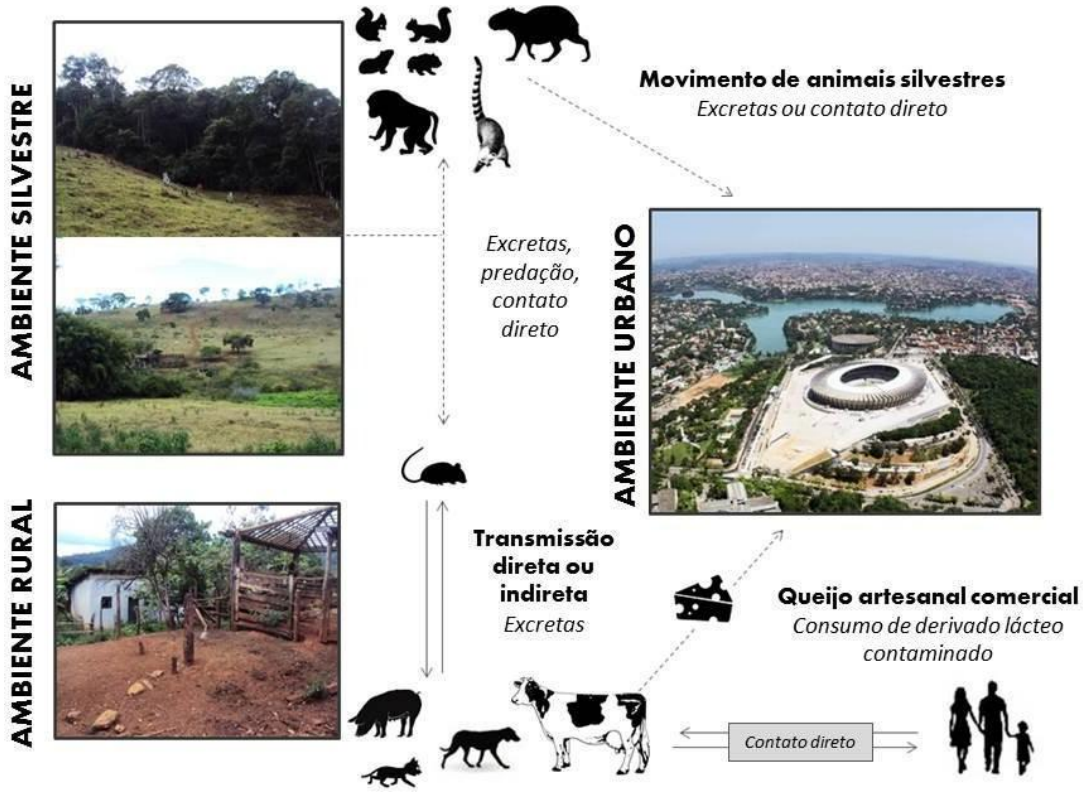


Figura 23. Modelo hipotético destacando a dinâmica da circulação do VACV e possíveis formas de transmissão em diferentes hospedeiros e ambientes.

Os roedores peridomésticos podem atuar como pontes entre o ambiente silvestre e rural, onde os surtos de VB são descritos. Roedores silvestres são apontados como potenciais reservatórios do VACV e transmissores do vírus para outros mamíferos, ou mesmo para roedores peridomiciliares, mantendo dessa forma o ciclo silvestre-rural. Por outro lado, em área urbana, a dinâmica também envolve animais silvestres, como capivaras e quatis, que podem entrar em contato com outros mamíferos. Os animais silvestres podem interagir com animais domésticos como cães e gatos que vive em região de transição (parques e reservas), isso pode favorecer a transmissão do VACV para outros animais domésticos e humanos. Além disso, supõe-se que VACV pode ser introduzido alternativamente ao ambiente urbano através da comercialização de produtos lácteos possivelmente contaminados.

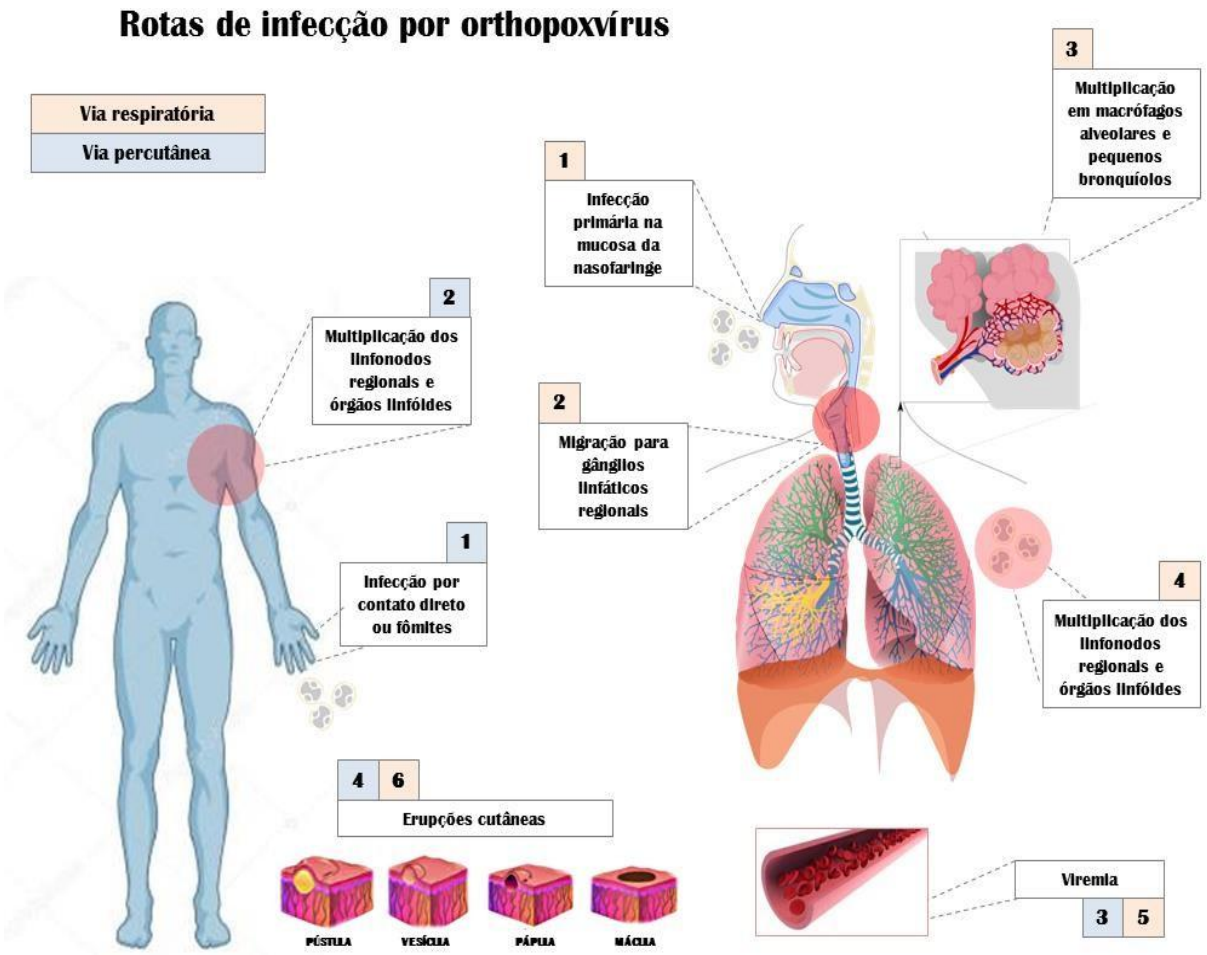


Figura 24.. Modelo hipotético das rotas de infecção dos OPV's em humanos

São estabelecidas duas rotas de infecção por OPV's. A transmissão respiratória, relatada para o VARV, se inicia com a infecção primária na mucosa da nasofaringe (1); seguida da migração do vírus para gânglios linfáticos regionais (2); multiplicação viral em macrófagos alveolares e pequenos bronquíolos (3); multiplicação nos linfonodos regionais e órgãos linfoides (4); viremia (5) e erupções cutâneas (6). A transmissão via percutânea /mucosa, relatada para VACV, pode ocorrer por meio de contato direto com erupções cutâneas de um animal ou humano infectado, ou por fômites (1); seguida de multiplicação viral nos linfonodos regionais e órgãos linfoides (2); viremia (3) e erupções cutâneas (4). Fonte: Oliveira, JS; dados não publicados.

7. CONCLUSÕES

Através de prospecção molecular, o VACV foi detectado em queijos artesanais comercializados e produzidos em Minas Gerais. Além de configurar um problema de saúde pública, esse pode ser um gargalo para o setor econômico.

O DNA do VACV foi detectado em 25 swabs de orofaringe humana (6,7%), o que indica a presença do VACV na região oral em indivíduos assintomáticos residentes na área urbana do Serro.

Embora não tenha sido demonstrada significância estatística entre os fatores de exposição e a detecção molecular do VACV na orofaringe da população amostrada no Serro, esse achado indica a chance de exposição através do consumo de alimentos lácteos possivelmente contaminados.

A partir de técnicas sorológicas foi detectada a prevalência de anti-OPV em uma população urbana de Minas Gerais. A prevalência global em relação a anticorpos neutralizantes na população analisada é menor do que a relatada em uma população rural amostrada na bacia leiteira do Serro anteriormente, onde a VB é endêmica.

O histórico vacinal atestado pela presença da marca da vacina antivariólica e a manipulação de leite *in natura* na produção de derivados são fatores de exposição estatisticamente associados à presença de anti-OPV na população urbana do Serro-MG. Este último reforça a caracterização da VB como uma zoonose ocupacional.

Evidência sorológica da circulação de VACV em indivíduos não vacinados e a detecção de marcador de fase aguda de infecção são indicativas da exposição ao VACV em área urbana, fora do contexto de surto e na ausência da transmissão clássica. Esses achados reforçam a hipótese da existência de rotas alternativas para a transmissão do VACV no Brasil.

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PARTICIPAÇÃO EM EVENTOS CIENTÍFICOS (2017-2021)

1. III Semana Acadêmica da Licenciatura em Ciências Biológicas da Universidade Federal de Viçosa campus Florestal. Palestrante sobre o tema "Doenças Emergentes". 2020
2. 55º Congresso da Sociedade Brasileira de Medicina Tropical e XXVI Congresso Brasileiro de Parasitologia. 2019.
3. Seminário do Instituto de Ciências Biológicas / UFMG: "*Animal Sex Determination by Genes, Chromosomes and the Environment.*" 2019.
4. Seminário do Instituto de Ciências Biológicas / UFMG: "*Biossegurança e OGMs.*" 2019.
5. Encontro Darwin Day - UFMG 2019.
6. Seminário do Instituto de Ciências Biológicas / UFMG: "*Vírus gigantes de DNA: expandindo os limites da virosfera*." 2019.
7. V Simpósio de Microbiologia da UFMG, Professor Eduardo Osório Cisalpino: Desafios Atuais no Enfrentamento de Doenças Microbianas. 2018.
8. Aula inaugural da Pós graduação do ICB: A Zika amarelou? Da dinâmica vetorial à biologia viral. 2017.
9. II Encontro Científico do Laboratório de Vírus - Comemoração dos 55 anos. 2017.
10. XXVIII Congresso Brasileiro de Virologia & XII Encontro de Virologia do Mercosul. 2017.

RESUMOS PUBLICADOS EM ANAIS DE EVENTOS E APRESENTADOS EM EVENTOS CIENTÍFICOS (2017-2021)

1. DUTRA, A. G. S.; **OLIVEIRA, J. S.**; COSTA, GB ; AMARAL, C. D. ; DRUMOND, B.P. ; KROON, E. G. ; de Oliveira, DB ; TRINDADE, G. S. . Soroprevalência de anticorpos neutralizantes contra o vírus da Febre Amarela (YFV) em uma população de área endêmica do estado de Minas Gerais. 2019. (Apresentação de Trabalho/55º Congresso da Sociedade Brasileira de Medicina Tropical e XXVI Congresso Brasileiro de Parasitologia, 2019).
2. DUTRA, A. G. S. ; **OLIVEIRA, J. S.** ; COSTA, GB ; KROON, E. G. ; ABRAHAO, J. S. ; DRUMOND, B.P. ; de Oliveira, DB ; TRINDADE, G. S. . Indivíduos Susceptíveis À Febre Amarela Em Área De Risco No Brasil: Inquérito Sorológico. 2019.

(Apresentação de Trabalho/ VI Simpósio de Microbiologia da UFMG - Conecta SIM: Microbiologia Interligada, 2019).

3. SILVA, P. H. B. ; **DE OLIVEIRA, J.S.** ; COSTA, P. S. P. M. ; DOMINGOS, I. J. S. ; STOFFELLA-DUTRA, A. G. ; AMARAL, C. D. ; OLIVEIRA, D. B. ; COSTA, GB ; TRINDADE, G. S. . Soroprevalência e fatores de exposição associados à anti-opv em área endêmica para Vaccinia virus no estado de Minas Gerais, Brasil. In: VI Simpósio de Microbiologia da UFMG - Conecta SIM: Microbiologia Interligada, 2019, Belo Horizonte. VI Simpósio de Microbiologia da UFMG - Conecta SIM: Microbiologia Interligada, 2019.
4. DOMINGOS, IJS ; **DE OLIVEIRA, J.S**. ; SILVA, PHB ; COSTA, P. S. P. M. ; LIMA, MT ; DUTRA, A. G. S. ; AMARAL, C. D. ; COSTA, GB ; KROON, E. G. ; TRINDADE, G. S. . Análise epidemiológica da vaccínia bovina como zoonose ocupacional: estudo de caso no município de Teófilo Otoni, Minas Gerais. 2019. (Apresentação de Trabalho/55º Congresso da Sociedade Brasileira de Medicina Tropical e XXVI Congresso Brasileiro de Parasitologia, 2019).
5. DOMINGOS, IJS **; DE OLIVEIRA, J.S.** ; SILVA, PHB ; COSTA, P. S. P. M. ; LIMA, MT ; DUTRA, A. G. S. ; AMARAL, C. D. ; COSTA, GB ; KROON, E. G. ; TRINDADE, G. S. . Análise Epidemiológica Da Vaccínia Bovina Como Zoonose Ocupacional: Estudo De Caso No Município De Teófilo Otoni, Minas Gerais. 2019. (Apresentação de Trabalho/VI Simpósio de Microbiologia da UFMG - Conecta SIM: Microbiologia Interligada, 2019).
6. **OLIVEIRA, J. S.;** COSTA, P. S. P. M. ; AMARAL, C. D. ; SILVA, N. I. O. ; COSTA, GB ; de Oliveira, DB ; DRUMOND, B.P. ; ABRAHAO, J. S. ; KROON, E. G. ; TRINDADE, G. S. . Rotas Alternativas De Transmissão Do Vaccinia Virus Em Uma Área Urbana Do Estado De Minas Gerais: Relato De Caso. 2018. (Apresentação de Trabalho/Simpósio).
7. COSTA, G. B.; ALMEIDA, L. R. ; SANTOS, A. G. R. C. ; **de OLIVEIRA, J. S.** ; MIRANDA, J. B. ; SARAIVA-SILVA, A. T. ; KROON, E. G. ; PEREIRA, P. L. L. ; SOARES, D. F. M. ; TRINDADE, G. S. . Vaccinia virus free circulation among dogs and coatis from wild and urban environments in Brazil: Insights into VACV emergence and risks for urban human populations. In: XXII International Poxvirus, Asfarvirus and Iridovirus Conference, 2018, Taipei. XXII International Poxvirus, Asfarvirus and Iridovirus Conference Daily Program, 2018. p. 121-121.
8. DUTRA, A. G. S. ; **OLIVEIRA, J. S.** ; FIGUEIREDO, P. O. ; AMARAL, C. D. ;

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9. FIGUEIREDO, P. O. ; SILVA, A. T. S. ; **OLIVEIRA, J. S.** ; AMARAL, C. D. ; BONJARDIM, C. A. ; ABRAHAO, J. S. ; KROON, E. G. ; DRUMOND, B.P. ; NOGUEIRA, M. L. ; DE OLIVEIRA, DB ; TRINDADE, G. S. . Detecção Molecular De Yellow Fever Virus Em Humanos E Primatas Não Humanos No Estado De Minas Gerais, 2017. 2018. (Apresentação de Trabalho/Simpósio).
10. COSTA, P. S. P. M. ; **OLIVEIRA, J. S.** ; DUTRA, A. G. S. ; SILVA, N. I. O. ; COSTA, GB ; de Oliveira, DB ; DRUMOND, B.P. ; ABRAHAO, J. S. ; KROON, E. G. ; TRINDADE, G. S. . Deteccção Molecular Do Vaccinia Virus Associada Ao Consumo De Queijo Artesanal: Um Relato De Caso. 2018. (Apresentação de Trabalho/Simpósio).
11. **de Oliveira, J.S. ;** COSTA, GB ; FIGUEIREDO, P. O. ; STOFFELLA-DUTRA, A. G. ; AMARAL, C. D. ; SARAIVA-SILVA, A. T. ; DRUMOND, B.P. ; ABRAHAO, J. S. ; KROON, E. G. ; TRINDADE, G. S. ; DUTRA, A. G. S. . Detecção de Vaccinia virus em queijo artesanal comercializado no Brasil. In: IV Simpósio de Microbiologia da UFMG ? Metabolismo Microbiano: Saúde, Ambiente e Biotecnologia., 2017, Belo Horizonte. Caderno de Resumos do IV Simpósio De Microbiologia Da UFMG., 2017.
12. **de Oliveira, J.S.** ; COSTA, GB ; FIGUEIREDO, P. O. ; AMARAL, C. D. ; STOFFELLA-DUTRA, A. G. ; SARAIVA-SILVA, A. T. ; DRUMOND, B.P. ; ABRAHAO, J. S. ; KROON, E. G. ; TRINDADE, G. S. . Anticorpos Neutralizantes Anti-Orthopoxvirus Em Indivíduos Não Vacinados De Uma População Urbana Do Estado De Minas Gerais, Brasil.. In: IV Simpósio de Microbiologia da UFMG - Metabolismo Microbiano: Saúde, Ambiente e Biotecnologia., 2017, Belo Horizonte. Caderno de Resumos do IV Simpósio De Microbiologia Da UFMG, 2017.
13. STOFFELLA-DUTRA, A. G.; COSTA, GB ; **de Oliveira, J.S. ;** FIGUEIREDO, P. O. ; AMARAL, C. D. ; SARAIVA-SILVA, A. T. ; BONJARDIM, C. A. ; ABRAHAO, J. S. ; DRUMOND, B.P. ; KROON, E. G. ; TRINDADE, G. S. . Análise Retrospectiva E

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ORGANIZAÇÃO DE EVENTOS CIENTÍFICOS (2017-2021)

1. 55º Congresso da Sociedade Brasileira de Medicina Tropical, o XXVI Congresso Brasileiro de Parasitologia, a 34ª Reunião de Pesquisa Aplicada em Doença de Chagas e a 22ª Reunião de Pesquisa Aplicada em Leishmanioses, o CHAGASLEISH 2019.. 2019.

CO-ORIENTAÇÃO DE TRABALHOS CIENTÍFICOS (2017-2021)

1. Jéssica Pauline Coelho Souza. O uso dos vírus herpes simplex oncolíticos como estratégia terapêutica no controle de células metastáticas do câncer de mama. Início: 2019. Monografia (Aperfeiçoamento/Especialização em Curso de Especialização em Diagnóstico e Controle Microbiológico) - Universidade Federal de Minas Gerais.

ANEXOS

Anexo 1 – QUESTIONÁRIO EPIDEMIOLÓGICO

Laboratório de Vírus UFMG

Projeto: Avaliação de rotas alternativas de transmissão do *Vaccinia virus* em áreas urbanizadas do estado de Minas Gerais, Brasil.

Entrevistador:_____Data da entrevista:__/___/____ Número do questionário:____

SEÇÃO 1 – INFORMAÇÃO GERAL OU DEMOGRÁFICA

1) Idade: _____ Data de nascimento:_____/_____/_____

2) Sexo:_____

3) Endereço:

_____

4) Qual seu nível educacional?

0. ( ) Nunca foi a uma escola 1. ( ) Ensino fundamental 2. ( ) Ensino médio 3. ( ) Graduação 4. ( ) Pós graduação 99. ( ) Ignorado

5) Qual sua profissão? _____

6) Origem étnica (ou Raça). Por favor, especifique sua etnia:

0. ( ) Pardo 1. ( ) Preto 2. ( ) Branco 3. ( ) Amarelo 4. ( ) Indígena 99. ( ) Ignorad

7) Qual é a sua renda familiar?

0. ( ) Menos que R$ 937,00 1. ( ) R$ 937,00 – R$ 1.874,00 2. ( ) R$ 1.874,00 – R$ 2.811,00 3. ( ) R$ 2.811,00 – R$ 3.748,00 4. ( ) R$ 3.748,00 – R$ 4.685,00 5. ( ) Mais que R$ 4.685,00 99. ( ) Ignorado

SEÇÃO 2 – HISTÓRICO SOBRE VACCÍNIA BOVINA

1. Você já ouviu sobre Vaccínia bovina antes?

0. Não ( ) 1. Sim ( ) 99. Ignorado ( )

2. Como você ouviu sobre Vaccínia bovina?

0. Nunca ouvi sobre a doença ( ) 1. Durante um surto ( ) 2. Através de fazendeiros e ordenhadores ( ) 3. Profissionais de saúde ou veterinários ( ) 4. Rádio ( ) 5. TV ( ) 6. Jornal local ( ) 7. Internet ( ) 99. Ignorado ( )

3. Você já teve Vaccínia bovina?

0. Não ( ) 1. Sim ( ) **Local da lesão**:_____**Duração das lesões**: _____99. Ignorado ( )

4. Conhece alguém que teve Vaccínia bovina?

0. Não ( ) 1. Sim ( ) **Local da lesão**:_____**Duração das lesões**: _____99. Ignorado ( )

5. Teve contato com algum indivíduo com Vaccinia bovina?

0. Não ( ) 1. Sim ( ) 99. Ignorado ( )

6. Exame clínico para marca vacinal (cicatriz de vacina contra varíola) no braço esquerdo do participante:

0. ( ) Ausência da marca vacinal 1. ( ) Presença da marca vacinal

7) Recentemente, você teve algum sintoma como: febre, dor de cabeça, dor no corpo, afta oral, linfadenopatia (íngua) algum tipo de lesão, sintoma respiratório?

0. Não ( ) 1. Sim ( ) **Qual? _____Quando?** __99. Ignorado ( )

SEÇÃO 3 – CONTATO COM ANIMAIS DOMÉSTICOS E SILVESTRES E HÁBITOS PESSOAIS

1. Você tem contato com animais domésticos?

0. Não ( ) 1. Sim ( ) - Qual? Cachorro ( ) Gato ( ) Cavalo ( ) Bovino ( ) Caprino ( ) 99. Ignorado ( )

2. Teve / tem contato com animais apresentando lesões?

0. Não ( ) 1. Sim ( ) **Quais**:_____99. Ignorado ( )

3. Já viu roedores na sua propriedade?

0. Não ( ) 1. Sim ( ) **Onde**:_____ 99. Ignorado ( )

4. Você tem contato com animais silvestres (macacos: micos, saguis, rato do mato)?

0. Não ( ) 1. Sim ( ) **Quais**:_____

99.

Ignorado ( )

5. Frequenta ambiente de mata?

0. Não ( ) 1. Sim ( ) 99. Ignorado ( )

6. Você frequenta propriedades rurais (fazenda)?

0. Não ( ) 1. Sim ( ) 99. Ignorado ( )

7. Você já viu macacos próximos à sua propriedade?

0. Não ( ) 1. Sim ( ) 99. Ignorado ( )

8. Foi vacinado contra Febre Amarela?

0. Não ( ) 1. Sim ( ) **Quantas vezes? _____Data da última vacinação:**

_____/_____/_____99. Ignorado ( )

9. Você já teve Febre Amarela?

0. Não ( ) 1. Sim ( ) **Quando? _____Quais foram os sintomas?**

_____99. Ignorado ( )

10. Você já teve Dengue?

0. Não ( ) 1. Sim ( ) **Quando? _____**99. Ignorado ( )

11. Você já teve Zika?

0. Não ( ) 1. Sim ( ) **Quando?** _____ 99. Ignorado ( )

12. Você tem hábito de consumir bebidas alcoólicas?

0. Não ( ) 1. Sim ( ) Uma vez na semana ( ) Até 2 vezes por semana ( )Entre 3 e 4 vezes por semana ( )Todos os dias ( ) 99. Ignorado ( )

13. Você fuma?

0. Não ( ) 1. Sim ( ) Uma vez na semana ( )Até 2 vezes por semana ( ) Entre 3 e 4 vezes por semana ( )Todos os dias ( ) 99. Ignorado ( )

14. Você sofre de alguma doença crônica (diabetes, hipertensão, asma, oesteoporose, colesterol alto)?

0. Não ( ) 1. Sim ( ) **Qual?** _____ 99. Ignorado ( )

SEÇÃO 4 – CONSUMO E MANIPULAÇÃO DE LEITE E DERIVADOS

1. Você faz o consumo de leite?

0. Não ( ) 1. Sim ( ) 99. Ignorado ( )

2. Qual a origem do leite consumido?

0. Não consome ( ) 1. Próprio ( ) 2. Outra propriedade do município ( ) 3. Industrial ( ) 99. Ignorado ( )

3. Você consome leite cru?

0. Não ( ) 1. Sim ( ) 2. Ás vezes ( ) 99. Ignorado ( )

4. Você manipula leite cru para produção de derivados?

0. Não ( ) 1. Sim ( ) 2. Ás vezes ( ) 99. Ignorado ( )

5.Os derivados são comercializados?

0. Não ( ) 1. Sim ( ) 2. Não se aplica ( ) 99. Ignorado ( )

6. Você consome queijo artesanal?

0. Não ( ) 1. Sim ( ) 99. Ignorado ( )

7. Realiza ordenha?

0. Não ( ) 1. Sim ( ) 99. Ignorado ( )

8. Tipo de ordenha?

0. Não realiza ( ) 1. Manual ( ) 2. Mecânica ( ) 99. Ignorado ( )

9. O leite obtido é distribuído?

0. Não ( ) 1. Sim ( ) 2. Não se aplica ( ) 99. Ignorado ( )

10. Utiliza algum produto para a limpeza das suas mãos durante a ordenha?

0. Não ( ) 1. Sim ( ) **Qual:** _____1. Antes da ordenha ( ) 2. Depois da Ordenha ( ) 3. Antes e depois ( ) 99. Ignorado ( )

11. Utiliza algum produto para a limpeza dos tetos durante a ordenha?

0. Não ( ) 1. Sim ( ) **Qual:** _____1. Antes da ordenha ( ) 2. Depois da Ordenha ( ) 3. Antes e depois ( ) 99. Ignorado ( )

Anexo 2 – TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Projeto: Avaliação de rotas alternativas de transmissão do *Vaccinia virus* em áreas urbanizadas do estado de Minas Gerais, Brasil.

(de acordo com Resolução Nº466, DE 12 DE DEZEMBRO DE 2012)

Você está sendo convidado pelo nosso grupo a participar de uma pesquisa que tem como finalidade analisar rotas alternativas de transmissão do *Vaccinia virus* (VACV). Pretendemos avaliar práticas que podem vir a facilitar o contato com o VACV. Assim, gostaríamos de convidá-lo a participar como voluntário de um estudo que nos auxiliará a compreender melhor a epidemiologia das infecções por VACV no Brasil, podendo auxiliar no desenvolvimento de medidas de prevenção.

O VACV é o agente causador da vaccínia bovina no Brasil, doença associado à formação de lesões ulcerativas em bovinos e ordenhadores, o que resulta em perdas econômicas e impactos relacionados à saúde pública. Desde 1999, vários casos de vaccínia bovina foram notificados, principalmente no estado de Minas Gerais, e um grande número de amostras de VACV foram isoladas durante esses surtos. O contato direto entre o homem e o animal infectado apresentando lesões no teto durante o manuseio sem uso de proteção, frequentemente resulta na transmissão do vírus para o homem e promove a disseminação do vírus dentro do rebanho, caracterizando essa doença como zoonótica e de caráter ocupacional.

Você está sendo convidado a participar como voluntário. Caso aceite, você responderá a um questionário simples, sobre seus hábitos, incluindo contato com animais domésticos e silvestres; e consumo e manipulação de leite cru e derivados. Além disso, será coletada uma amostra de sangue para detectar se você possui imunidade contra o vírus, no entanto, essa coleta não é obrigatória para a sua participação na pesquisa. Esse exame poderá provocar um leve ardor causado pela picada da agulha, e, muito raramente, hematoma (mancha roxa). Esses são os mesmos efeitos que qualquer exame de sangue pode causar. Também será coletada uma amostra de swab oral e swab de lesão, caso você possua alguma no momento. Todos os testes serão acompanhados por profissional habilitado e medidas para diminuir os problemas citados, como aplicação de gelo após a coleta, serão realizadas. Serão retirados 5 ml de sangue. Todo material utilizado é descartável ou estéril, portanto isento de risco de contaminação. Serão utilizadas seringas descartáveis individuais para cada paciente. Todo material utilizado será

destruído em frente ao paciente. Todos os exames a serem realizados serão gratuitos e os resultados serão enviados gratuitamente aos pacientes doadores.

Sua participação neste estudo possibilitará o entendimento sobre as práticas que podem vir a facilitar a infecção pelo VACV. Este estudo possibilitará o desenvolvimento de medidas de prevenção contra o VACV.

Para a realização deste projeto, os pesquisadores o submeteram ao Comitê de Ética em Pesquisas (COEP) envolvendo seres humanos da Universidade Federal de Minas Gerais. O COEP poderá ser contatado em caso de dúvidas éticas no TCLE, basta solicitar uma cópia ao comitê através do seguinte contato: Endereço - Av. Antônio Carlos, 6627, Pampulha - Belo Horizonte - MG - CEP 31270-901 Unidade Administrativa II - 2º Andar - Sala: 2005 Telefone: (031) 3409-4592 - E-mail: coep@prpq.ufmg.br.

Você tem a liberdade de se recusar a participar e ainda se recusar a continuar participando em qualquer momento da pesquisa, sem qualquer prejuízo. A pesquisa será realizada no Laboratório de Vírus, Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais.

Nenhum dos procedimentos usados oferece riscos à sua dignidade, e em nenhum momento usaremos seu nome. Desta forma, as identidades serão preservadas. Esclarecemos também que ao participar desta pesquisa você poderá não ter nenhum benefício direto, assim como não terá nenhum tipo de despesa para participar desta pesquisa, bem como nada será pago por sua participação. Entretanto, esperamos que este estudo nos forneça informações importantes sobre a Vaccinia Bovina, de forma que possamos contribuir para evitar infecções futuras.

Após estes esclarecimentos, solicitamos o seu consentimento de forma livre para participar desta pesquisa. Portanto, preencha, por favor, os itens que se seguem.

Termo de consentimento livre após esclarecimento

Eu,_____

li e/ou ouvi o esclarecimento acima e compreendi para que serve o estudo e qual o procedimento a que serei submetido. As informações esclarecem riscos e benefícios do estudo, deixando claro que sou livre para interromper minha

participação a qualquer momento, sem justificar minha decisão. Sei que meu nome não será divulgado, que não terei despesas e não receberei dinheiro para participar do estudo.

Assim sendo, concordo em participar do estudo.

_____, _____/_____/_____

Assinatura do voluntário	Nome: Identidade: Telefone pessoal: Telefone para contato: Nome do contato:
Giliane de Souza Trindade Pesquisador responsável	Endereço: Universidade Federal de Minas Gerais - Instituto de Ciências Biológicas / Departamento de Microbiologia /Laboratório de Vírus - Av Antonio Carlos 6627 - Pampulha CEP: 31 270-901 Belo Horizonte MG Brasil Correio eletrônico: giliane@icb.ufmg.br Telefones para contato: (31) 3409-3002

Marca do polegar

Anexo 3 – PUBLICAÇÕES (2017 – 2021)

viruses

MDPI

Review

Vaccinia Virus Natural Infections in Brazil: The Good, the Bad, and the Ugly

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Abstract: The orthopoxviruses (OPV) comprise several emerging viruses with great

importance to human and veterinary medicine, including vaccinia virus (VACV), which causes outbreaks of bovine vaccinia (BV) in South America. Historically, VACV is the most comprehensively studied virus, however, its origin and natural hosts remain unknown. VACV was the primary component of the smallpox vaccine, largely used during the smallpox eradication campaign. After smallpox was declared eradicated, the vaccination that conferred immunity to OPV was discontinued, favoring a new contingent of susceptible individuals to OPV. VACV infections occur naturally after direct contact with infected dairy cattle, in recently vaccinated individuals, or through alternative routes of exposure. In Brazil, VACV outbreaks are frequently reported in rural areas, affecting mainly farm animals and humans. Recent studies have shown the role of wildlife in the VACV transmission chain, exploring the role of wild rodents as reservoirs that facilitate VACV spread throughout rural areas. Furthermore, VACV circulation in urban environments and the significance of this with respect to public health, have also been explored. In this review, we discuss the history, epidemiological, ecological and clinical aspects of natural VACV infections in Brazil, also highlighting alternative routes of VACV transmission, the factors involved in susceptibility to infection, and the natural history of the disease in humans and animals, and the potential for dissemination to urban environments.

Keywords: orthopoxvirus; smallpox vaccine; vaccinia virus; zoonosis; public health; ecology; host range; natural infections

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1. Introduction

The *Poxviridae* family is comprised of large DNA viruses, capable of infecting a variety of organisms. It is divided into two subfamilies: the *Entomopoxvirinae*, which are invertebrate viruses, and the *Chordopoxvirinae*, which are those that infect vertebrates. *Orthopoxvirus* (OPV) is a genus of the *Chordopoxvirinae* subfamily that is significant due to its impact on global public health. Among the

10 species in OPV genus, the variola virus (VARV) is arguably the most significant, due to its role as the etiological agent of smallpox, a devastating human disease [1].

Variola virus (VARV) emerged in the human population concomitantly with the establishment of the first agricultural settlements, probably between 8000 and 10,000 years ago [2]. As the human population began to grow and spread, VARV became endemic in virtually every area of the globe. Smallpox had a profound impact on the human population; responsible for killing approximately 500 million of people in the last century alone [2–5]. In May 1980, smallpox was declared eradicated due to a herculean effort promoted by the World Health Organization (WHO), which established the global smallpox eradication program. This conquest only became possible due to the worldwide distribution of a live vaccine composed of a related virus from the OPV genus, the vaccinia virus (VACV) [1,3,4,6–8]. VACV is remarkably important to the history of vaccination, immunology, and virology. However, despite being an extremely well-studied virus, its origin and natural history remain poorly understood [4].

1.1. A Little Bit of History

The concept of vaccination was first explored by Edward Jenner, an English physician, in 1796 [9]. Jenner showed that inoculation of purulent samples from lesions of a pox-infected sick cow (Cowpox) produced a local lesion in the inoculated human, consequently protecting against smallpox. The vaccination process was then created and the virus used, cowpox virus (CPXV), was arm-to-arm transported to different countries and continents [3,4,10]. Stimulated by the fear of vaccinal syphilis transmission from human sources of the vaccine, the arm-to-arm vaccination was then replaced by propagation of the vaccine in calves in the mid-19th century [3]. Calves were first used for vaccine production in Italy, and this process gradually spread throughout Europe [3]. By the end of the 19th century, there several "backyard" factories had been established in Europe where vaccine production was carried out as an unregulated activity. Due to the lack of quality control and regulation unsurprisingly, the "vaccine virus" was, in fact, a miscellaneous mixture of different virus strains of different origins, passage histories, and properties [3,4].

The first description of VACV appeared in historical descriptions of the smallpox vaccination process, and date back to the 1930s, when Allan Downie demonstrated that the material used for the smallpox vaccination (vaccinia virus) at that time had distinct biological

properties that were distinct from the cowpox virus (CPXV) [3,11–13]. VACV had been introduced randomly over the course of vaccine manufacture in the 18th or 19th centuries [13]. At that time, arm-to-arm vaccination had already been replaced by propagation in calves.

The development of a heat stable vaccine was only achieved in the 20th century. By 1950, the vaccine was produced by vaccine manufacturers who infected the flanks of calves with vaccinia [4]. In 1953, the global eradication program was proposed by the WHO, however, no country expressed interest in eradicating smallpox worldwide until 1958. It was only in 1966, during the 19th WHO World Health Assembly, that the global eradication program took off due to increased investment in this effort [3,4,14].

By February 1967, there were 77 vaccine manufacturers distributed throughout 52 countries. At that time the vaccine was produced by methods which included multiple hosts for virus growth including calf, sheep, water buffalo, chick embryo and tissue culture (bovine embryo fibroblasts culture). The vaccines produced on the skin of animals were by far the most extensively used by manufacturers throughout the world, including developed and developing countries. During the Intensified Smallpox Eradication Programme, launched in 1967, several VACV strains were used worldwide, which included the Lister, New York City Body of Health and Paris strains, and it is probable that some of these strains shared a common ancestry. These vaccine strains were chosen based on the low virulence profile and their distribution was varied between the different continents [3]. Among the orthopoxviruses, VACV is the most comprehensively studied. During the time of mass vaccination, it was assumed that VACV could never establish itself in nature due to the fact that it had presumably become laboratory attenuated and because its origin and natural hosts remained obscure [1,13,15–18]. However, several VACV strains have been described from different locations throughout the world. In India, a VACV sub-lineage, named buffalopox virus (BPXV), is a re-emerging zoonotic viral infection affecting mainly buffaloes, but also bovines, and humans that have come into direct contact with those infected animals [19–21]. The first recorded incidence of BPXV infection in buffaloes was in 1934 [22–26], and since then several outbreaks have been described in India but also in other countries such as Pakistan, Egypt, Nepal, and Bangladesh [19,20,27,28]. In addition, another VACV strain, named rabbitpox virus (RPXV), has been associated with infections in domestic rabbits in the Netherlands and the United States [29,30]. The disease ecology and virus transmission chain for these strains are still poorly understood. Similarly to the Indian subcontinent, the circulation of VACV has been described in some South American countries including: Argentina [31]; Uruguay [32];

Colombia [33], and; especially in Brazil [34] (Figure 1). In this article, we present a comprehensive review of the literature combined with some recent data obtained by our research group focused on the emergence of VACV in Brazil due to the preponderance of cases reported, and also because of the impact that its natural circulation is causing in this country.

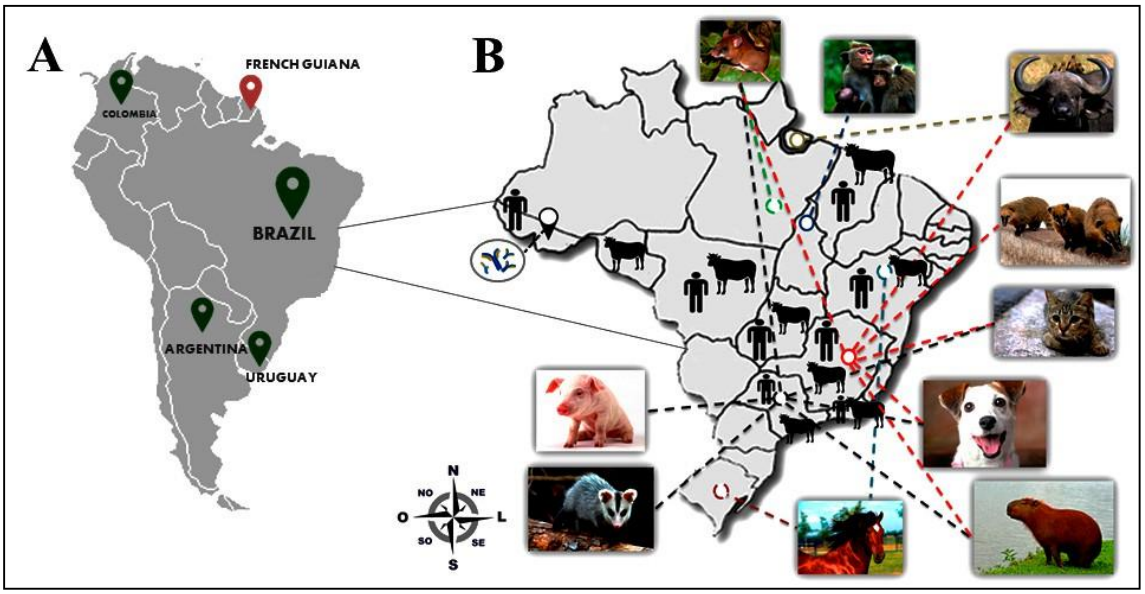

Figure 1. Detection and distribution of vaccinia virus (VACV) in South America. (**A**) A map of South America is shown on the left and the green pins indicate countries where VACV has been detected in recent years. The red pin indicates the absence of VACV detection in French Guiana; (**B**) A map of Brazil highlighting the distribution of VACV in different regions and the detection of VACV in a broad range of hosts. The antibodies (blue) indicate serological evidence of VACV circulation in humans in rural areas of Acre state. Dashed lines in different colors represent VACV circulation in different Brazilians states, i.e. red dashed lines represent VACV circulation in Minas Gerais State, black dashed lines represent circulation in São Paulo State, and green dashed lines represent circulation in Pará State.

The circulation of zoonotic VACV was first reported in Southeast Brazil in 1999 [35,36]. The infection was associated with several exanthematous outbreaks that have been described in Brazilian rural areas affecting mainly milking cattle and their handlers [37–46]. In Brazil, the disease caused by VACV is popularly known as "bovine vaccinia" (BV), probably due to the fact that most cases have been described in dairy cattle. In this review, we will focus

on the emergence of VACV in South America, mainly in Brazil, addressing the clinical, evolutionary and eco-epidemiological aspects.

1.2. The Disease Named Bovine Vaccinia

Bovine vaccinia (BV) is the name used to describe a vesiculopustular exanthematous disease in milking bovine herds and dairy workers who are in direct contact with these animals. BV is characterized by exanthematic lesions on the teats and udders of affected cows [38,47–50]. In naturally infected cows, BV lesions appear as red papules mainly located on the skin of the teats and udder. Papules progress to vesicles and vesicles become umbilicated pustules surrounded by inflammatory tissue. These pustules progress to ulcers until complete wound healing occurs (Figure 2). Calves that feed on infected cows often present with lesions on the lips, muzzle, and mouth [38,51]. A recent study has shown that in experimentally infected dairy cows, vesicles and papules appear around 3–4 days post infection (d.p.i.), and ulcers appear around the 5th d.p.i. and can last up to the 15th d.p.i. Scab formation begins on the 6th d.p.i., and healing commences on the 18th d.p.i. Infected dairy cows can develop well-defined ulcers in the oral mucosa and the mammary lymph nodes can appear enlarged by the 18th d.p.i. [47–49,52].

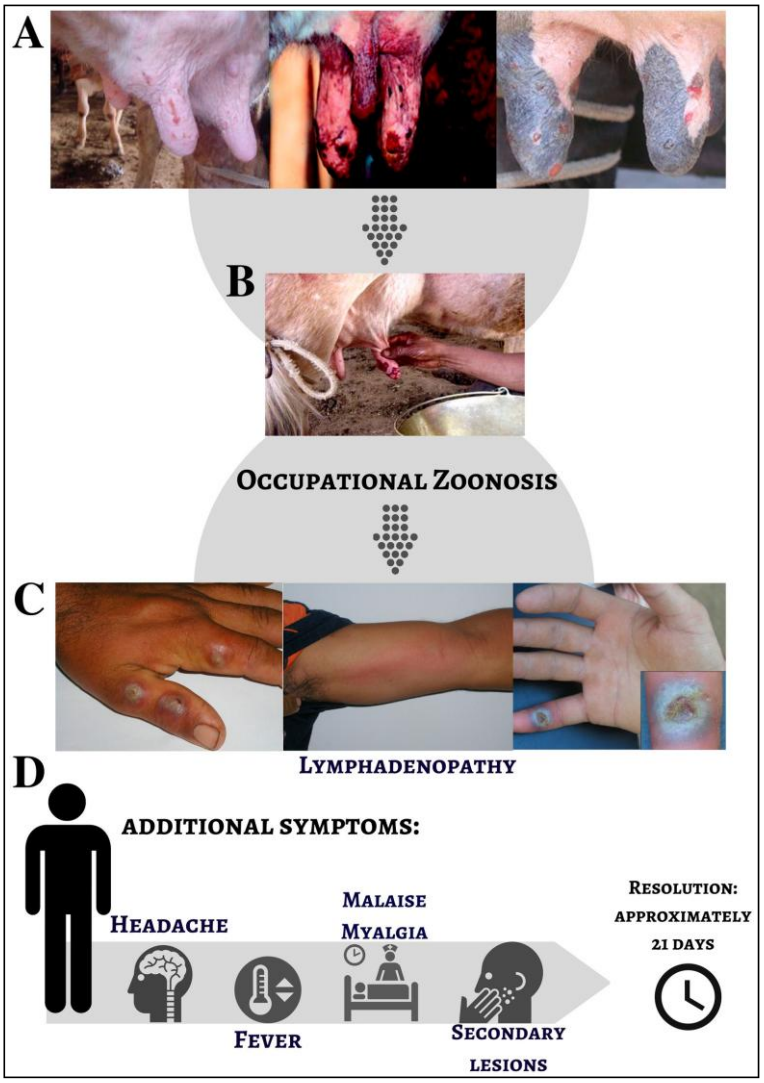

Figure 2. Clinical presentation of bovine vaccinia infection in dairy cattle and humans. (**A**) Nodular and ulcerative lesions on the udder and teats of dairy cows; (**B**) The classical transmission of VACV involves direct contact between dairy workers and infected cows; (**C**) Nodular and ulcerative lesions on hands and forearms of rural workers; (**D**) Additional systemic symptoms present during VACV infection in humans (Source: [43,53–55]).

BV is considered an occupational zoonosis, with dairy workers and farmers being the main risk group. Studies reporting BV outbreaks have shown that affected individuals generally have direct contact with dairy cattle, often due to the activity of milking. In fact, the processes involved in milking process and basic cattle management represent the main route of VACV transmission [34]. Individuals who have direct contact with infected dairy cows will develop cutaneous localized punctuate lesions on the skin accompanied by itching and followed by local

edema and vesicular lesions [38,40,42,43,56–58]. Systemic symptoms such as fever, headache, malaise, myalgia, inguinal lymphadenopathy and the development of secondary lesions are also observed (Figure 2) [34,40,42,43,59].

During the intensified vaccination campaign for smallpox eradication in the 1970s, several outbreaks linked to domestic animals derived from vaccinated individuals were reported not only in Brazil but also in other South America countries [3]. Only at the end of the 90s, almost 20 years after the discontinuation of smallpox vaccination in Brazil, BV outbreaks started to emerge (Figure 2) [15,34,60,61].

2. The Good: Uncovering the Eco-Epidemiological and Evolutionary Aspects of Natural Infections with Vaccinia Virus

While the emergence of VACV in Brazil has had profound negative impacts on the dairy industry and public health in general, the natural circulation of this virus in the country offers a good opportunity to expand our understanding of its eco-epidemiological and evolutionary aspects. The first Brazilian VACV isolate dates back to the 1960s and was isolated from wild and sentinel rodents through the efforts of the Rockefeller Institute for Research on Arboviruses located in Brazil [62–64] (Figure 1). However, BV outbreaks and VACV isolates from humans and dairy cattle appear in the literature from the late 1990s, from studies based in rural areas of the Southeast region of Brazil (Rio de Janeiro, São Paulo, and Minas Gerais states). While VACV circulation has been reported throughout the country, this southeast region of Brazil remains the epicenter of BV outbreaks, with Minas Gerais state being the most affected region. This is probably due to the fact that Minas Gerais state is the largest producer of milk in the country and possesses some of the largest dairy cattle herds in Brazil [34,65–67]. Despite its increasing significance, BV notification is not mandatory to Brazilian health authorities and reports of outbreaks and case studies are still restricted to few research groups in Brazil. While many aspects of natural VACV infection have been described in the last two decades, much is still unknown about its circulation in nature, alternative routes of infection and its natural hosts/reservoirs.

Concomitant with its wide geographical spread, VACV has been detected in a broad range of host species including farming/production animals (bovines, equids, swine, and buffaloes) and companion animals (dogs and cats) [17,34,61,68–71]. VACV has also been reported in wildlife (Supplementary Table S1) (capybaras, primates, and marsupials) [69,72–74] and in synanthropic (*Mus musculus* and *Rattus* sp.) and wild rodents. Despite

VACV detection in several mammalian species, viral circulation in sylvatic cycles and the identification of potential reservoirs still represent an aspect of its natural history that requires further exploration [15,18,74–76].

In an attempt to elucidate the origin of BV outbreaks and a possible VACV reservoir, some investigators have demonstrated through serological or molecular evidence, OPV and VACV circulation in animals that naturally transit between rural and sylvatic environments. The detection of anti-OPV antibodies and VACV DNA in primates (*Cebus apella* and *Alouatta caraya*) from the Amazon region [72] and procyonids in São Paulo state are good examples [77]. Molecular and serological evidence of VACV circulation has also been observed in several species of marsupials (*Didelphis* sp., and *Caluromys philander*) and wild rodents (*Calomys* sp., *Akodon* sp., *Necromys lasiurus*, *Trinomys setosus*, *Cerradomys subflavus*, *Oligoryzomys* sp., and *Nectomys squamipes*) collected in regions with and without previous BV history [18]. It is important to emphasize that many of the captured rodents are generalists by habit, and therefore, easily adapt to anthropic environments [18]. Indeed, the importance of wild rodents in the VACV transmission chain in rural areas has been suggested, also in conjunction with interactions between wild rodents and marsupials, which could maintain natural VACV circulation and trafficking between forests and peridomestic environments [18]. Furthermore, data presented by Miranda and colleagues reinforce the findings proposed by Abrahão et al., who proposed an ecological model to explain how the rodents could act as a link for VACV spread between wild and anthropic environments [56].

Since the isolation of the first presumably zoonotic VACV isolates in Brazil, two controversial hypotheses have arisen relating to BV outbreaks and the origin of VACV [15,17,75,78]. The first of these proposes that Brazilian vaccinia viruses (Br-VACV) originate from a vaccine strain, which could have escaped to the field. The second hypothesis proposes that these isolates had been circulating among wild animals before the introduction of VACV, though have recently emerged as zoonotic agents. Several studies have confirmed a remarkable two-type population structure following analysis of several VACV isolates, distinguishable by their genetic and biological features, such as molecular signatures (single nucleotide polymorphism (SNP) and insertion or deletion of bases (indels) in hallmark genes), and their virulence in vivo and in vitro [17,68,79,80]. Recently, Medaglia and colleagues [17] proposed a new evolutionary relationship between the Br-VACV isolates and VACV vaccine strains based on molecular analysis of Serro 2 virus (S2V) and Cantagalo virus (CTGV), two Br-VACV isolated during BV outbreaks [35,43]. In that study, the S2V and CTGV were genetically related to VACV Instituto Oswaldo Cruz (VACV-IOC) strain, and

not horsepox virus, which is believed to be related to an ancestor of the VACV lineages [13,17]. However, the absence of whole genome sequences from Br-VACV isolates represents a significant gap that would help to elucidate the origin and evolutionary history of the viruses circulating in South America. On the other hand, the characterization of VACV isolated in Colombia during the outbreak in 2014 could provide clues to better understand the evolutionary history of VACV, and also its natural transmission cycle. Usme-Ciro et al. demonstrated that the VACV identified in Colombia were related to Br-VACV group 1 isolates, although phylogenetic analysis suggests that the strains from Brazil and Colombia diverged long ago or independently arose [33].

Based on the canonical gene marker A56R (viral hemagglutinin) [81,82] phylogenetic analysis showed the clustering of Br-VACV isolates in two distinct clades (Figure 3) corroborating previous studies [15,17,18,33]. Following naming conventions already proposed in the literature these Br-VACV clades have been designated as Group 1, comprised of S2V, CTGV, Guarani P2 virus (GP2V), Passatempo virus (PSTV) and other non-virulent strains; and Group 2, comprised of Guarani P1 virus (GP1V), Serro virus 2011 (SH2V), Pelotas 1 virus (P1V) and other virulent strains.

Smallpox eradication remains one of the most important achievements in science and public health and, during that time, many different VACV strains were used as vaccines around the world. Consequently, its escape to the field is a plausible event. However, the presence of two biological and genetically divergent groups suggests a distinct evolutionary history for Br-VACV. The lack of complete VACV genome sequences derived from naturally circulating VACV isolates means that we can only speculate on the origins of Br-VACV strains and their relationship to vaccine strains. Despite the fact that other studies using gene markers for phylogenetic inference have consistently confirmed the two-type population structure of Br-VACV isolates, our analysis (Figure 3) only represents a small portion of the entire genome, used here as the A56R gene has been largely used in the molecular characterization of most VACV isolates during BV outbreaks [68,75,83]. An alternative hypothesis worth considering is the possible misdiagnosis of cowpox virus in Brazil in past decades. Veterinarian textbooks describe cowpox lesions in cattle and in 1985 there was, for example, a publication reporting the detection of cowpox biologically identified as cowpox virus, but with characteristics of what we now know as bovine vaccinia [84]. This could indicate the circulation of zoonotic Br-VAVC well before the Br-VAVC outbreaks that emerged during the late 1990s [85–87].

Our data reinforce the previous reports that indicate the circulation of two distinct Br-VACV

groups. These data support the notion that both scenarios, that is, the emergence of wild isolates and vaccine escape, may have contributed to the emergence of the two distinct zoonotic Br-VACV groups in circulation. Lastly, looking beyond the intricate evolutionary history of Br-VACV, it is reasonable to be concerned that the emergence of new genetic variants could give rise to more virulent strains in the future, thereby having a greater impact on public health and in the environment.

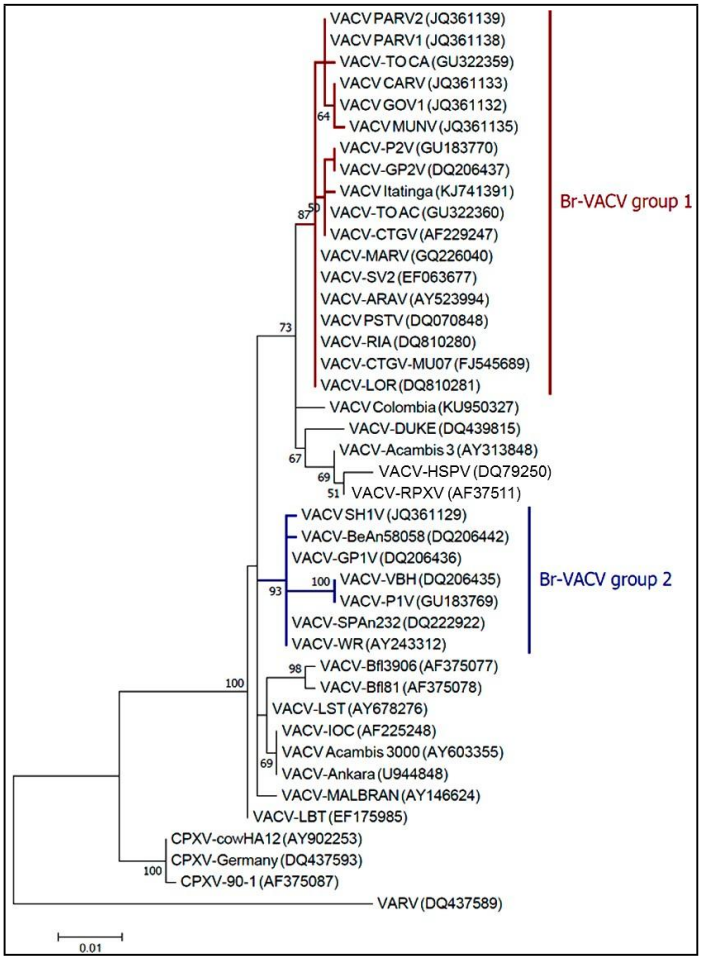

Figure 3. Phylogenetic analysis based on the A56R gene of VACV vaccine and wild isolates, with cowpox virus (CPXV) sequences included as an outgroup. These sequences are available in the NCBI nucleotide database under the GenBank Accession Numbers provided in brackets on the tree. The sequences were aligned by using ClustalW algorithm and the evolutionary history was inferred by using the Maximum likelihood (ML) method, using Mega 7.0 (GE Healthcare, Buckinghamshire, UK) software and the Jukes-cantor model was selected for ML inference by the program JmodelTest

2.1.6 (Free Software Foundation, Inc., Boston, MA) The evolutionary distances were computed using the Maximum Composite Likelihood method with 1000 Bootstrap replicates. The analysis involved 65 nucleotide sequences with a total of 734 positions in the final dataset. Evolutionary analyses were conducted in Mega 7.0 software.

3. The Bad: The Economic and Public Health Burden Associated with Bovine Vaccinia and the Alternative Routes of Zoonotic VACV Transmission in Brazil

3.1. The Burden of Bovine Vaccinia for Agricultural Industry in Brazil

The agricultural industry is extremely important to the Brazilian economy. Brazil is ranked as the fourth largest dairy producer in the world, and around 35 billion liters of milk were produced in 2015, generating 30 billion US dollars, and employing 4 million people [88,89]. Minas Gerais state is traditionally known for the production of milk and milk-derived products and accounts for 25.5% of the total volume of milk produced in Brazil, leading the national average [88]. As BV outbreaks mainly affect dairy cattle and milkers, the disease has a great impact on the dairy economy. Because infected individuals experience a 21-day period of illness, they became temporarily unavailable to work due to the painful vesiculopustular lesions developed, and also further systemic symptoms (Figure 2). Hence, with the rural workers sick and temporarily away from work, there is a need to acquire new dairy professionals [15,34,38,40,43,53,90,91]. Importantly, during the onset of illness, the interaction between humans and animals favors the spread of infection to the cattle herd, as well as the introduction of sick cattle to naïve herds through trade between farms or through sick dairy workers who often work at more than one property per day [36].

Regarding dairy cattle, the presence of painful vesiculopustular lesions, followed by secondary bacterial infections (Figure 2), makes the milking process difficult, resulting in a decreased milk production. The attack rate in lactating cows is generally very high, ranging from 80–100%, which can lead to a rapid viral dissemination throughout the cattle herd, also affecting calves that feed on sick lactating cows [34,53]. The vesiculopustular lesions present on the cows' teats are very painful, and the action of milking action can lead to the loss of teats thereby reducing milk production. There is currently no evidence that milk contaminated with VACV particles can cause disease, that is, lesions on the mouth. Furthermore, there are no studies demonstrating the presence of anti-VACV antibodies in people who ingested raw milk and cheese but did not handle infected dairy cattle [92,93]. As a consequence, the small properties that depend exclusively on the dairy economy as the

main source of income are largely affected. Furthermore, there are high financial costs associated with medical and veterinary expenses during the quarantine period, which is mainly a burden to small farmers [34,38,53]. Another important aspect associated with BV burden is the under-reporting of most outbreaks,

and probably the absence of a differential diagnosis that could promptly improve surveillance efforts [43,92,94]. BV can easily be confused with other vesicular diseases that affect cattle, such as foot-and-mouth disease, vesicular stomatitis, pseudocowpox, bovine papular stomatitis virus (BPSV) and herpetic mamilitis [45,76,95]. Control measures can be implemented to reduce and prevent the spread of VACV, such as the suspension of trade and/or traffic of the dairy herd, as some authors have demonstrated the spread of VACV through cattle movement [96,97].

3.2. Bovine Vaccinia: A Neglected Public Health Concern

In natural VACV infections, the classical form of transmission is through direct contact between rural workers (milkers and farmers) and infected bovines, making BV an occupational zoonosis [34,78,91]. During the milking process, rural workers that handle infected dairy cows without adequate personal protective equipment (i.e., gloves) can easily be infected with VACV. Infected individuals commonly develop vesicular lesions on their hands and forearms, and, as VACV is epitheliotropic, the infection can easily disseminate throughout the body by self-inoculation [40,53,98]. BV outbreaks have high morbidity, with active infections reported in approximately 80% of humans working on affected properties and has been attributed to immune modulation by VACV [99]. Despite the impact of BV outbreaks on the dairy economy and public health, epidemiological surveillance is not sufficient to monitor and control the disease, and the number of human cases is still underestimated [92]. The incidence and prevalence of VACV infections are poorly studied in Brazil. Recently, Costa and colleagues detected neutralizing antibodies against OPV at a prevalence of 30.8% in a rural population from an important dairy basin in Minas Gerais state, where several BV outbreaks have been recorded [43,68,93]. Although 32.1% of individuals in that study were vaccinated against smallpox, almost 20% of non-vaccinated individuals were exposed to zoonotic VACV infections [92]. Several risk factors for VACV infection were noted in the study group including being employed as rural workers, direct contact with bovines and equids, milking, contact with raw milk for cheese production, and the occurrence of previous BV outbreak in the area [92]. Likewise, Mota et al. observed an overall seroprevalence of 27.9% in individuals from

Amazonian rural villages,
with 23.4% of non-vaccinated individuals exposed to zoonotic VACV infections.

Another important concern associated with BV outbreaks is the inadequate treatment of infected individuals, related to the difficulty of healthcare professionals in recognizing the disease [34,43,94]. Clinically, human VACV infection can be confused with other similar vesiculopustular infections such as parapoxviruses, leishmaniosis, mycosis, staphylococcal or *Bacillus anthracis*, making an accurate diagnosis difficult [54,94,100,101]. Concerning the similarity between the infections caused by parapoxvirus and orthopoxvirus, it is important to highlight that cases of co-circulation and co-infection between parapoxvirus and VACV have already been described in Brazil, making the possibility of a clinical diagnosis even more difficult [54,94,100,101].

A recent study evaluated the knowledge of healthcare professionals from a BV endemic area, and confirmed that 43.1% of participants were unaware of BV and aspects related to zoonotic VACV infections [94]. A common theme in the literature is that person-to-person VACV transmission can easily occur due to the direct contact with individuals recently vaccinated against smallpox [102–107], and direct contact with individuals naturally infected with zoonotic VACV [93,104,108]. Hence, person-to-person transmission should not be neglected during zoonotic BV outbreaks as infected individuals could act as possible sources of infection for healthcare professionals, increasing the burden to public health [94]. Furthermore, the possibility of nosocomial VACV spread should not be neglected [109], and further attention should be given to these infections, mainly due to the possible fatal complications that can occur in immunocompromised patients [102,110].

4. Alternative Routes of Zoonotic VACV Infections

As already mentioned, BV outbreaks and VACV infections have been described in all Brazilian territories (Figure 1, Supplementary Table S1), and most affected hosts are humans and bovines [34,91]. However, many other hosts have been suggested to participate in the VACV transmission chain [18,34,69,70,74,91], as well as additional forms of zoonotic VACV transmission [93]. Since the detection of anti-OPV neutralizing antibodies in residents of rural settlements in the Brazilian Amazon (Acre state), a region without any reports of BV outbreaks previously, some authors have discussed other possible VACV exposure routes to humans [111]. No correlation was observed between antibody detection and contact with cattle in this region. These data point towards alternative routes of OPV exposure and the hypothesis proposed is the close interaction between these individuals and the wild environment [111].

However, Costa and colleagues, upon evaluating a rural population from a BV endemic area, did not observe a correlation between anti-OPV neutralizing antibodies and contact with wild environments. Despite this, access to wild areas and contact with wild animals should be better explored as a possible route of human infection [92].

Corroborating the hypothesis that alternative routes of VACV transmission exist in Brazil, a family cluster was investigated for a possible case of person-to-person VACV infection [93]. Neutralizing antibodies and VACV DNA were detected in the blood of subjects, and manual milking was excluded as the main source of exposure. It was assumed that transmission had occurred via direct contact between the test subject and the farmer who had VACV lesions on his hands [93]. Previous studies of intrafamilial transmission were described in which VACV was transmitted by milkers to other residents on their properties through direct contact [104,108]. In addition, VACV was isolated from domestic utensils in the home environment of an infected patient during a BV outbreak [58]. Indeed, VACV particles are resistant in the environment, remaining viable across a range of temperatures or associated with organic matter [112,113]. However, in the family cluster study mentioned previously, participants reported the usual consumption of raw milk and artisanal cheese [93]. Taking this into account, the possible role of milk and dairy products as a source of infection has raised interesting questions regarding VACV epidemiological cycle [50,76,114,115]. Some reports support the hypothesis that milk is a potential source of VACV exposure and/or transmission. The first evidence in support of this was the isolation of VACV from milk samples during BV outbreaks in Minas Gerais [76]. Viral particles could remain viable even after contaminated milk being submitted to different thermal treatments [114]. Recently, Rehfeld and colleagues evaluated the ripening process applied to reduce cheese contamination and modify its physical and chemical characteristics. However, VACV infectious particles persisted throughout and following the ripening process and were isolated 60 days after the ripening period. Additionally, VACV DNA was detected in milk from symptomatic and asymptomatic dairy cows, including properties where BV outbreaks had not reported [50].

In Minas Gerais state, artisanal cheese has been recognized as an intangible heritage item, and is traditionally made using raw milk. To better understand the role of artisanal cheese as a possible source of VACV infection, we analyzed commercial artisanal cheese samples produced in Minas Gerais State. A total of 38 samples were collected from June 2015 to June 2017 in Belo Horizonte city, however, the samples were produced on dairy basins, corresponding to the following cities or state regions: Serro, Araxá, Alto Paranaíba/ Cerrado

and Canastra (Figure 4).

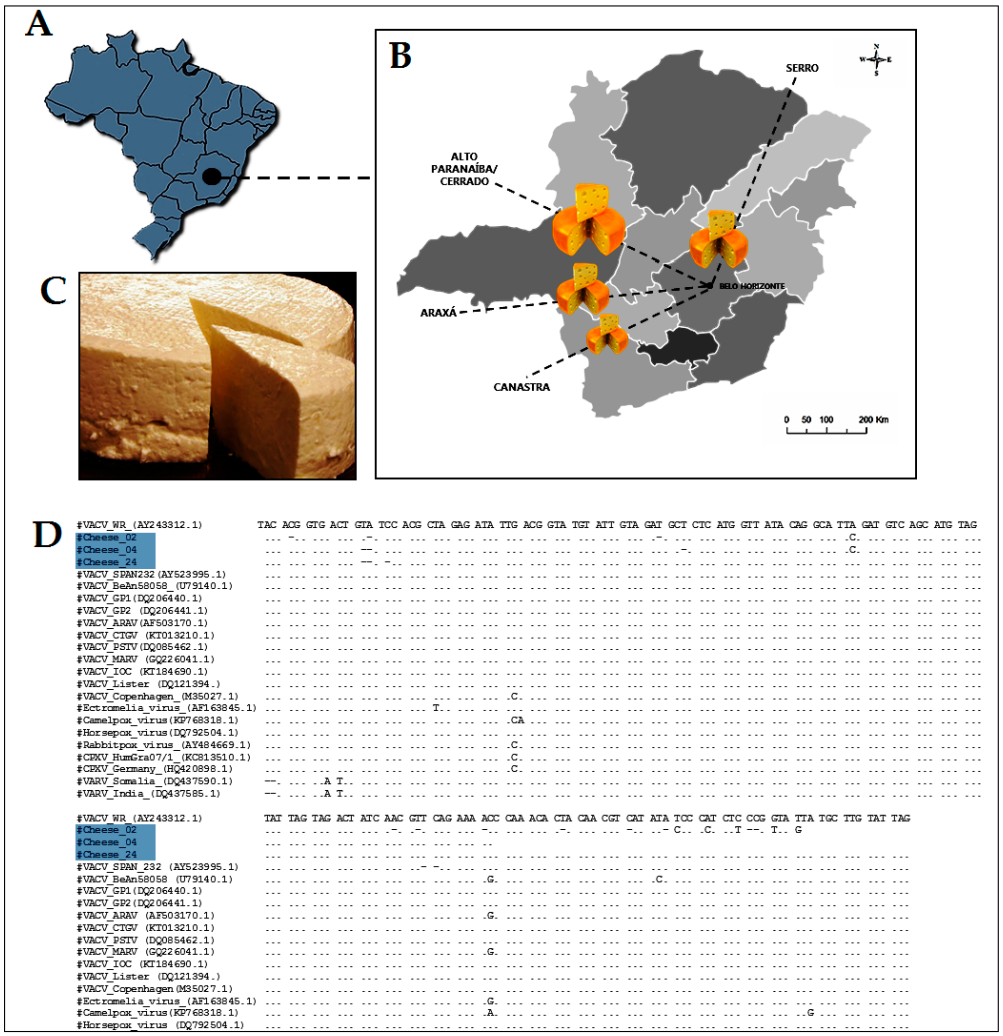

Figure 4. Distribution of artisanal cheese samples in four different dairy basin in Minas Gerais state.

(A) Map of Brazil highlighting where Minas Gerais state and Belo Horizonte city are located;

(B) All samples were collected in Belo Horizonte city, in the central area of Minas Gerais; (**C**) An example of artisanal cheese produced in Minas Gerais state; (**D**) Nucleotide sequence of the VACV detected in commercial artisanal cheese samples (blue) C11R (viral growth factor) gene compared with homologous sequences of several other orthopoxviruses. The amplified fragments were sequenced in both orientations by the dideoxy method in an ABI3130 platform (Applied Biosystems, Foster City, CA, USA), and sequence quality was analyzed by using Sequence Scanner Software 1.0 (Applied Biosystems, Foster City, CA, USA). Sequences were aligned (ClustalW (http://www.genome.jp/tools/clustalw). Information regarding chesse samples processing has been included as Supplementary Material.

OPV-specific nested-PCR was performed targeting the C11R gene. Real-time PCR targeting A56R gene was also performed [51,80,116]. Eight samples (21.0%) tested positive for C11R gene, and three (7.9%) for A56R (Table 1). Only one sample (2.6%) tested positive for both targets. The alignment of the C11R fragments showed high similarity to the other VACV isolates from Brazil (Figure 4).

Table 1. Commercial artisanal cheese samples produced in four different dairy basins in Minas Gerais state tested for the presence of vaccinia virus DNA, 2015–2017.

Sample	Date (Month/Year)	Dairy Basin	Nested-PCR C11R	Real-Time PCR A56R
01	June/2015	Serro	+	—
02	June/2015	Serro	+	—
03	June/2015	Serro	—	—
04	June/2015	Serro	+	—
05	June/2015	Serro	—	—
06	June/2015	Serro	+	—
07	February/2016	Serro	+	—
08	March/2016	Serro	—	—
09	April/2016	Serro	—	—
10	April/2016	Serro	—	—
11	April/2016	Alto do Paranaíba/Cerrado	+	+
12	May/2016	Araxá	+	—
13	August/2016	Araxá	—	+
14	September/2016	Araxá	—	—
15	September/2016	Araxá	—	+
16	September/2016	Alto do Paranaíba/Cerrado	—	—
17	October/2016	Araxá	—	—
18	March/2017	Serro	—	—
19	March/2017	Alto do Paranaíba/Cerrado	—	—
20	March/2017	Araxá	—	—
21	April/2017	Araxá	—	—

22	April/2017	Serro	—	—
23	April/2017	Serro	—	—
24	April/2017	Serro	+	—
25	May/2017	Araxá	—	—
26	May/2017	Serro	—	—
27	May/2017	Alto do Paranaíba/Cerrado	—	—
28	May/2017	Serro	—	—
29	May/2017	Canastra	—	—
30	June/2017	Araxá	—	—
31	June/2017	Serro	—	—
32	June/2017	Serro	—	—
33	June/2017	Araxá	—	—
34	June/2017	Serro	—	—
35	June/2017	Serro	—	—
36	June/2017	Araxá	—	—
37	June/2017	Serro	—	—
38	June/2017	Araxá	—	—

+: Positive samples; −: negative samples.

To our knowledge, this is the first report on the detection of VACV DNA in commercial artisanal cheeses in Brazil. Previous studies also analyzed viral viability in experimentally contaminated milk and its derivatives [50,76,114]. The consumption of artisanal cheese has already been identified as a possible new route of VACV transmission [92,93]. This relationship was also discussed during a buffalopox virus outbreak in India [98]. However, it was not clear in that study whether oral lesions resulted from milk consumption or the autoinoculation process [91]. Furthermore, Rehfeld and colleagues presented data on the transmission of VACV through the ingestion of contaminated milk using a mouse model. In addition, systemic viral spread with molecular detection of VACV DNA in the oral mucosa and feces was observed, even though all animals remained asymptomatic [117].

A study by de Oliveira et al. has demonstrated that VACV is not inactivated after thermal treatment [114]. Combined with the results presented here we believe that viral particles remaining viable and infectious throughout the ripening process is plausible and the consumption of artisanal cheeses may represent a real route of human exposure to the virus. It should be emphasized that the artisanal cheeses produced in the Minas Gerais are commercialized all over Brazil and other countries. No clinical cases of VACV infection have been directly linked to the consumption of milk or milk-derived products despite their widespread consumption in Brazil and elsewhere. Consequently, additional studies are necessary to clarify the role of raw milk and its derivatives in the VACV transmission chain.

5. The Ugly: Spreading of VACV to Urban Environments

The emergence of VACV in Brazil has had a significant impact on both the dairy economy and public health. Moreover, recent findings related VACV circulation in urban areas have raised a greater concern due to the risks and burden that could be associated with human infections.

Dutra and collaborators recently detected VACV DNA in capybaras (*Hydrochoerus hydrochaeris*) in wild areas in Pantanal and Minas Gerais state, but also in urban areas of Belo Horizonte city, Minas Gerais [69]. Capybaras are the largest wild rodent in the world, restricted to the Americas, adapt easily to anthropic environments, and can easily transit between rural and urban environments [74,118]. Hence, some investigators have suggested that these animals could transfer VACV between rural and domestic environments as they transit between farms and urban areas.

Another study describes the detection of VACV in domestic cats from urban areas in Brazil [70]. OPV neutralizing antibodies and VACV DNA were detected in domestic cats from Belo Horizonte city, Minas Gerais state [65]. Two important aspects of this study should be highlighted in the context of VACV ecology: (1) the geographic areas in which VACV-positive cats were detected comprise areas with little verticalization and the presence of green areas containing domestic animals such as bovines and equids, and wild animals (rodents, coatis, etc.); (2) the highest number of VACV-positive cats were detected in the region of Pampulha, the same region in which we had demonstrated VACV circulation in capybaras [69]. These data demonstrate that the virus is circulating in the urban environment and that the

detection of VACV in capybaras in this area was not an isolated phenomenon. Considering the presence of bovines, equids and also wild rodents in this region, we may be facing the establishment of a new and still poorly understood urban cycle of VACV transmission [65]. Also, these findings highlight the possible threat this situation poses to non-vaccinated individuals and to public health in the region. If we consider the absence of vaccination and the lack of specific treatments together with the high population contingent that can be exposed to the virus, the circulation of VACV in urban environments certainly poses an ugly scenario. Also, another aspect regarding VACV emergence in large human populations that should be explored in further studies is the potential for increased virulence, associated with human adaptation and host range changes, as has been demonstrated for the variola virus and recently for monkeypox virus [2,119].

We propose a hypothetical model based on a previous study by Abrahão et al. [56]. Based on current trends, this hypothetical model could illustrate the dynamic of VACV circulation in urban, rural and wild areas (Figure 5), and also considers important information regarding the role of domestic animals and wildlife in the VACV natural cycle. Initially, VACV outbreaks were described in rural environments affecting dairy cattle and humans. Later, it was demonstrated that other farming animals such as equids could be implicated in the VACV transmission chain. No VACV transmission has been described from equids to humans until the recently. It has been hypothesized that wild rodents could be VACV reservoirs, and peridomestic rodents could act as the link for VACV spread between wild and rural environments, promoting the transmission among wild mammals and dairy cattle, and other farm animals and humans. The circulation of VACV in wild animals such as capybaras and coatis in urban areas could favor the direct spread of VACV between wild and urban environments. Another alternative route of VACV spread in urban areas is through contaminated commercial artisanal

cheese. However, while VACV could be detected in dairy food, this alternative route is still poorly understood and further studies are necessary to clarify the role of dairy products in the transmission of VACV.

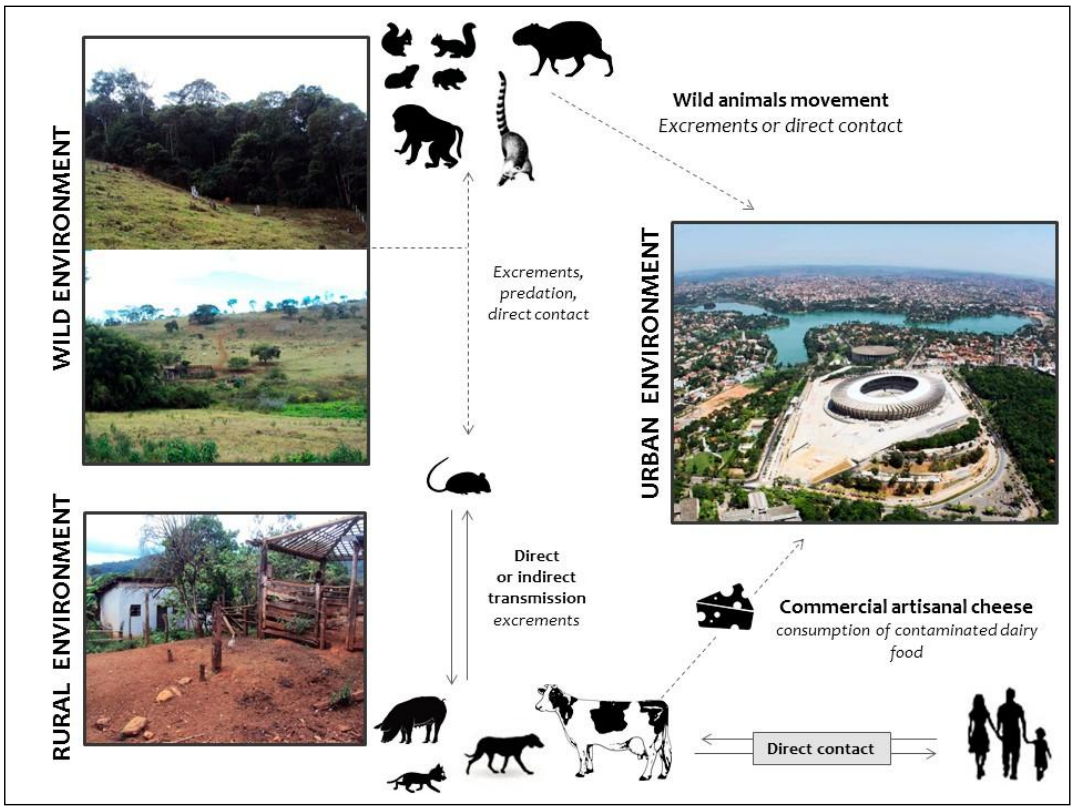

Figure 5. Hypothetical model highlighting the dynamic of vaccinia virus circulation in different hosts from wild, rural and urban environments. VACV outbreaks have been largely described in rural areas, affecting mainly dairy cattle and milkers. Equids have also been affected, although there are no human cases associated with direct contact with horses. Peridomestic rodents have been postulated as the link between bovine vaccinia (BV) outbreaks in dairy farms and VACV circulation in wildlife. Wild rodents could act as VACV reservoirs and transmit the virus to other small mammals, as well as peridomestic rodents, thus maintaining the wild-rural cycle. In urban areas, the dynamic also involves wild rodents that could be in contact with other mammals such as capybaras and coatis. These wild species can interact with domestic animals such as dogs and cats that live in regions bordering green areas (natural parks and forest reserves), which could favor VACV spread and transmission to other domestic animals and humans. Alternatively, VACV could be disseminated to urban areas through contaminated dairy products. Solid lines represent hypotheses already described. Dashed lines indicate new hypotheses pointed out by our group.

6. Concluding Remarks

Twenty years have passed since the first report confirming the circulation of vaccinia virus in Brazil. At that time, the researchers involved in the isolation and characterization of these wild and/or zoonotic isolates found it difficult to convince the scientific community that these isolates were real sylvan isolates and not mere laboratory contaminants. Today, however, the circulation of VACV in wild, rural and even urban environments in Brazil is a scientific fact. In response, a new line of discussion is necessary, at the very center of which lies an important question: how should public health authorities respond to VACV circulation to protect public health interests?

This review seeks to establish what we consider the positive and negative aspects related to the emergence of VACV in Brazil. We highlight the possibility of broadening our understanding about the ecological and evolutionary aspects of the virus as a positive aspect of VACV emergence in the region (the good), though we also discuss the negative impact that bovine vaccinia virus has had on the milk economy and on public health (the bad). This situation becomes ugly when we consider the evidence for VACV circulation in urban environments, which could have extremely dire consequences as the virus encounters a large non-immune population.

In Brazil, as in most of the world, vaccination against smallpox was terminated in the 1970s and the last case of smallpox in the country was described in April 1971. Therefore, a large part of the population is no longer immune to poxvirus infections, either because immunity has waned over the last 40 years or simply because the majority of people are too young to have been vaccinated. As of today, smallpox vaccines, composed of less virulent strains of vaccinia virus such as the Modified vaccinia virus Ankara (MVA) or the ACAM2000™—a plaque-purified derivative of the US Dryvax Vaccine—are available in many countries as a result of the heightened fear of smallpox reintroduction [10,120,121]. Nonetheless, would it be feasible to vaccinate Brazilians against vaccinia virus? From an immunological point of view, there are few doubts about the efficacy of smallpox vaccines against those VACV isolates (although no studies on the matter have been systematically conducted). Thus the real question is whether it would truly be necessary. This question is far more difficult to answer. At this point, there is an unequivocal pattern of virus spread in the country, as a growing number of isolates have been obtained all over Brazil, from different hosts, and in different environments (Figure 1). Likewise, increased contact between these viruses and people has been documented [58,93,108]. Furthermore,

many would say that the difficulties—or even risks—posed by a mass vaccination campaign using poxvirus vaccines in people is simply not justifiable in terms of the number of individuals at risk of infection. Indeed, due to the fact that the notification of BV is not compulsory in the country, we do not have access to the real number of cases in humans. Besides, we also do not have access to official data regarding adverse effects that arise from smallpox vaccination in the country, which prevents us from considering the real benefit of resuming vaccination. Others may say that the availability of new anti-poxvirus drugs, such as the ST-246 and CMX-001 [121,122], could circumvent the constraints of mass vaccination against orthopoxviruses as the number of affected people has not yet been considered indicative of an epidemic. Moreover, vaccinia immunoglobulin therapies also represent a feasible approach to treatment or prophylaxis that could undermine the need of mass vaccination [123]. However, even if we have drugs and immunoglobulin therapies available to treat VACV infections, Brazil still has issues related to treatment costs and implementation policies.

While this picture is alarming, the occurrence of BV in Brazil still represents a big question mark from a public health point of view, as scientists and officials alike are uncertain of the true public health significance of this disease and, consequently, how to manage it.

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Short communication

Cross-sectional study involving healthcare professionals in a Vaccinia virus endemic area

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A B S T R A C T

Orthopoxviruses (OPV) are emerging viruses with great importance in human and veterinary medicine, such as Vaccinia virus (VACV), which causes outbreaks of bovine vaccinia (BV) in South America. The clin- ical aspects of BV are similar to other vesicular infections, complicating the clinical diagnosis. This cross- sectional study evaluated the knowledge of Healthcare Professionals about BV and revealed their unpre- paredness about BV in a VACV hyper-endemic area in Brazil, highlighting the public health issues asso-ciated with VACV infections. This study presents an opportunity to discuss the importance of vaccination for healthcare professionals who work in areas of VACV circulation and brings an educational measure on VACV infections for health professionals around the world.

The *Orthopoxvirus* genus (OPV) comprises some emerging viruses that stand out by their importance in human and/or veteri- nary medicine, including *Cowpox virus* (CPXV), *Monkeypox virus* (MPXV) and *Vaccinia virus* (VACV). VACV has gained notoriety since it was used as a vaccine during the smallpox eradication campaign successfully conducted by the World Health Organization (WHO) [1]. After the smallpox eradication in 1980, mass vaccination was suspended, however, it is still recommended for military person- nel, health assistance, and laboratory workers in the United States and some parts of Europe [2]. Eventually, accidental infections were reported with VACV being transmitted from vaccinees to others, sometimes causing serious and even fatal adverse reactions [3,4].

VACV emerged in Brazil in the late 90s and has been associated with naturally acquired infections in humans, cattle and other ani- mals. Currently, the expansion of VACV has been observed in South

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America, Argentina [5], Uruguay [6], and Colombia [7]. VACV causes vesiculopustular disease outbreaks in cattle, usually called bovine vaccinia (BV), being an occupational zoonosis [8]. During these outbreaks, it have been mainly reported that milkers who have direct contact with infected dairy cows, present cutaneous localized punctual lesions on the skin accompanied by itching and followed by local edema and vesicular lesions. Systemic symp- toms such as fever, headache, malaise, myalgia, inguinal lym- phadenopathy are also observed [8,9]. BV has high morbidity and infected individuals often have secondary lesions due to self- inoculation [8]. Clinical aspects of VACV infection are similar to other infections such as leishmaniosis, mycosis, staphylococcal or *Bacillus anthracis*, often accompanied by secondary bacterial infec- tion making it difficult to clinically diagnose [9,10].

The classic route of VACV transmission is with direct contact

with infected animals [8,9]. However, cases in which infected patients reported no direct contact with cattle had been docu- mented, highlighting the possibility of alternative infection routes [11–13]. Though BV has been so far described in rural environ- ments, VACV has been recently detected in urban rodents and cats [8,14,15].

BV has been described in several Brazilian states, more common in particular regions where there are dairy basins, especially the

Southeast region where the most important milk production

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micro-region is located [8]. Even in regions with more cases, many cases still go undetected, or are clinically misdiagnosed as other vesicular diseases. As misdiagnosis can lead to incorrect treatment, BV becomes a real burden for veterinary and human public health. In the health system units, there is the possibility of nosocomial infections occurrence and complications for immunocompromised patients as well, especially due to the potential for person-to- person transmission [4,13,16].

A cross-sectional study was carried out during September 2014–2015 in Serro city (18°36^{0}17^{00} S 43°22^{0}46^{00} W), Minas Gerais State, Brazil (Fig. 1) to evaluate BV knowledge and experience level of health care professionals and also to assess OPV exposure through serological evidence. Serro is an endemic region for VACV circulation [9,13,17,18] and has a population of 20,833 inhabitants, being 12,895 residents in the urban area and 7938 in the rural area
(IBGE, 2010). The public health system of Serro consists of one hos- pital, nine health units, one municipal polyclinic and one epidemi- ological surveillance service. The municipality health service comprises 103 health professionals.

All 103 health professionals working in Serro were invited to participate in this study. A semi-structured questionnaire (Supple- mentary material 1) was applied to elicit demographic data (age, gender, self-reported skin color, occupation, and educational level) and knowledge about BV (clinical symptoms, transmission routes, diagnosis, host chain, treatment and prevention measures). A descriptive analysis of the results was performed using the statis- tical software EPI-INFO version 7.2 (www.cdc.gov/epiinfo). Chi- square test and Fisher exact test were used to analysis. This study was approved by the Research Ethics Committee of Universidade Federal de Minas Gerais under the registration protocol FR – 413704. An informed consent was obtained from all participants. In order to assess serological evidence of OPV exposure and anti- OPV protective immunity of healthcare professionals enrolled in this study, we also collected serum samples and tested them by ELISA IgG and plaque reduction neutralization test (PRNT) [19]. Although in Brazil BV outbreaks have been associated with VACV, serological assays performed (ELISA and PRNT) do not discriminate for any OPV specific species due to cross reactivity. Hence, the anti- bodies detected are described as IgG and neutralizing anti-OPV antibodies. Additionally, we defined vaccinated individuals as those ≥38 years old, who were born before the smallpox vaccina- tion campaign stopped. As previously described, successful vacci- nation against smallpox is usually determined by the development of a vesicular skin lesion called ‘‘take” [20]. In the

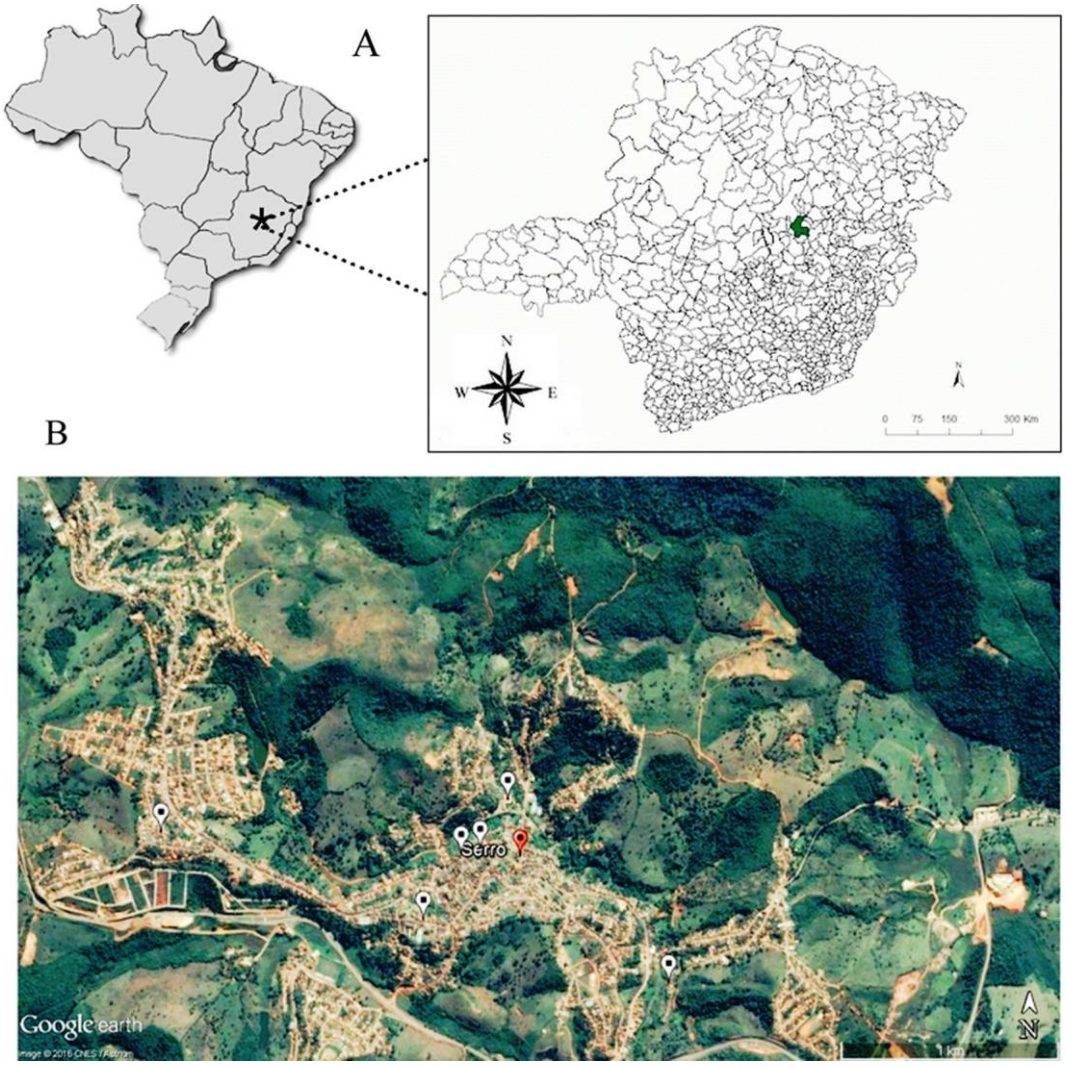

Fig. 1. Overview of Minas Gerais State, Brazil: (A) Location of Minas Gerais State in Southeast region (darkish states) of Brazil with a detailed close-up of Minas Gerais State municipals. Serro City is highlighted in green. (B) Locations of the health units sampled during the investigation are marked with white points. (Google Earth, 2017). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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vaccinated group, we also sought for the presence of vaccine ‘‘take”, which is strongly correlated with protection [21].

A total of 51 individuals (49.5% of healthcare professionals in Serro) were enrolled in this study (Table 1). Women represented 92.1% of the participants and 64.7% self-reported mixed skin color. The median age was 36 years (ranging from 19 to 60 years), and 19 individuals (37.3%) were ≥38 years old, which we considered vaccinated according to our cut-off age. In the vaccinated group, 9 individuals (47.3%) presented the vaccine take on their left arm. Twenty-seven participants were nursing professionals (53.0%), and 18 were community health agents (35.4%). Other healthcare professionals (11.6%) comprise dentists and professionals who work in health care units as pharmacy attendants, laboratory secretaries, or in general services.

BV knowledge questions are summarized in Table 2. Twenty-nine (56.9%) of participants have heard about BV. Eleven individuals (21.6%) had witnessed an outbreak of BV and only five (9.8%) said they knew that the etiologic agent is a virus. Thirty-six individuals (70.6%) said that BV does not occur only in humans, but also in other animals (cows, horses, dogs, cats and rodents), and 29 (56.9%) knew unspecific BV clinical signs and symptoms, such as fever, headache, myalgia, nausea, jaundice, vomiting, chills, disseminated lesions, and lymphadenopathy. However, 17 individuals (33.3%) did not know about BV transmission routes and 47.1% did not know about preventative measures. Forty-three participants (84.3%) did not know about laboratory diagnosis, and most of them (94.1%) did not know if the health unit where they work has access to BV diagnosis. Only 3 individuals (5.9%) indicated that samples from BV suspected cases are forwarded to the state reference laboratory (Fundação Ezequiel Dias – FUNED) and 68.6% were unaware of the proper clinical management and treatment for BV

suspected cases. It has been reported that in outbreak sites the common recommended treatment includes the use of corticos- teroids, which is usually associated with the worsening of a patient's condition [8,9].

Among the 51 participants, 22 were positive for IgG antibodies, representing an overall prevalence of 43.1% (95% Confidence Inter- val [CI]: 30.5–56.7) (Table 1). The IgG prevalence among those individuals with age ≥38 years was 52.6%, whereas for those with age <38 years was 37.5%. On the other hand, the prevalence of IgG antibodies in individuals with vaccine take was 77.8% and among those participants without a take was 35.7%. In terms of neutraliz- ing antibodies, which are correlated with protection [11,18,20,21], 12 individuals were positive in the PRNT test (23.5%, 95% CI: 13.9– 36.9), with antibody titers ranging from 100 to 6400 neutralizing units/ml (Table 1). The prevalence of neutralizing antibodies among those individuals with age ≥38 years was 63.2%, whereas all individuals with age <38 years had no detectable neutralizing antibodies. Considering the vaccine take group, the prevalence rates among those with a take were 66.7%, while among the non- take group were 14.3%.

Demographic characteristics that significantly associated with positive serology (neutralizing antibodies) were age ≥38 years ($p < 0.00001$), educational level ($p = 0.008$), time working in healthcare service ($p = 0.01$), and vaccine take. All associated with both IgG and neutralizing antibodies ($p = 0.03$ and $p = 0.0003$, respectively) (Table 1).

Here, we report healthcare professionals' knowledge and per- ceptions about BV in Brazil, a country largely affected by BV, a zoo- notic disease. BV outbreaks and VACV infections are a burden for human and animal health in Brazil, directly impacting the activi- ties of rural workers making it an occupational disease and also

Table 1

Demographic variables related to the presence of anti-OPV IgG and neutralizing antibodies for the 51 healthcare professionals from bovine vaccinia endemic area, Brazil.

Demographics	ELISA IgG	Odds ratio (95% CI)	Neutralizing antibodies	Odds ratio (95% CI)

Total of participants = 51	antibodies					
	Positive (%)[y]	Negative (%)		Positive (%)	Negative (%)	
Gender						
Male	3 (75.0)	1 (25.0)		2 (50.0)	2 (50.0)	
Female	19 (40.4)	28 (59.6)		10 (21.3)	37 (.7)	
Self-reported skin color						
Brown	14 (42.2)	19 (57.8)	5 (15.2)		28 (84.8)	
Black	6 (54.5)	5 (45.5)	4 (36.4)		7 (63.6)	
White	2 (28.6)	5 (71.4)	3 (42.8)		4 (57.2)	
Age (years)						
<38	12 (37.5)	20 (62.5)	0 (0.0)		32 (100.0)	Reference
≥38	10 (52.6)	9 (47.4)	12 (63.2)		7 (36.8)	53.1 (5.9–479.0)
Presence of vaccine take[A]						
Yes	7 (77.8)	2 (22.2)	6.3 (1.2–34.3)	6 (66.7)	3 (33.3)	12.0 (2.3–61.4)
No	15 (35.7)	27 (64.3)	Reference	6 (14.3)	36 (86.7)	Reference
Educational level						
Elementary school or less[B]	4 (80.0)	1 (20.0)	4 (80.0)		1 (20.0)	19.0 (1.9–193.3)
High school or	18 (39.1)	28	8 (17.4)		38	Reference

more[C]		(60.9)		(82.6)	
Occupation					
Nursing professionals	11 (40.7)	16 (59.3)	8 (29.6)	19 (70.4)	
Community health agent[D]	8 (44.4)	10 (55.6)	2 (11.1)	16 (88.9)	
Others[E]	3 (50.0)	3 (50.0)	2 (33.3)	4 (66.7)	
Time working in healthcare service					
≤10 years	15 (40.5)	22 (59.5)	5 (13.5)	32 (84.5)	6.4 (1.6–26.2)
>10 years	7 (50.0)	7 (50.0)	7 (50.0)	7 (50.0)	Reference

[y] Percent frequencies are shown by row.

[A] The vaccine take is a skin lesion developed after successfully smallpox vaccination, visualized on left arm of vaccinated individuals. The presence of vaccine take was determined only for those individuals aged ≥ 38 years, whom have been enrolled during WHO smallpox eradication campaign.

[B] Elementary school or less (≤8 years of study).

[C] High school or more (>8 years of study).

[D] this group includes nursing technician, nursing assistant and nurse.

[E] this group includes other professions such as dentist, pharmacy attendant, laboratory secretary and general services.

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Table 2
Frequency distribution of variables evaluated to assess the extent of bovine vaccinia (BV) knowledge of the healthcare professionals in Serro city, Minas Gerais, Brazil, 2014–2015.

Knowledge questions about BV	n (%)
Total of participants = 51	
Heard about BV before	
Yes	29 (56.9)
No	22 (43.1)
Etiological agent	
Bacteria	0
Fungi	0
Virus	5 (9.8)
Other	0
Don't know	46 (90.2)
Which hosts can get BV[y‡]	
Cows	34 (66.7)
Horses	7 (13.7)
Dogs	1 (1.9)
Cats	2 (3.9)
Rodents	10 (19.6)
Others	0
Don't know	15 (29.4)
People can get BV from[‡]	
Cows	32 (62.7)
Horses	3 (5.9)
Dogs	2 (3.9)
Cats	2 (3.9)
Rodents	3 (5.9)
Humans	8 (15.7)
Raw milk	3 (5.9)
Artisanal cheese	2 (3.9)
Others	0

protect themselves against the disease. The unpreparedness could be related to the limited knowledge about BV and Vaccinia or Orthopoxvirus infections, such that individuals have never heard about BV or VACV and are unaware of the clinical manifestations indicative of BV, forms of treatment and prevention that are essen- tial information used to recognize and manage BV cases. These data point to the need to spread information about BV among

Don't know (33.3)	17
Recognized BV clinical signs[‡A]	
Yes (56.9)	29
No (43.1)	22
Knew laboratory tests[B]	
Yes (15.7)	8
No (84.3)	43
Knew prevention measures[C]	
Yes (52.9)	27
No (47.1)	24
Know treatment forms[‡]	
There is no specific treatment	0
Pain treatment/palliative	6 (11.7)
Secondary or disseminated infections treatment	8 (15.7)
Others	0
Don't know	35 (68.6)

[y] Other hosts listed besides humans.

[‡] Multiple responses allowed, totals may not add up to 100%.

[A] Correct answers were defined if respondents specified at least lymphadenopathy or skin lesions.

[B] Correct answers were defined if respondents specified at least serological or molecular tests.

[C] Correct answers were defined if respondents specified at least using personal protective equipment and isolation of infected patients.

affecting the dairy economy [8,18]. An accurate perception of risk during patients' management, who can also act as possible sources of infection, could lead to prompting an appropriated reaction dur- ing outbreaks [16] and helping to reduce the burden and dissemi- nation of the disease.

Our results showed that 43.1% of healthcare professionals enrolled in this study have never heard about BV, which could be attributed to not being well-informed enough to recognize and therefore report clinical cases accurately. In fact, the same propor- tion of participants (43.1%) also did not know the correct clinical signs and symptoms of BV (Table 2). These findings suggest that healthcare professionals are unprepared to handle effected individ- uals during BV outbreaks. In terms of OPV surveillance, this shows a gap for community members with the lack of basic knowledge to

healthcare professionals working in Serro, where a high number of healthcare professionals were unaware of BV (Table 2) and an endemic region where BV outbreaks have been officially reported since 2005 [9,13,17,18]. In fact, those participants who have worked in the healthcare service for a period of ≤10 years are 6.4 times more likely to have neutralizing antibodies than those who have been working for >10 years (Table 1).

Some participants said they know that BV is a zoonotic disease, however, this response might be attributable to the name BV which indicates that dairy cattle can be involved. In addition, a large majority had poor knowledge about other hosts that could be infected and transmit the virus to humans. Taking into account VACV can infect a broad host range, other hosts such as dogs and cats [15,22] and alternative routes of VACV transmission should also be kept in mind.

The presence of IgG antibodies in unvaccinated individuals (those who are <38 years old), and in persons without a vaccine take, suggest that these individuals could be continuous exposed to OPV and corroborates the hypothesis of alternative routes of VACV transmission [11–13,18], which needs to be better investi- gated. Furthermore, lack of knowledge about BV can increase the chances of exposure through direct contact with infected patients and also facilitate the viral spread into a hospital environment due to the incorrect management of patients [16]. Indeed, those individuals who had a basic educational level are 19 times more likely to have neutralizing antibodies than those with a higher edu- cational level.

As expected, participants who had a vaccine take were 6.3 more likely to have IgG antibodies and 12 times more likely to have neu- tralizing antibodies than those who did not have a take. The asso- ciation between the presence of antibodies and vaccine take was expected, since the vaccine take is strongly correlated with protec- tion [20,21]. Moreover, this result shows that vaccinated individu- als have protective immune response against OPV, while younger non-vaccinated professionals, do not have protective immunity against OPV, having the potential of getting infected during a virus exposure in their work environment.

In the United States and some parts of Europe, healthcare pro- fessionals treating patients with VACV infections and laboratory personnel who directly work handle VACV or other OPV receive vaccination as a preventive measure to occupational exposure [2,23]. Even though in Brazil VACV infections are not necessarily reported to the health surveillance system, healthcare profession- als are under great risk to acquire infections due to direct contact with infected patients during management and treatment. Thus, vaccination for healthcare

professionals should be considered, especially in those endemic areas for VACV circulation and BV outbreaks.

This study has some limitations. A relatively small number of health professionals were sampled (almost 50.0% were enrolled), even though all 103 professionals were invited to participate in our survey. Furthermore, no physicians were available to partici- pate. This fact could indicate that the study may be underpowered to better evaluate the knowledge of health professionals regarding BV. Although we choose to sample a population in a BV endemic area, the data collected here might not be representative of the healthcare professional population in other Brazilian States.

Considering that BV is an endemic disease and a burden for public health in Brazil, healthcare professional outreach and edu-

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cation can also stimulate community participation in surveillance activity and provide basic knowledge to the community to protect themselves against the disease. Therefore, educational material was produced in order to provide knowledge about BV. Educational measures aimed at health professionals proved to be effective in terms of emerging diseases surveillance. In Africa, educational intervention of community members stimulated Monkeypox pre- vention and was essential to ensure a better proper diagnosis of suspected cases [24–26]. Indeed, educational approaches applied to MPXV infections had considerable impacts on disease reduction through early case identification, decreasing the chances for MPXV transmission [24–26]. Furthermore, the enhanced educational training demonstrated that health care workers ensure measures on clinical knowledge and surveillance actions.

The material is written in Portuguese, Spanish and English and

was included in this article as supplementary material (Supple- mentary material 2). We believe that educating the affected popu- lation, veterinarians and public health assistance staff about the different aspects of BV could reduce the burden on public and vet- erinary health.

After smallpox eradication, the importance of poxviruses has decreased in human medicine, not being unusual, the unprepared- ness of health professionals about the clinical and epidemiological aspects related to OPV have also decreased. Our data show that most of the public healthcare professionals, who are in direct con- tact with infected individuals, have poorly knowledge about VACV natural infection even in an endemic area. Pointing to the impor- tance of knowledge about BV among healthcare professionals, data presented here enhances the efforts for BV control and prevention.

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Appendix A. Supplementary material

Supplementary data associated with this article

can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2017.04.048.

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Serologic and Molecular Evidence of Vaccinia Virus Circulation among Small Mammals from Different Biomes, Brazil

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Vaccinia virus (VACV) is a zoonotic agent that causes a disease called bovine vaccinia, which is detected mainly in milking cattle and humans in close contact with these ani- mals. Even though many aspects of VACV infection have been described, much is still unknown about its circulation in the environment and its natural hosts/reservoirs. To in- vestigate the presence of *Orthopoxvirus* antibodies or VACV DNA, we captured small rodents and marsupials in 3 areas of Minas Gerais state, Brazil, and tested their samples in a laboratory. A total of 336 animals were tested; positivity ranged from 18.1% to 25.5% in the 3 studied regions lo- cated in different biomes, including the Atlantic Forest and the Cerrado. Analysis of nucleotide sequences indicated co-circulation of VACV groups I and II. Our findings rein- force the possible role played by rodents and marsupials in VACV maintenance and its transmission chain.

irus species belonging to genus *Orthopoxvirus* (OPV) receive great attention because of *Variola virus* (VARV), which is associated with smallpox (*1*). Smallpox caused many deaths worldwide and was eradicated after a massive vaccination campaign developed by the World Health Organization (*2*). Because OPVs have very similar antigenic structure (*1*), cross-protection enabled the use of cowpox virus (CPXV) and later vaccinia virus (VACV) as anti-smallpox vaccine agents (*2*).

Given its widespread use, VACV has been studied for many years, and these efforts shed light on various as- pects regarding virus biology. After smallpox eradication,

vaccination was discontinued (*2*), and only select institu- tions in the United States (e.g., the military and certain pub- lic health facilities) receive the vaccine for their efforts to prevent the use of VARV as a biologic weapon. Household transmission from vaccinees and eczema vaccinatum are some of the negative aspects of vaccinating and have been responsible for severe outcomes (*3*). Although VARV is now restricted to laboratory facilities, other OPVs have been emerging as zoonotic pathogens in different geographic ar- eas, namely CPXV in Europe, monkeypox virus (MPXV) in Africa, and VACV in Asia and South America (*4*).

In Brazil, natural infections with VACV are called bo- vine vaccinia (BV) and are reported in rural areas, mainly in milking cattle and in men who are in close contact with these animals. The first officially recorded reports of BV date from the early 2000s and occurred in the southeastern region of Brazil (*5*,*6*). Currently, there is evidence of virus circula- tion in all regions of Brazil (*7*,*8*); however, the southeast is still the epicenter of registered BV cases, with Minas Gerais state being one of the most affected. Studies have shown that mammal species in addition to bovids and humans could be naturally infected by (or at least exposed to) VACV (*9*–*19*). VACV was isolated from samples from a rodent from the Amazon region in the 1960s (*9*), and now there are other documented incidents of virus circulation in these animals (*10*–*12*,*15*,*17*). By taking into account virus detection in small rodents, the fact that CPXV (*20*) and probably MPXV have rodents as reservoirs (*21*), and the frequent reports of

these animals' presence during BV outbreaks, an

ecologic

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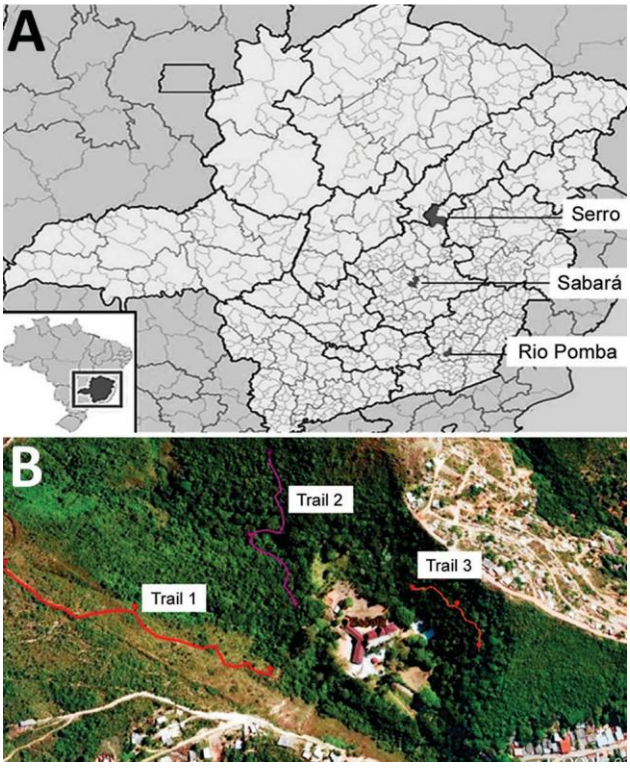
model was created to propose the participation of rodents in the VACV transmission chain (*12*). Because some species of native rodents could have ecologic advantages in areas with anthropic disturbance, they could work as bridges between natural and human/domestic habitats, bringing viruses from wild animals to domestic ones and vice versa (*12*). This model is reinforced by studies of virus transmission between mice and from experimental infection through contaminated



milk (*22*). To better evaluate the circulation of VACV in small rodents, we undertook comprehensive collection cam- paigns in 3 areas of Minas Gerais with or without confirmed BV outbreaks. Animals were evaluated for the presence of VACV DNA and antibodies against OPV. Because marsupi- als were often captured and previous studies have detected OPV antibodies and VACV DNA in these animals (*17*,*19*), their samples were also tested.

Methods

Collection Sites

We collected small mammals in 3 areas of Minas Gerais. Brazil was chosen because of its history of BV outbreaks and its different biomes and conservational status (Figure 1, panel A). The 3 municipalities where collections were performed were Sabará, Serro, and Rio Pomba.

Sabará is a city located in an anthropic area situated in the transition from savannah (the Cerrado biome) to the Atlantic Forest. The study site (19°53′9″S, 43°48′45″W) was delimited on the grounds of a former educational

Figure 1. Locations of study areas, Minas Gerais state, Brazil. A) Locations of the 3 municipalities where collections were performed: Sabará, Serro, and Rio Pomba. Inset shows location of Minas Gerais state in southeastern Brazil. B) Identification of 3 sample transects
in Sabará. Trail 1 has savannah vegetation, and trails 2 and 3 have Atlantic Forest vegetation. Sources: panel A, Scribble Maps; panel B, T.M.F. de Ázara. A color version of this figure is available online (http://wwwnc.cdc.gov/EID/article/23/6/16-1643-F1.htm).

institution in a previous study (Figure 1, panel B). Three sampling transects were demarcated: 1 in savannah veg- etation with intense anthropogenic disturbance and the other 2 in forest vegetation (with 1 of the 2 having more disturbance than the other) (Figure 1, panel B). In each transect, 15 sampling points were established with 2 live traps in each, 20 m apart, where captures of small mam- mal took place.

In Rio Pomba, the field site (21°16′29″S, 43°10′45″W) has characteristic Atlantic Forest vegetation. Animal trap- ping was performed in the area around the Instituto Feder- al de Educação, Ciência e Tecnologia (Figure 2, panels A, B). Animal collections were performed in forest, pasture, and peridomicile areas (Figure 2, panels A–G). In each transect, 10 traps were placed at a distance of 10 m from each other and in alternating positions (on forest floor or on tree trunks).

Serro, a city whose economy is based on milk and cheese production, has seen many cases of BV since 2005 (*23*). The capture of small mammals was carried out in 2 farms (Figure 2, panels C, D). The study site (180°36′21.16″S, 43°23′12.89″W) is situated in the Cer- rado biome and has some intersections of Atlantic Forest. Animal collections were carried out in forest, pasture, and peridomicile areas (Figure 2, panels A–G), and traps were positioned in the same manner as in Rio Pomba.

Animal Trap and Sample Collection

Captures lasted from April 2011 through May 2012 for Sabará (12 campaigns) and from September 2012 through September 2013 in Serro (5 campaigns) and Rio Pomba (6 campaigns). Small mammals were captured in size- selective live cages by using pineapple chunks and cot- ton balls soaked in cod liver oil as baits. Each sampling section lasted for 4 nights, and baits were replaced after 2 nights. After capture, animals were anesthetized with ketamine (70 mg/kg) and xylazine (12 mg/kg) for serum collection. Animals were weighed, measured for size, and visually evaluated for clinical signs of disease, such as skin lesions. For organ collection, animals were eutha- nized by intracardiac injection of 3 times the anesthetic dose according to guidelines of the American Society of Mammalogists (*24*). Collections were authorized by the Environment Ministry of Brazil through the SISBIO sys- tem (license no. 20807–2).

Biosafety

All collections were performed by trained professionals (either veterinarians or biologists) according to US Centers for Disease Control and Prevention recommendations (*25*). During animal

manipulation, personal protective equip- ment (disposable coveralls, surgical gloves, goggles, and N98 masks) was used.

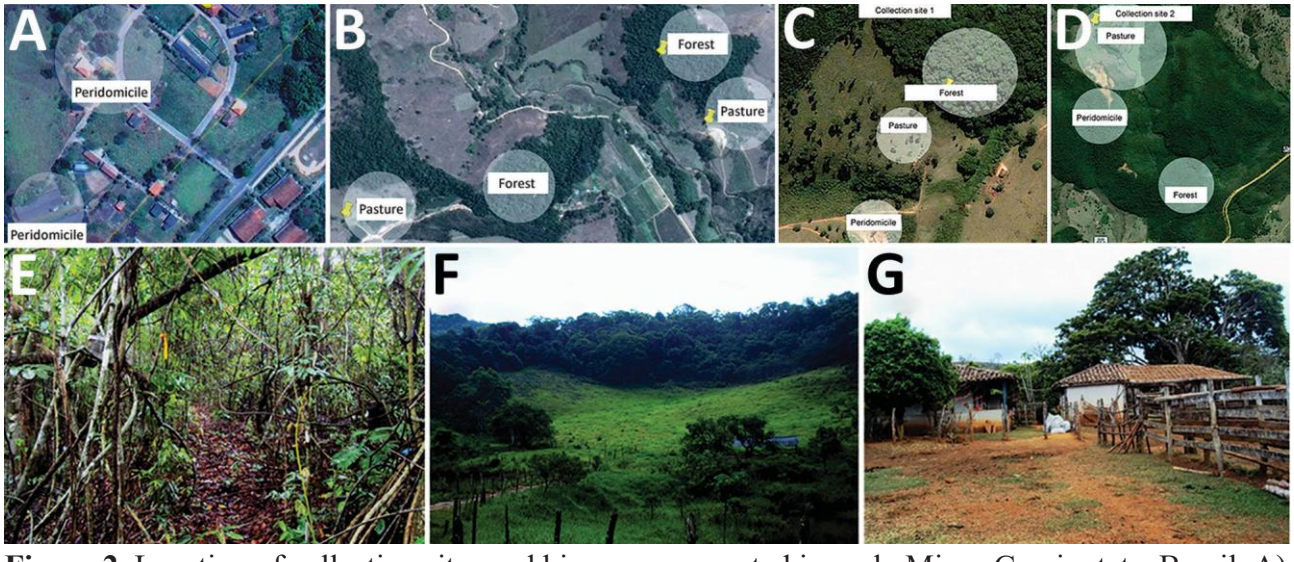


Figure 2. Location of collection sites and biomes represented in each, Minas Gerais state, Brazil. A) Collection site 1 in Serro. B) Collection site 2 in Serro. C) Peridomicile collection areas in Rio Pomba. D) Forest and pasture collection areas in Rio Pomba. E) Example of a forest area where animals were captured. F) Example of peridomicile area. G) Example of pasture area. In panels A–D, circles represent areas where transects for capture were demarcated. Sources: panels A,–D, Google Maps, modified by F.V. Nunes; panels E–G, F.V. Nunes.

A color version of this figure is available online (http://wwwnc.cdc.gov/EID/article/23/6/16-1643-F2.htm).

DNA Extraction from Organs

In addition to serum, which was tested by real-time PCR (rPCR) targeting the C11R viral growth factor gene with- out DNA extraction, liver was the chosen organ for rPCR trials. The organs were macerated with mortar and pestle after liquid nitrogen was added, and DNA was extracted with PureLink Genomic DNA Mini Kit (Invitrogen, Carls- bad, CA, USA) as recommended by the manufacturer. The same protocol was applied for other organs tested, includ- ing intestine, bladder, heart, gonads (ovary/testicles), bone marrow, spleen, lung, diaphragm, and kidney.

Cells and Virus

A VACV Western Reserve strain was used as a positive control. BSC-40 cells were grown in Eagle's minimum essential medium (Invitrogen) supplemented with 5% fe- tal bovine serum (Cultilab, São Paulo, Brazil); 25 mg/mL Fungizone (amphotericin B) (Cristália, São Paulo, Brazil); 500 U/mL penicillin; and 50 mg/mL gentamicin (Schering- Plough, São Paulo, Brazil).

rPCR Assays

All rPCR experiments were performed in 48-well plates in Step One machines (Applied Biosystems, Foster City, CA, USA) by using SYBR Green Master Mix (Applied Biosystems). DNA from liver samples was diluted in wa- ter for a final use concentration of 10 ng/□L and 50 ng/

□L. For serum samples, a 1:10 or 1:100 dilution was per-

formed, and samples were tested without previous DNA extraction. For both liver and serum samples, amplifica- tion of the C11R gene was tested, and liver samples were

additionally tested for amplification of the A56R hemag- glutinin gene (primer sequences available upon request). For C11R, an amplicon of 82 bp and a melting tempera- ture of 74.99°C were expected, and for A56R, a sequence of 160 bp and a melting temperature of 74.41°C were expected. All reactions had a final volume of 10 □L, and samples were tested in duplicates. Reaction steps com- prised initial DNA denaturation at 95°C for 10 min, 40 cycles of denaturation (95°C for 15 s), annealing/exten- sion (60°C for 60 s), and a melting curve (95°C for 15 s, 60°C for 60 s, and 95°C for 15 s). Samples were consid- ered positive when melting temperature varied only up to 1°C compared with a positive control (10 ng of DNA ex- tracted from purified VACV Western Reserve strain) and had amplification in duplicate or for >1 target. Samples with a single amplification were retested and considered equivocal when no more amplification was observed.

Nucleotide Sequencing and Sequence Analyses

Positive samples that could be reamplified (A56R-posi- tive) or amplified by a conventional PCR targeting C11R (C11R-positive) (*26*) were chosen for sequencing. For A56R, product from the previous reaction was reamplified in a conventional PCR reaction using 1 □L of the first reac- tion as input and 0.2 nmol/L (A56R) rPCR primers. PCR cycling for A56R gene consisted of 10 min at 95°C for de- naturation, 30 cycles of denaturation (95°C for 10 min), annealing (60°C for 60 s), extension (72°C for 60 s), and a final extension of 10 min at 72°C. Products with single bands were directly sequenced, and products with multiple bands had the target gene extracted from acrylamide gels

stained with SYBR Gold Nucleic Acid Gel Stain (Invit- rogen) and had its DNA purified. Nucleotide sequencing was performed by dideoxy method in an ABI3130 platform (Applied Biosystems), and sequence quality was analyzed by using Sequence Scanner Software 1.0 (Applied Bio- systems). Sequences were aligned with other reference se- quences from the BLAST nucleotide database (http://blast. ncbi.nlm.nih.gov/Blast.cgi) by using MEGA 6.0; the same program was used for identity matrix construction (*27*).

Plaque-Reduction Neutralization Test

The plaque-reduction neutralization test (PRNT) protocol has been described previously by Geessien Kroon et al.
(*28*). Samples were considered positive when a reduction of ≥50% in virus plaque numbers was observed.

ELISA

ELISA was performed for rodent blood samples following a protocol also described previously (*28*). For each plate, 1 positive control (serum from *Mus musculus* experimen- tally infected with VACV-Guarani P1) (*29*) and 3 negative controls (serum of noninfected *M. musculus*) were added. Cutoff was established as the mean of negative controls op- tical density units plus 3 times their SD. Samples with an optical density 10% above or below the cutoff were consid- ered equivocal.

Interaction Networks

Interaction networks are useful to help with understanding of virus-host dynamics. Each species is represented by a vertex, and the link between 2 vertices represents the inter- action between 2 different species, making it possible to an- alyze their interdependence (*30*). The networks also show which species have the higher number of positive samples for virus detection and the area where they were collected. By using data from VACV-positive small mammal species, we created weighted networks with the program Pajek 4.07
(*31*). Accordingly, adjacency matrices were generated for each study area in which hosts were represented by lines and VACV by columns.

Results

rPCR Amplification of VACV DNA from Free-living Small Mammals

A total of 325 animals had their samples tested by rPCR targeting the C11R gene, the A56R gene, or both. Of these animals, 21 (6.4%) tested positive (i.e., amplification in du- plicates or in >1 sample/target) and 58 (17.8%) equivocal (online Technical Appendix Table 1, https://wwwnc.cdc. gov/EID/article/23/6/16-1643-Techapp1.pdf). The cycle thresholds varied from 28.42 to 39.33. From the total ani- mals tested by rPCR, 114 had samples

available for all tests

(C11R targeted in liver and serum and A56R in liver). One animal was positive in the 3 tests performed, 11 in 2 tests, and 5 in only 1 test (data not shown). For the remaining positive animals, ≥1 assays could not be performed, and 1 sample type was positive in 1 test.

Of all the animals from the different collection sites in Sabará, 11/48 (22.9%) rodents and 3/76 (3.9%) marsupi- als were positive by rPCR. For Serro, no marsupials were positive but 4/25 (16.0%) rodents were positive. For Rio Pomba, 2/137 (1.4%) of rodents and 1/18 (5.5%) of mar- supials were positive (online Technical Appendix Table 1). Four rPCR-positive animals (2 rodents and 2 marsu- pials) were selected for viral DNA detection in different organs by rPCR targeting the C11R and A56R genes. Posi- tivity was found for heart, spleen, intestines, bladder, lungs, kidneys, and gonads (online Technical Appendix Table 2). No amplification was observed in any bone marrow or dia-
phragm samples tested.

OPV Antibodies in Serum from Free-living Small Mammals Tested by PRNT

PRNT tests were performed in a total of 314 serum samples,
and from these, 33 were considered positive, corresponding to 10.5% of the animals. The reduction percentages var- ied from 50.5% to

95.6%. For the Sabará collection, posi- tivity was 9.0% (10/111), 14.3% (6/42) for rodents and 5.8% (4/69) for marsupials. For Serro, positivity was 4.2% (2/47), and only rodent samples were positive, correspond- ing to 8.0% (2/25) of rodents tested. For Rio Pomba, posi- tivity was 13.4% (21/156), 14.3% (20/139) for rodents and 5.8% (1/17) for marsupials (online Technical Appendix Table 1).

OPV Antibodies in Serum from Free-living Small Mammals Tested by ELISA

ELISA tests were performed on 189 rodent serum sam-

ples; a control serum for marsupials was not available. Of the animals tested, 19/189 (10.0%) were positive and 11/189 (5.8%) equivocal (online Technical Appendix Table 1). By location, 3/35 (8.5%) animals from Sabará, 9/25 (36.0%) from Serro, and 7/129 (5.4%) from Rio Pomba were positive.

Sequencing

Two PCR amplicons, amplified from Sabará animals, were sequenced with C11R primers and resulted in se- quences of 168 bp that were aligned with VACVs in Bra- zil and other OPVs (online Technical Appendix Figure 1). These 2 sequences had 100% similarity with each other; similarity with VACVs in Brazil ranged from 98.2% to 100% and with CPXV from 87.3% to 89.1%, whereas similarity with VARV was 94.5% and with MPXV 90.9% (data not shown). For A56R, sequencing was performed

in positive rPCR samples and resulted in 6 sequences of 102 bp. When compared with OPV sequences, 2 samples from Sabará had an 18-nt deletion shared by Brazil VACV group I, whereas the other 4 samples from Sabará, Serro, and Rio Pomba did not have that deletion, being more similar to Brazil VACV group II and other OPVs (online Technical Appendix Figure 2).

Geographic and Species Distribution of Positivity

For all areas studied, 336 animals belonging to 18 genera had their samples tested by rPCR, PRNT, and/or ELISA, and 65 (19.3%) were positive in ≥1 of these tests. Total positivity was 18.1% for Sabará, 25.5% for Serro, and 18.5% for Rio Pomba (online Technical Appendix Table 1). A higher positivity was observed for rodents (25.7%) than for marsupials (7.6%) (data not shown).

Species identified among the test-positive rodents were *Calomys* sp., *Akodon* sp., *Necromys lasiurus*, *Trinomys se- tosus*, *Cerradomys subflavus*, *Oligoryzomys* sp., *Nectomys squamipes*, *Mus musculus*, and *Rattus rattus*. For marsu- pials, the positive animals were characterized as species/ genera *Didelphis* sp., and *Caluromys philander* (online Technical Appendix Table 3).

Test-positive animals were captured in all sample ar- eas in Sabará (savannah and forest) and Serro (pasture, forest, and peridomicile areas), whereas test-positive ani- mals in Rio Pomba were captured in pasture and forest. The interaction network for Sabará illustrates that 4 spe- cies had positive samples, with 3 of them found either in forest or savannah and the other in both areas. *N. lasiurus* (the hairy-tailed bolo mouse) had the highest number of positive samples in Sabará (Figure 3, panel A). For Serro, evidence of VACV circulation was found in 6 species; of these, 2 were captured in forest, 2 in pasture, and 2 in peridomicile areas. The species with the largest number

of positive samples in Serro was *T. setosus* (the hairy Atlantic spiny rat; Figure 3, panel B). For Rio Pomba, 5 genera were positive, including *Akodon* sp. mice captured in pasture and forest, *Calomys tener* and *N. lasiurus* mice captured in forest, and *C. philander* and *Didelphis aurita* opossums captured in pasture. The species with the high- est number of positive animals in Rio Pomba was *C. tener* (the delicate vesper mouse), followed by *N. lasiurus* and *Akodon* sp. mice (Figure 3, panel C).

Discussion

In our study, we analyzed different biomes in an area where BV infections are common, and the positivity rates found for VACV were 25.7% for rodents and 7.6% for marsupials. Even though VACV is known to circulate in Brazil and cause a disease that leads to economic, social, and public health effects, few studies have been conducted with the aim of clarifying the VACV transmission chain and potential natural hosts (*9–19*,*22*,*32*). Previous stud- ies showed antibody positivity of 8.7%–17.9% for wild rodents captured in places with or without documented BV (*15*,*17*) and seropositivity of 8.2% for *Didelphis* spp. marsupials (*17*).

The higher positivity rate found for rodents in our study could be attributable to the use of 3 different tech- niques, including 2 techniques for detecting antibodies (PRNT and ELISA) and 2 targets for DNA detection (rPCR).The 3 techniques used to assess virus circulation provide distinct responses about infection stages. Where- as rPCR indicates the presence of viral DNA, the ELISA used in our study reveals the presence of IgG (indicative of previous infection) (*33*), and PRNT detects neutral- izing antibodies that can be of different types, including IgG and IgM, which are produced early in the infection process (*34*). Because we found positive animals for ≥1

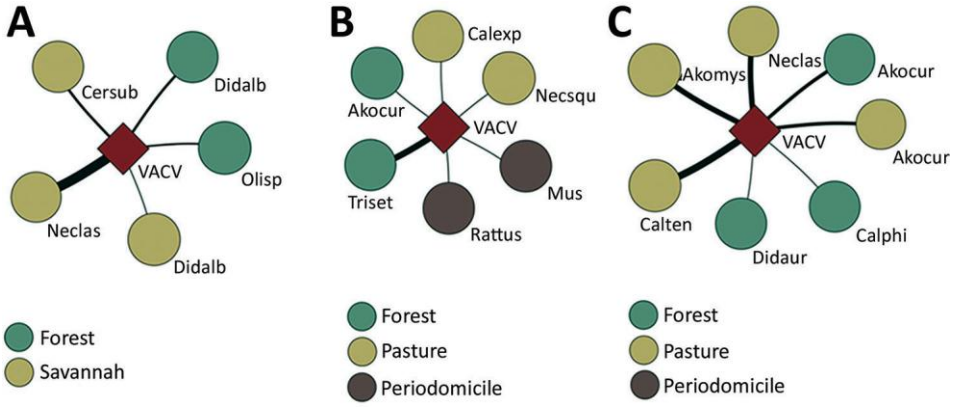


Figure 3. Interaction networks for vaccina virus among small mammals in Sabará (A), Serro (B), and Rio Pomba (C) in Minas Gerais state, Brazil. The square represents vaccinia virus. Circles

represent small mammal species (labeled). The color in the circles represents the area where mammals were collected. The thickness of lines increases with the number of positive samples from a species. Acokur, *Akodon cursor* mouse; Akomys, *Akodon* cf; mystax; Calexp, *Calomys expulsus*; Calphi, *Caluromys philander*; Calten, *Calomys tener*; Cersub, *Cerradomys subflavus*; Didalb, *Didelphis albiventris*; Didaur, *Didelphis aurita*; Mus, *Mus musculus*; Neclas, *Necromys lasiurus*; Necsqui, *Nectomys squamipes*; Olisp, *Oligoryzomys* sp.; Rattus, *Rattus rattus*; Triset, *Trinomys setosus*; VACV, vaccinia virus. A color version of this figure is available online (http://wwwnc.cdc.gov/EID/article/23/6/16-1643-F3.htm).

techniques, we can speculate that an active transmission cycle is happening in all 3 study areas. Additionally, only Serro has recurrent reports of BV outbreaks (*23*,*29*), which could be a result of the presence of positive animals in the peridomicile area, where they could infect other an- imals, such as cows, and cause disease. Furthermore, be- cause large-scale milk production occurs in Serro, many livestock animals, including bovines, could work as infec- tion amplifiers.

The rPCR technique has been used for MPXV detec- tion in rodents in Africa, where samples were considered equivocal when repeatability of results was not achieved
(*21*). In our study, we made this same observation, which might be attributable to a low virus load in the samples. In turn, the low virus load could be related to the late cycle threshold in which amplification occurred and a lack of clinical signs in animals with a positive result.

Sequencing of A56R rPCR amplicons revealed the co- circulation of Brazil VACVs belonging to groups I and II, a fact that reinforces previous data on Brazil VACV virus diversity (*11*,*35*–*37*). Again, even when infected with virus belonging to group II, which were found to be virulent in a mice model (*38*), wild rodents and marsupials tested in our study did not have clinical signs detected. Also, these animals infected with VACV group I or II had viral DNA in many organs, as indicated by rPCR. It is not possible to assert that virus is replicating in these tissues, given that virus could be present in blood that circulates through these organs; however, previous in vivo infection experiments have found virus in different mice organs (*32*), probably because of systemic infection. This observation also was made in mice infected with milk (a possible route of natu- ral infection) contaminated with VACV-Guarani P2 virus. This virus was found to be nonvirulent in a mice model; animals shed viral DNA and produced OPV antibodies but did not show clinical signs (*22*). The detection of vi- rus DNA in intestines, bladder, and gonads could reinforce previous data suggesting that virus transmission occurs through feces (*39*) and support the hypothesis of alterna- tive transmission through urine and sexual contact. Mari- ana virus has been isolated from the gonads of mice (*14*), so it could be speculated that the sexual transmission route is involved.

Among the positive rodent species, *Akodon* sp., *N. squamipes*, *Oligoryzomys* sp. (*15*,*17*), and *M. musculus*
(*12*) have already been found to be positive in previous studies, reinforcing evidence of its participation in the VACV transmission cycle; however, the exact role played by these animals is not yet known. In addition to *M. mus- culus*, *C. subflavus*, *N. lasiurus*, *T. setosus*, *C. tener*, and
R. rattus rodents were also found to be positive in our study, indicating the role of multiple hosts in VACV trans- mission in Brazil. Among the

marsupials, *Didelphis* spp.

opossums had already been found to be positive (*17*,*19*), and *C. philander* opossums also had positive samples.

Although only 1 virus (VACV) was analyzed for in- teraction network construction and no interactions between different species were observed, the networks created illus- trate the participation of the small mammals for each stud- ied area and the areas where these positive animals were collected (Figure 3). The networks also suggest an impor- tant role of *N. lasiurus* mice for the VACV transmission chain in Sabará, *T. setosus* rats in Serro, and *C. tener*, *N. lasiurus*, and *Akodon* sp. mice in Rio Pomba. Most of these species are generalist animals that can be adapted to a dis- turbed environment. However, the *C. philander* opossum is an arboreal species that lives in forests (*40*), which could indicate that a wild cycle is being maintained and that other animals could be transporting the virus between forests and peridomicile areas. These findings corroborate the models proposed by Abrahão et al. (*12*) in which rodents and other small mammals could work as links between natural and anthropic environments.

In conclusion, our findings reinforce evidence of par- ticipation of rodents and marsupials in the VACV trans- mission cycle and the possibility that these animals might work as links between natural and anthropic

environments. These findings also further illustrate the multi-host charac- teristic of VACV infection in Brazil.

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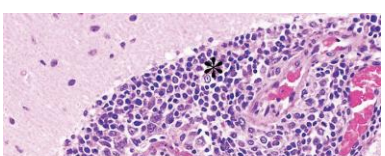
December 2015: Zoonotic Infections



- Biological Warfare Plan in the 17th Century—the

- Identifying and Reducing Remaining Stocks of Rinderpest Virus
- Opportunistic Pulmonary *Bordetella hinzii* Infection after Avian Exposure
- Zoonotic Leprosy in the Southeastern United States
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- Influenza A(H6N1) Virus in Dogs, Taiwan
- Methicillin-Resistant *Staphylococcus aureus* Prevalence among Captive Chimpanzees, Texas, USA, 2012
- Novel *Waddlia* Intracellular Bacterium in *Artibeus intermedius* Fruit Bats, Mexico
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- Increased Number of Human Cases of Influenza Virus A(H5N1) Infection, Egypt, 2014–15
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- Hendra Virus Infection in Dog, Australia, 2013
- No Evidence of Gouléako and Herbert Virus Infections in Pigs, Côte d'Ivoire and Ghana
- Aquatic Bird Bornavirus 1 in Wild Geese, Denmark
- Vectorborne Transmission of *Leishmania infantum* from Hounds, United States
- Porcine Deltacoronavirus in Mainland China

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We investigated possible vaccinia virus (VACV) in urban house cats in Brazil. Serum samples from 6 cats were positive for VACV by PCR, indicating likely VACV circula- tion among house cats in urban areas of Brazil. This finding highlights the importance of epidemiologic surveillance to avoid outbreaks among urban human populations.

Detection of Vaccinia Virus in Urban Domestic Cats, Brazil

Galileu Barbosa Costa, Júlia Bahia Miranda, Gregório Guilherme Almeida, Jaqueline Silva de Oliveira, Mariana Siqueira Pinheiro, Stefanne Aparecida Gonçalves, Jenner Karlisson Pimenta dos Reis, Ricardo Gonçalves, Paulo César Peregrino Ferreira, Cláudio Antônio Bonjardim, Jônatas Santos Abrahão, Erna Geessien Kroon, Giliane de Souza Trindade

Vaccinia virus (VACV) outbreaks, first reported in Brazil in 1999, affect dairy cattle and humans in rural areas (*1*). Although studies have shown evidence of VACV circulation among several mammal species (*1*–*3*), no consensus exists regarding the role of these animals in the VACV transmission chain or which animal is the natural reservoir. In fact, domestic or wild mam- mals could be asymptomatic hosts and also contribute to VACV transmission (*3*).

In contrast to VACV, cowpox virus (CPXV) circulates in urban environments in Europe but also in surrounding wild and rural areas (*4*). CPXV is transmitted to humans mainly by cats, which play a link between the natural res- ervoirs and humans in the urban environment (*4,5*). In

cats, the clinical course of CPXV infection varies from no symp- toms to widespread skin necrotic lesions and can ultimately lead to death (*6*). Some studies have shown serologic evi- dence of orthopoxvirus infection in cats from Europe and have addressed the role of these animals in orthopoxvirus transmission to humans (*7,8*).

Because VACV and CPXV share some epidemiologic features and cats have a prominent role in the urban CPXV transmission chain, we decided to investigate whether ur- ban domestic cats have evidence of exposure to VACV in Brazil. This study was approved by the Animal Experi- ments Committee of the Universidade Federal de Minas Gerais (registration protocol 315/2014).

We performed a retrospective study of serum samples from 277 house cats, collected during September 2012–De- cember 2014 in 5 states in Brazil (online Technical Appen- dix Figure 1, https://wwwnc.cdc.gov/EID/article/23/2/16-1341-Techapp1.pdf). The states in this study were those whose veterinary clinics agreed to submit samples. We screened serum samples for neutralizing antibodies by using a ≥70% plaque-reduction neutralization test (*9*). To detect VACV DNA in serum samples, we performed real- time PCR targeting the C11R and A56R genes (*9*). We directly sequenced A56R fragments in both orientations and in triplicate by using the Mega-BACE sequencer (GE Healthcare, Buckinghamshire, UK). We used ClustalW (http://www.genome.jp/tools/clustalw) and MEGA7 soft-

ware (http://www.megasoftware.net) to align nucleotide sequences and construct a phylogenetic tree (neighbor- joining method with 1,000 bootstraps).

The cats' ages ranged from 3 months to 15 years; 150 (54.2%) of the cats were female. Thirteen cats (4.7%) had outdoor access, and 37 (13.4%) were admitted to the vet- erinary clinic for ≥1 night. Some cats had clinical illness inconsistent with orthopoxvirus infection, which can overlap with other common dermatologic diseases affecting cats (online Technical Appendix Table). Most (8/53 [15.1%]) seropositive cats were from the Pampulha region of the city of Belo Horizonte (Minas Gerais State) (online Tech- nical Appendix Figure 1), followed by the eastern region of the city. We detected neutralizing antibodies in 16 animals (5.8%), with titers ranging from 100 to 1,600 neutralizing units/mL; of these, 13 (4.7%) were positive for C11R gene and 6 for A56R gene (online Technical Appendix Table). Alignment of the A56R fragments showed high similar- ity to the homologous gene of VACV isolates from Brazil (online Technical Appendix Figure 1). For the phylogenetic tree, we grouped sequences with VACV group 1 and 2 iso- lates (Figure).

We describe evidence of VACV circulation in cats in an urban environment in Brazil. Many studies have attempted to elucidate VACV outbreaks and risk factors in rural and wild areas (*1*–*3*). Our findings reveal a seropositivity rate of

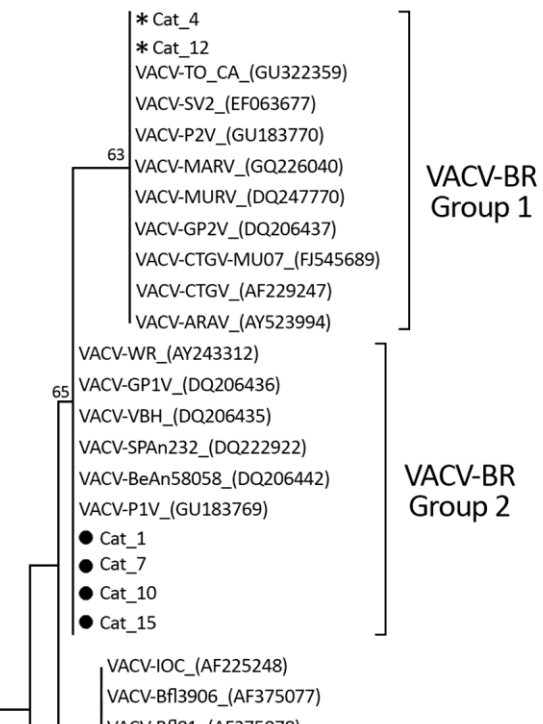

Figure. Phylogenetic tree constructed based on nucleotide sequences of orthopoxvirus A56R (hemagglutinin) genes detected in serum samples of 6 house cats house cats with neutralizing antibodies for vaccinia virus, Belo Horizonte, Brazil, September 2012–

December 2014. The tree was constructed with A56R gene sequences by using the neighbor-joining method with 1,000 bootstrap replicates and the Tamura 3-parameter model in MEGA7 (http://www.megasoftware. net). Asterisks indicate group 1 vaccinia virus isolates (nonvirulent strains) detected in 2 cats. Black circles indicate group 2 vaccinia virus isolates (virulent strains) detected in 4 cats. Numbers along branches are bootstrap values. GenBank accession numbers are shown for reference isolates. Scale bar indicates nucleotide substitutions per site.

5.8%, which is lower than the rate observed in a previous study from Norway (*8*) and higher than the rate observed in a study of cats in Austria (*7*). Notably, the Pampulha region, where most seropositive animals were detected, corresponded to areas of relatively low elevation that fea- ture houses with green areas, cottage houses, and ecologic parks, with forested areas making up the remaining portion of the land (online Technical Appendix Figure 1).

Recent data from our research group revealed that capy- baras (*Hydrochoerus hydrochaeris*) from the Pampulha re- gion tested positive for VACV (*10*). These data, corroborated by molecular detection of VACV groups 1 and 2 in house cats from Belo Horizonte, further indicate the presence of VACV in an urban environment (online Technical Appendix Figure 2). In this study, PCR-positive cats showed no clinical signs that would indicate orthopoxvirus infection at the time of sample collection (online Technical Appendix Table), un- like what was observed among cats infected with CPXV in Europe (*4,5*). Furthermore, cats 4, 10, and 15 (online Techni- cal Appendix Table), in which we detected ongoing VACV DNA, had no clinical signs. Although we detected group 2 VACV (virulent strains) in 4 samples, our findings corrobo- rate the results of Bennett et al. (*6*), which showed that cats infected with VACV had asymptomatic infection.

Limitations of our study include selection bias of animals; it was not possible to use a convenience sample from the 5 Brazilian states. We were also unable to obtain detailed clinical information of all animals and unable to collect additional clinical samples to better understand the clinical course of VACV infection in cats. In Brazil, no records of VACV-like detection in urban populations are available, despite the fact that VACV was recently found in urban areas (*10*). In fact, potential sources of infection for cat populations (e.g., small rodents) should be considered. Cats could possibly seroconvert without the onset of classi- cal illness. Hence, VACV could be circulating in cats from urban environments. The potential role of cats in infecting humans should be investigated further to determine wheth- er VACV can emerge in urban human populations and pose a threat to public health.

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Vaccinia Virus among Domestic Dogs and Wild Coatis, Brazil, 2013–2015

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To determine their potential role as a source of human infec- tion, we tested domestic dogs (urban) and wild coatis (wild) in Brazil for vaccinia virus. Our findings of positive neutralizing antibodies and quantitative PCR results for 35/184 dogs and 13/90 coatis highlight a potential public health risk.

Since smallpox was declared eradicated in 1980, after a massive effort led by the World Health Organization, other orthopoxviruses have gained notoriety as zoonotic agents worldwide (*1*). Over the past 17 years in Brazil, many zoonotic outbreaks of vaccinia virus (VACV) infec- tion have been recorded throughout the country, becoming a burden for the dairy industry and public health (*2*). The most affected hosts during outbreaks are dairy cattle and humans (*2*). Recent studies assessing the role of wildlife in the maintenance cycle of VACV in nature have corroborated previous findings that rodents and marsupials serve as links between natural and anthropic environments (*2–4*). Indeed, the increased frequency of reported VACV de- tection in several species of mammals points toward new insights into the circulation and maintenance of VACV in wild (forest) and rural (farm) environments (*2,5–8*). Stud- ies conducted in Latin America suggest that wildlife, espe- cially small and medium-sized mammals, plays a role in virus transmission and maintenance of orthopoxviruses in nature (*9*). Furthermore, some studies have shown the presence of VACV in urban environments, emphasizing the risks for humans (especially those not vaccinated against smallpox) (*10,11*).

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To determine the potential role of domestic and wild animals as a source of VACV infection for humans, we investigated VACV circulation among domestic dogs and wild coatis, animals that live at the intersection of urban and wild environments in Brazil. The capture of wild animals was authorized by the Brazilian Institute of Environ- ment and Renewable Natural Resources, and the study was approved by the Ethics Committee in Animal Experimenta- tion of Universidade Federal de Minas Gerais.

The Study

We analyzed serum and anal swab samples collected dur- ing 2013–2015 from 184 domestic dogs and 90 wild coatis in the city of Belo Horizonte (19°55′15″S, 43°56′16″W) in the state of Minas Gerais, Brazil (Figure 1). Swab samples of lesions, if present, were also collected. To determine the presence of neutralizing antibodies in serum, we used an orthopoxvirus plaque reduction neutralization test as previously described (*12*). Serum titers were defined as the high- est dilutions that inhibited ≥70% of virus plaques compared with negative controls.

To detect VACV DNA from serum and anal swab samples, we performed real-time PCR targeting the C11R or A56R gene (*12*). We directly sequenced A56R fragments in both orientations and in triplicate by using the ABI3130 platform (Applied Biosystems,

Waltham, MA, USA). Se- quences were aligned with other reference sequences from GenBank by using MEGA 7.0 (http://www.megasoftware. net). Statistical analyses were conducted by using Epi Info software version 7.2.1.0 (https://www.cdc.gov/epiinfo); χ^2 and Fisher exact tests were applied with significance set at 5%. We also calculated relative odds ratios (ORs) and 95% CIs.

We detected orthopoxvirus neutralizing antibodies in 35 dogs (prevalence rate 19.0%, 95% CI 14.0%–25.5%; ti- ters 100–400 neutralizing units/mL) and in 13 coatis (prevalence rate 14.4%, 95% CI 8.5%–23.3%; titers 100–800 neutralizing units/mL) (Table 1). Univariate analyses indi- cated significant associations between presence of neutral- izing antibodies and the following: male dogs (OR 2.6; p = 0.02), dogs 6–10 years of age (OR 5.2; p = 0.04), coatis captured in 2013 (OR 11.2; p = 0.002), juvenile coatis (<1 y of age) (OR 35; p = 0.001), and adult coatis (>2 y of age) (OR 5.1; p = 0.04).

Samples from all seropositive animals were submitted for quantitative PCR (qPCR) to detect VACV DNA (Table

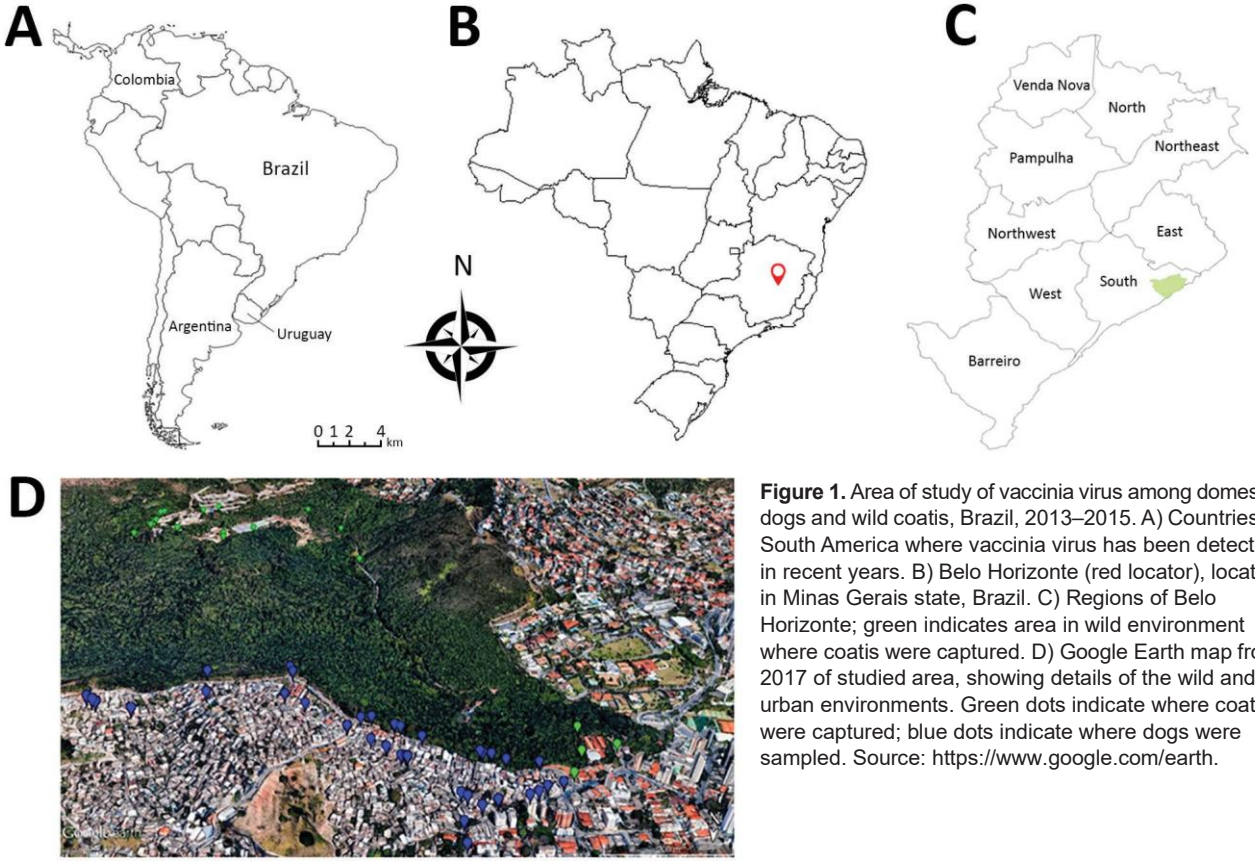

Figure 1. Area of study of vaccinia virus among domestic dogs and wild coatis, Brazil, 2013–2015. A) Countries in South America where vaccinia virus has been detected in recent years. B) Belo Horizonte (red locator), located in Minas Gerais state, Brazil. C) Regions of Belo Horizonte; green indicates area in wild environment where coatis were captured. D) Google Earth map from 2017 of studied area, showing details of the wild and urban environments. Green dots indicate where coatis were captured; blue dots indicate where dogs were sampled. Source: https://www.google.com/earth.

2). Overall, serum samples from 7 dogs and 6 coatis were positive for the C11R gene; of these, anal swab samples were positive for this gene for

3 dogs and 4 coatis. Samples from the C11R-positive animals were submitted for an- other qPCR targeting the A56R gene. Serum samples were positive for the A56R gene for 5 dogs and 4 coatis; of these, anal swab samples were positive for A56R for 1 dog and 3 coatis. No lesion swab samples were positive by qPCR for both C11R and A56R genes.

Alignment of the amplified A56R fragments showed high similarity to the homologous gene of VACV isolates from Brazil (online Technical Appendix Figure, https://wwwnc.cdc.gov/EID/article/24/12/17-1584-Techapp1. pdf). Furthermore, 5 sequenced samples (from 4 dogs and 1 coatis) showed an 18-nt signature deletion, which is present in sequences of mouse nonvirulent VACV strains from Brazil (group 1 VACV). This deletion was not present in samples from 4 animals (1 dog and 3 coatis), grouping with mouse virulent VACV strains from Brazil (group 2 VACV).

Conclusions

We assessed VACV exposure of 2 interacting species of animal: domestic dogs from an urban area and coatis from a bordering wild area. In contrast to VACV infections, human cowpox virus infections have mostly occurred in urban areas of Europe. Cowpox virus is transmitted to hu- mans mainly by domestic cats that are in contact with ro- dents, the natural cowpox virus reservoirs (*13*). However, some authors have hypothesized that domestic dogs could be implicated in the transmission cycle of VACV, acting as a link between the natural reservoirs and humans in urban environments (*14,15*). Indeed, our molecular findings sup- port exposure and possible VACV infection of these ani- mals, thereby indicating that they are a potential source of VACV exposure for humans in urban areas.

The seroprevalence of orthopoxvirus neutralizing an- tibodies in dogs in Brazil has been described. Peres et al. found that, along with other farm animals, 22.8% of 114 dogs tested were seropositive for orthopoxviruses (*7*); this seroprevalence differs from that observed in our study, which was 3.8% lower. In addition, most animals tested by Peres et al. were from rural areas where no bovine vaccinia outbreaks had been officially reported, and 96% of farm- ers declared that their domestic animals have contact with wild animals (*9*). These findings indicate that dogs could be exposed to VACV through contact with wild animals, corroborating our hypothesis.

We also detected orthopoxvirus neutralizing antibod- ies in wild coatis, which is consistent

with results of a

Table 1. Associations between neutralizing antibodies against *Orthopoxvirus* and demographic characteristics of domestic dogs and wild coatis, Belo Horizonte, Brazil, 2013–2015*

Variable	No. (%)†	No. positive‡	(%)No. negative‡	(%) p value	Odds ratio (95% CI)
Domestic dogs Year of sampling	123 (66.8)	24 (19.5)	99 (80.5)		
2014					
2015	61 (33.2)	11 (18.0)	50 (82.0)	1.00	
Sex					
F	85 (46.4)	23 (27.1)	62 (72.9)	Reference	
M	96 (52.5)	12 (12.5)	84 (87.5)	**0.02**	**2.6 (1.2–5.6)**
Age, y					
≤1	24 (13.1)	7 (29.2)	17 (70.8)	Reference	
2–5	82 (44.8)	16 (19.5)	66 (80.5)	0.4	
6–10	41 (22.4)	3 (7.3)	38 (92.7)	**0.04**	**5.2 (1.2–22.6)**
>10	18 (9.8)	4 (22.2)	14 (87.8)	0.9	
Size					
Small	75 (41.0)	13 (18.8)	56 (81.2)	Reference	
Medium	69 (37.7)	11 (20.0)	44 (80.0)	1.00	
Large	30 (16.4)	4 (14.3)	24 (85.7)	0.8	
Confinement status					
Always inside home	41 (22.4)	8 (19.5)	33 (80.5)	Reference	
Always in backyard	115 (62.8)	18 (15.6)	97 (84.3)	0.7	
Home and backyard	25 (13.7)	9 (36.0)	16 (64.0)	0.2	
Outdoors access†					
Yes	83 (45.3)	19 (22.9)	64 (77.1)	0.3	

No	98 (53.6)	16 (16.3)	82 (83.7)	Reference		
Access to MMP						
Yes	18 (9.8)	6 (33.3)	12 (66.7)	0.2		
No	101 (55.2)	18 (17.8)	83 (82.2)	Reference		
Wild coatis						
Year of capture 2013	57 (52.8)	12 (21.0)	34 (59.6)	**0.002**	**14.8 (1.8–119.8)**	
2014	51 (47.2)	1 (1.9)	42 (82.3)	Reference		
Sex						
F	64 (59.3)	10 (15.6)	44 (68.7)	Reference		
M	44 (40.7)	3 (6.8)	32 (72.7)	0.3		
Age group						
Juvenile, ≤1 y	44 (40.7)	1 (2.3)	35 (79.5)	Reference		
Subadult, 1–2 y	18 (16.7)	5 (27.8)	10 (55.6)	**0.01**	**0.05 (0.006–0.5)**	
Adult, >2 y	46 (42.6)	7 (15.2)	31 (67.4)	**0.04**	**5.1 (1.2–22.6)**	

*Totals may not add up to 100% because of missing information. Boldface indicates significance; odds ratios are shown only for significant results. MMP, Mangabeiras Municipal Park.

†Includes access beyond backyard.

‡By plaque reduction neutralization test.

previous study that described the seroprevalence of or- thopoxviruses in procyonids from Mexico (*9*). Our detec- tion of VACV DNA in anal swab samples from coatis indicate that these animals could act as a source of virus for domestic dogs and humans and serve as a link between wild and urban environments. However, future studies to determine if viable virus is shed are needed to confirm this possibility.

Table 2. Diagnostic results for 7 domestic dogs and 6 wild coatis with neutralizing antibodies for

vaccinia virus, Belo Horizonte, Brazil, 2031–2015*

Animal	PRNT70 titer (NU/mL)	qPCR C11R		qPCR A56R		Strain
		Serum sample	Anal swab	Serum sample	Anal swab	
Dog 2	1:40 (100)	+	–	+	–	Group 1
Dog 58	1:80 (200)	+	+	+	–	Group 1
Dog 41	1:40 (100)	+	–	–	–	
Dog 77	1:80 (200)	+	+	+	–	Group 1
Dog 86	1:40 (100)	+	–	–	–	Group 1
Dog 121	1:160 (400)	+	–	+	–	
Dog 128	1:160 (400)	+	+	+	+	Group 2
Coati 5	1:40 (100)	+	+	+	+	Group 2
Coatis 17	1:40 (100)	+	–	–	–	
Coatis 27	1:80 (200)	+	–	–	–	
Coatis 39	1:160 (400)	+	+	+	+	Group 2
Coatis 48	1:40 (100)	+	+	+	–	Group 1
Coatis 50	1:80 (200)	+	+	+	+	Group 2

*NU, neutralizing units; PRNT70, 70% plaque reduction neutralization test; qPCR, quantitative PCR.

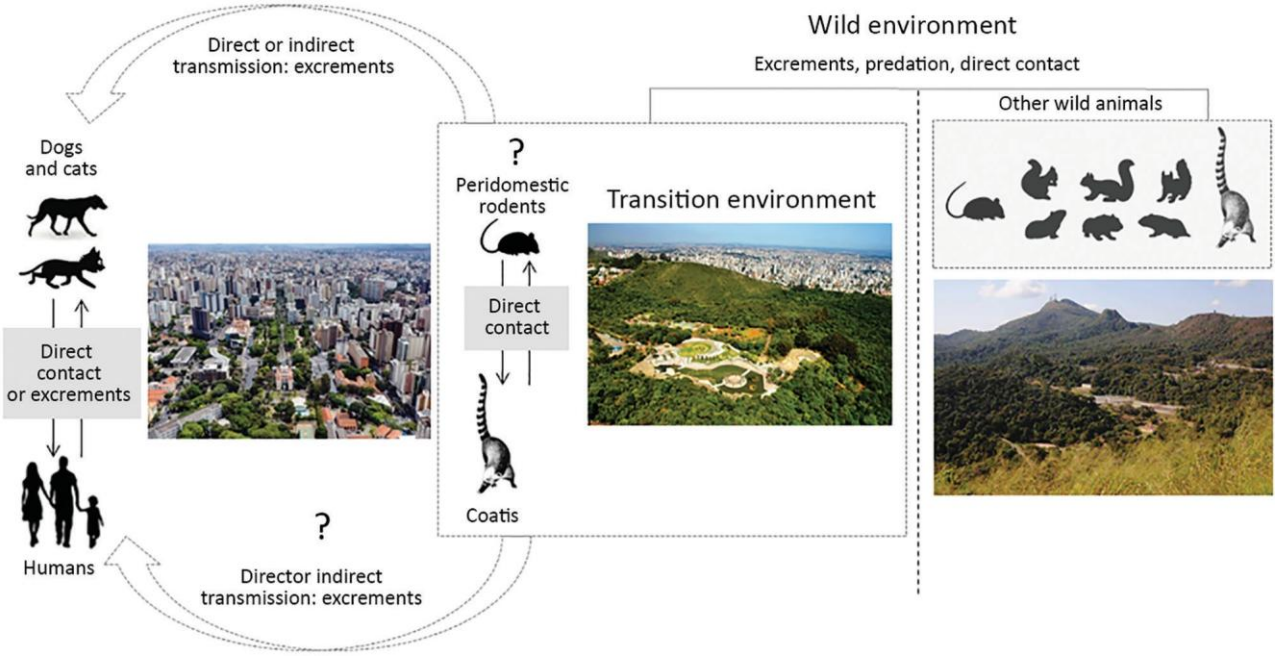

Figure 2. A hypothetical model developed to visualize the role of domestic animals and wildlife in the natural cycle of vaccinia virus (VACV). The model illustrates the dynamics of VACV circulation in urban and wild areas of Brazil. In urban areas, wild coatis could promote the transmission of VACV between domestic animals or humans because they are in direct contact with domestic dogs and circulate among urban residences. Domestic dogs could also promote the transmission of VACV to humans because
of direct contact or possibly indirect contact thought contaminated feces. In the wild environment, coatis can interact with other mammals such as wild rodents, which are believed to be VACV reservoirs, and acquire the infection (this potential interaction is still under investigation).

To impart information about the role of domestic ani- mals and wildlife in the natural cycle of VACV, we de- veloped a hypothetical model based on previous studies (*2,3,5*), which could illustrate the dynamics of VACV cir- culation in urban areas (Figure 2). Because coatis can cir- culate in wild environments and surrounding urban areas, they could act as a bridge promoting the transmission of VACV between wild animals (mainly rodents) and dogs or humans. Domestic dogs could transmit VACV directly to humans through close contact or indirectly thought con- taminated feces (Figure 2). These data raise questions about VACV

circulation in Brazil and open discussions about the role of dogs and coatis in the VACV epidemiologic cycle.

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e Tecnológico.

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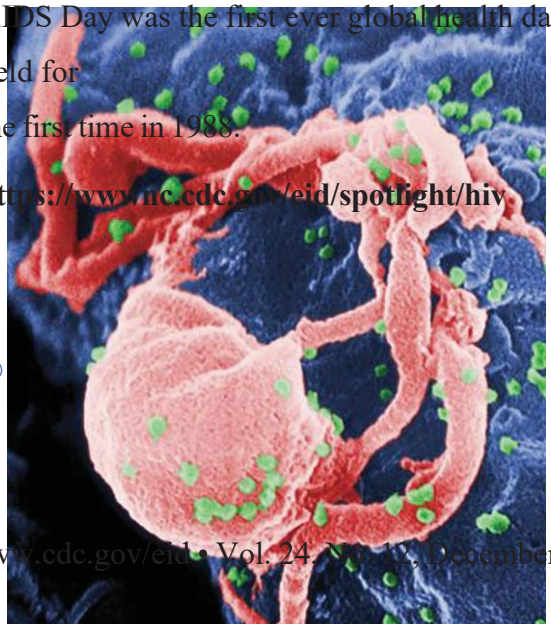
Spotlight: HIV-AIDS

World AIDS Day is held on December 1 of each year and is an opportunity for people worldwide to unite in the fight against HIV, show their support for people living with HIV, and commemorate people who have died. World AIDS Day was the first ever global health day, held for the first time in 1988.

https://wwwnc.cdc.gov/eid/spotlight/hiv



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RAPID COMMUNICATION

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Circulation of vaccinia virus in southern and south-eastern wildlife, Brazil

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Abstract

We evaluated 345 wild animals from southern and south-eastern Brazil to understand their role in vaccinia virus (VACV) transmission cycle. VACV DNA was detected in rodents, marsupials, chiroptera and cingulate, expanding the knowledge of VACV host range in wildlife that could potentially act as source of infection in rural and urban areas.

KEYWORDS

ecological maintenance, molecular detection, vaccinia virus, VACV epidemiology, wildlife

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1 | INTRODUCTION

Vaccinia virus (VACV) is a zoonotic agent of wide geographic dis- tribution in several countries of South America, especially in Brazil where it is responsible for a disease called bovine vaccinia (BV) (Oliveira et al., 2017). BV has been predominantly reported in rural areas, affecting mainly dairy cattle and humans, being a burden

to public health and local dairy economies (Oliveira et al., 2017). Additionally, VACV has also been detected in other species present in the rural environment (equids and buffaloes) (Lima et al., 2019) and domestic animals in urban areas (dogs and cats) (Costa et al., 2018; Oliveira et al., 2017). However, VACV natural history and circulation in wildlife are still poorly explored (Miranda et al., 2017; Oliveira et al., 2017).

Pedro Starling Pereira Martins da Costa and Jaqueline Silva Oliveira contributed equally to this study.

Few studies have documented the circulation of VACV in synan- thropic and wild rodents (Abrahão et al., 2009; Miranda et al., 2017), and also in other sylvatic animals such as primates, marsupials and coatis (Abrahão et al., 2009; Costa et al., 2018; Lima et al., 2019; Miranda et al., 2017; Oliveira et al., 2017). Aiming to include the wildlife in the VACV transmission cycle, ecological models have been proposed (Costa et al., 2017, 2018; Lima et al., 2019; Oliveira et al., 2017). According to the models, sylvatic animals could potentially act in VACV transmission between rural, wild and even urban envi- ronments (Abrahão et al., 2009; Costa et al., 2018; Lima et al., 2019; Oliveira et al., 2017). Furthermore, VACV could also circulate silently in areas with very few cattle herd and anthropogenic disturbance (Kurth et al., 2008). Hence, studies focused on VACV circulation in wildlife could add information regarding its ecoepidemiology, as- sessing the risks of VACV spreading from the wild to rural and urban areas, as well documented for Cowpox virus in Europe (Essbauer, Pfeffer, & Meyer, 2010; Kroon et al., 2016).

2 | MATERIALS AND METHODS

We retrospectively analysed a total of 345 DNA samples extracted from the liver of wild animals captured during 2007–2011 in southern and south-eastern areas of Brazil (Figure 1). In these sampled areas, VACV outbreaks are frequently reported in São Paulo state and Rio Grande do Sul state. This study was approved by the Animal Ethics Committee of the Instituto Adolfo Lutz (protocol no. 2/2015 and

2/2017) and Animal Ethics Committee of the Faculdade de Medicina Veterinária e Zootecnia (protocol no. 211/2008). Moreover, it is in ac- cordance with the Brazilian Institute of Environment and Renewable Natural Resources' (IBAMA) normative statement n. 119 of 11 October 2006, chapter VI, art 0.26. Liver samples were manually frac- tionated with disposable surgical blades or scalpels and processed me- chanically by using Mini-Beadbeater-24 BioVortexerTM homogenizer (Biospec Products, Bartlesville, USA). Samples were submitted for DNA extraction using the Illustra Tissue & Cells genomic Prep Mini Spin kit (GE Healthcare, Chicago, USA) in Instituto Adolfo Lutz and send to Laboratório de Vírus at Universidade Federal de Minas Gerais (UFMG). To detect VACV DNA, we performed a semi-nested PCR tar- geting the C11R gene (viral growth factor; vgf) and a real-time PCR to A56R gene (hemagglutinin) has been used in genetic analyses for VACV differentiation (Kroon et al., 2016; Peres et al., 2018).

We directly sequenced the amplified fragments in both orien-

tations and in duplicate by using the ABI3130 platform (Applied Biosystems—Thermo Fisher Scientific, Foster City, USA). Sequences were aligned with other reference sequences from GenBank by using MEGA 7.0 software.

3 | RESULTS AND DISCUSSION

A large diversity of wild animals such as rodents, non-human pri- mates, marsupials, carnivores, chiropterans, placental mammals, lagomorph, cingulate and artiodactyl mammals were screened

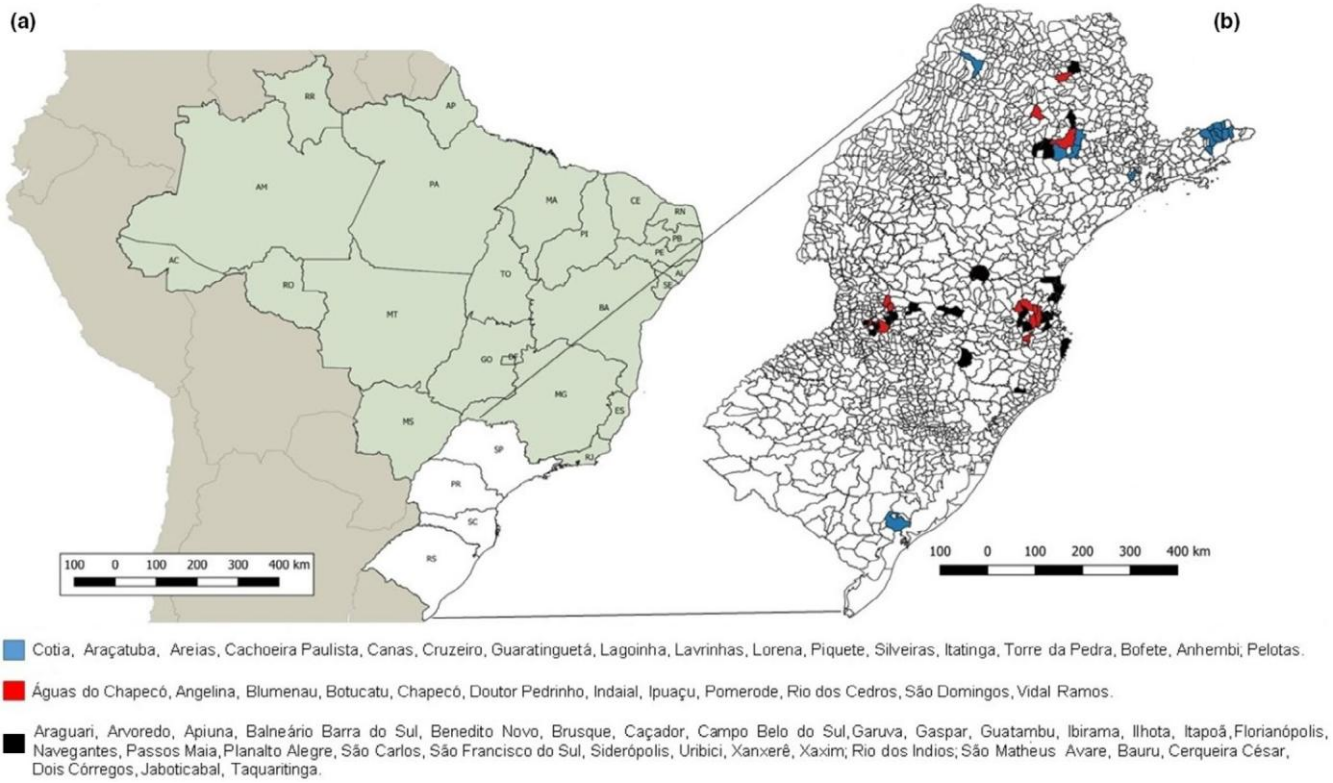

FIGURE 1 (a) Area of vaccinia virus study in wild animals, Brazil. States of southern and south-eastern regions of Brazil where samples were collected. (b) The municipalities sampled present a black colour. Red indicates municipalities where animals were positive

(Table 1). A total of 12 animals (3.5%) being rodents, non-human primates, marsupials and cingulate tested positive for C11R gene, in which *Euphractus sexcintus* and *Akodon* sp. had amplicons se- quenced. Alignment of the C11R fragments showed highly similarity (90%–100%) to the homologous gene of orthopoxviruses, including VACV strains isolated from Brazil (Figure 2). Additionally, 18 animals (5.2%) tested positive for A56R gene. Of these, we detected the VACV in two bats (*Molossus rufus* and *Eumops perotis* species), both sampled in Botucatu city, São Paulo state. The VACV circulation in bats has never been explored in Brazil before. Alignment of the am- plified A56R fragments showed 100% similarity to the homologous gene of VACV isolates from Brazil (Figure 3) and the presence of an 18-nt signature deletion, which is present in sequences of mouse non-virulent Brazilian-VACV strains (group 1 Brazilian VACV).

These findings reinforce the hypothesis that VACV can widely circulate in wild environments in Brazil, potentially infecting a wide range of hosts. The detection of VACV in *Akodon montensis*, a species with generalist habits, corroborates the participation of rodents in VACV epidemiological cycle as previously described (Abrahão et al., 2009; Miranda et al., 2017). Furthermore, the detection of VACV in *Molossus rufus*, a forest bat that also possesses synanthropic habits, reinforces the hypothesis that these wild animals could act as sources of transmission of wildlife viruses (Abrahão et al., 2009; Costa et al., 2018). It is important to emphasize that the wildlife animals included

TABLE 1 Molecular detection of vaccinia virus in wildlife in southern and south-eastern Brazil

Order	Families	Species	No. of tested animals	No. of positive animals	
				C11R	A56R
Artiodactyla	Cervidae		1	0	0
Carnivora	Felidae	*Cerdocyon thous, Procyon cancrivorus, Nasua nasua,*	23	0	0
	Canidae	*Galictis cuja*			
	Mustelidae	*Leopardus trigrinus, Puma yagouaroundi*			

	Procyonidae	*Leopardus wiedii, Puma concolor* *Lontra longicaudis*			
Chiroptera	Molossidae Phyllostomidae	*Eumops perotisŁ,Tadarida brasiliensis* *Molossus molossus, Molossus rufusŁ* *Eumops glaucinus,* *Artibeus obscurus Artibeus lituratus*	20	0	2
Cingulata	Dasypodidae	*Euphractus sexcintusŁ* *Dasypus novemcinctus*	3	1	0
Didelphimorphia	Didelphidae	*Didelphis albiventris, Monodelphis* sp.	31	2	3
	Marmosidae	*Micoureus paraguayanus* *Philander frenatus, Didelphis aurita* *Gracilinanus microtarsusŁ* *Lutreolina crassicaudataŁ*			
Lagomorpha	Leporidae	*Lepus europaeusŁ*	5	0	1
Pilosa	Myrmecophagidae	*Tamandua tetradactyla*	12	0	0
		Myrmecophaga tridactyla			
Primates	Cebidae Atelidae Callitrichidae Cercopithecidae	*Saimiri sciureusŁ, Alouatta guariba* *Callithrix* sp., *Alouatta belzebul* *Alouatta seniculus, Ateles chamek* *Ateles marginatus, Ateles paniscus* *Cebus albifrons, Erythrocebus patas* *Lagothrix lagotricha, Alouatta caraya* *Mandrillus sphinx, Alouatta fusca* *Papio hamandryasŁ, Papio papio*	47	1	1
Rodentia	Echimyidae Muridae Cricetidae Caviidae	*Nectomys* sp., *Akodon montensisŁ* *Hydrochoerus hydrochaerisŁ* *Oligoryzomys nigripes, Cavia aperea* *Thaptomys nigritaŁ, Delomys* sp.,	203	8	11

Sciuridae	*Oxymycterus* sp., *Scapteromys* sp.
Erethizontidae	*Brucepattersonius iheringi*
	Euryoryzomys russatusǂ
	Sooretamys angouya
	Guerlinguetus aestuansǂ
	Sooretamys angouya
	Nectomys squamipesǂ
	Sphiggurus spinosusǂ, Rattus rattusǂ

Note: ǂ: Positive species in molecular screening

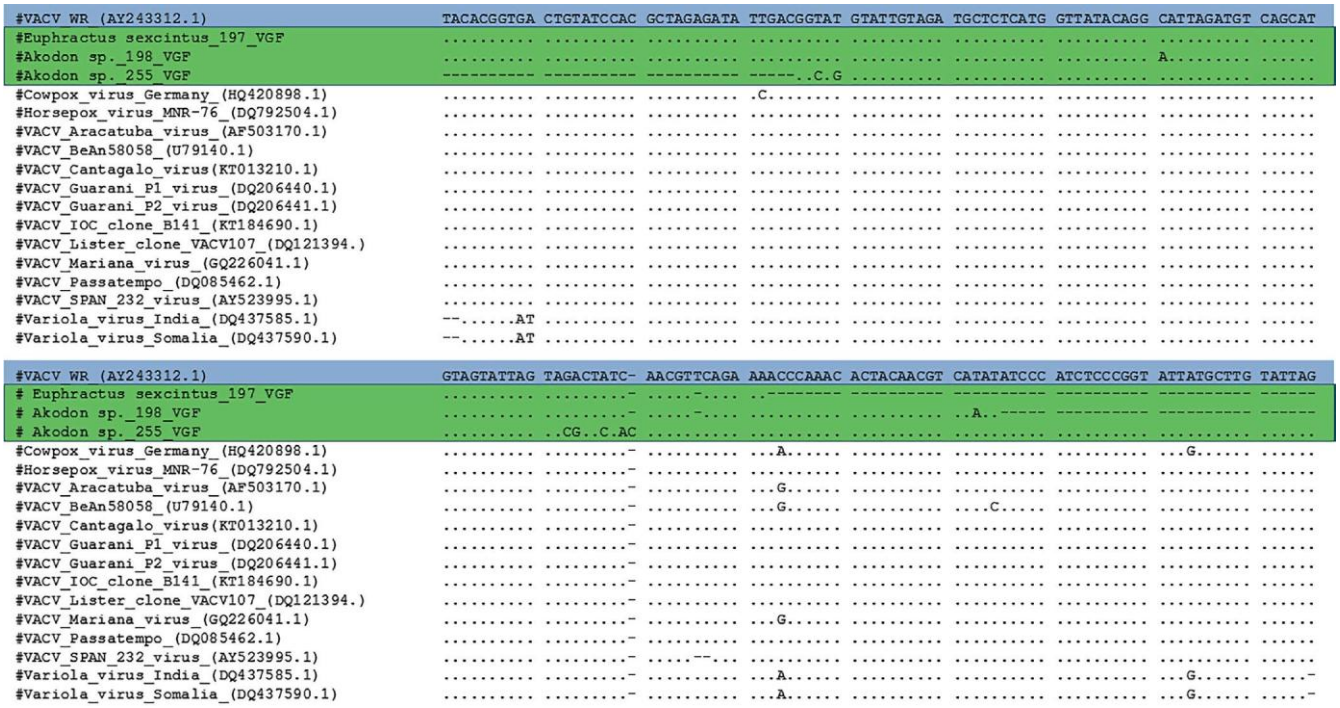

FIGURE 2 Green indicates nucleotide sequence of the vaccinia virus C11R gene found in two rodents (*Akodon* sp.) and one cingulate (*Euphractus sexcintus*) and in blue the sequence of VACV-WR compared with homologous sequences of several other orthopoxviruses

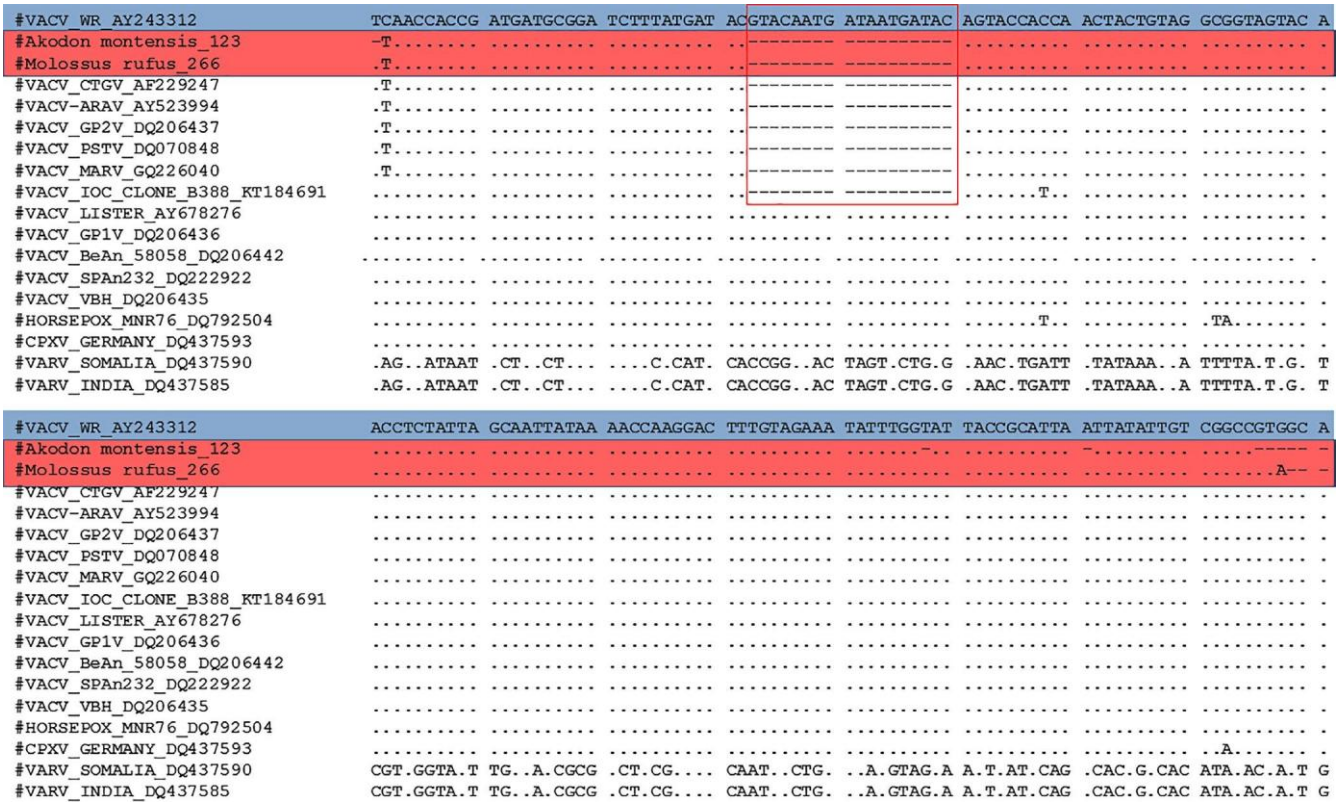

FIGURE 3 Red indicates nucleotide sequence of the vaccinia virus A56R (hemagglutinin) gene found in *Akodon montensis* and *Molossus rufus* species of the marsupials and chiropterans, respectively, compared with homologous sequences of several other orthopoxviruses. Strains containing the conserved deletion region (red) were grouped with other vaccinia viruses (groups 1 and 2) isolated in Brazil

in this study were sampled in areas with ecosystem alterations due to anthropic actions, such as agricultural activity and hydroelectric dam plant construction. These factors could enhance the occurrence of generalist and/or peridomestic species, whose VACV circulation has already been described (Miranda et al., 2017), as well as bringing other wild animals closer to rural and urban areas, thereby expand- ing their potential to act as sources of VACV exposure to humans and domestic animals. A similar dynamic is well established about the natural circulation of Cowpox virus (CPXV) in Europe. The dy- namic comprise wild rodents identified as natural hosts and felines act as intermediate hosts enabling the transmission of CPXV to other animals and even humans (Essbauer et al., 2010; Kurth et al., 2008).

Limitations of our study include lack of clinical information from the sampled animals aiming to evaluate the presence of clinical signs suggestive of VACV infection, absence of serum samples that could allow us estimate the seroprevalence in the wildlife and the absence of viable clinical material for virus isolation. However, our findings provide important information regarding the circulation of VACV in wild environments, contributing to understand the VACV mainte- nance in the wildlife in the absence of outbreaks. Additional studies should investigate the potential of transmission of specific groups of wild animals (other than rodents) to fully elucidate their role in VACV epidemiological cycle.

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CONFLIC T OF INTEREST

None to declare.

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Article

Educational Approach to Prevent the Burden of Vaccinia Virus Infections in a Bovine Vaccinia Endemic Area in Brazil

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Abstract: Bovine vaccinia (BV), caused by Vaccinia virus (VACV), is a zoonotic disease characterized by exanthematous lesions on the teats of dairy cows and the hands of milkers, and is an important public health issue in Brazil and South America. BV also results in economic losses to the dairy industry, being a burden to the regions involved in milk production. In the past 20 years, much effort has been made to increase the knowledge regarding BV epidemiology, etiologic agents, and interactions with the hosts and the environment. In the present study, we evaluated milking prac- tices that could be associated with VACV infections in an endemic area in Brazil and proposed an educational tool to help prevent VACV infections. In our survey, 124 individuals (51.7%) from a total of 240 had previously heard of BV, 94 of which knew about it through BV outbreaks. Although most individuals involved in dairy activities (n = 85/91) reported having good hygiene practices, only 29.7% used adequate disinfecting products to clean their hands and 39.5% disinfected cows' teats before and after milking. Furthermore, 46.7% of individuals reported having contact with other farm and domestic animals besides dairy cattle. We also evaluated the presence of IgG and IgM antibodies in the surveyed population. Overall, 6.1% of likely unvaccinated individuals were positive for anti-*Orthopoxvirus* IgG antibodies, and 1.7% of all individuals were positive for IgM antibodies. Based on our findings, we proposed educational materials which target individuals with permanent residence in rural areas (mainly farmers and milkers), providing an overview and basic information about preventive measures against VACV infections that could enhance BV control and prevention efforts, especially for vulnerable populations located in endemic areas.

Keywords: Vaccinia virus; bovine vaccinia; dairy practices; public health; prevention; educational

1. Introduction

The Orthopoxvirus (OPV) genus affects multiple species and carries great importance in human and veterinary medicine [1–3]. Among the members of this genus that are pathogenic to humans and capable of significant impact on global public health are: *Variola virus* (VARV), the cause of the lethal and terrifying smallpox disease, which impacted humanity and challenged public health [4,5]; *Monkeypox virus* (MPXV), endemic in Africa and the most significant the most significant threat to public health since the eradication of smallpox [6,7]; *Cowpox virus* (CPXV), a zoonotic virus with rodent reservoirs, has an increasing number of human case reports in Europe recently [8,9]; and *Vaccinia virus*

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(VACV), the primary component of the smallpox vaccine, and responsible for natural human and animal infections in southern Asia and South America [1,2,10].

Forty years after smallpox eradication, the emergence of zoonotic OPVs has increased worldwide [1,2,8,10,11]. In Asia and South America, VACV has been described in recurrent outbreaks of vesiculopustular disease, affecting mainly dairy cattle, buffaloes, and milkers, creating a burden to public health and the dairy economy [2,10]. In Brazil, VACV outbreaks were recorded at the end of the 1990s in the Southeast region of the country, which con- centrates the largest number of dairy farms and bovine herds [12]. Furthermore, VACV infections have been reported in other South American countries in recent years [13,14], with outbreaks described in Colombia [15].

VACV infections in humans are usually associated with mild disease. Infected indi- viduals develop maculopapular lesions on the fingers, hands, or forearms [2]. The lesions initially appear as itchy focal points on the affected skin, followed by the appearance of local edema and the formation of vesicles, which tend to ulcerate into pustules, merging into focal areas of inflammation. Systemic symptoms such as fever, headache, myalgia, and lymphadenopathy are also reported [2,16]. Although lesions are usually found on fingers and hands of affected individuals, the spread to other body areas such as face, eyes, mucosa, chest, and genitals have been reported, possibly as a result of autoinocula- tion [17,18]. A case of progressive VACV infection has recently been documented in an HIV positive patient in Colombia, which draws attention to the severity of VACV disease in immunosuppressed individuals [19].

Although VACV circulation has been reported throughout Brazil, awareness of the disease remains low [2,20]. A recent study involving healthcare professionals in a VACV endemic area revealed that unpreparedness related to management of VACV infections in humans could be associated to the limited knowledge about OPV infections [20]. Healthcare professionals were unaware of clinical manifestations, treatment, and prevention; which is essential information to recognize and manage VACV infections [20].

Educational measures aimed at health professionals proved to be effective in terms of OPV surveillance [21,22]. Community participation in the

recognition and reporting of diseases is an interesting approach that helps to increase surveillance, reduce disease spread, and decrease the impact on public health. Furthermore, educational initiatives have been used to improve the knowledge and behavior toward infectious diseases, which can also work as a prevention method. The goal of this study is to describe the basic hygiene measures adopted during the dairy handling practices in an endemic area in Brazil, as well as to understand the seropositivity of anti-OPV IgG and IgM antibodies among individuals with permanent residence in rural areas, also providing evidence to guide preventive and control measures. We further aimed to produce educational materials that could be used as tools to aid in the prevention of VACV infections.

2. Results

In our survey, 124 individuals (51.7%) from a total of 240 had previously heard of BV (Table 1), 94 of which (75.8%) became aware of it during outbreaks in their farms. Nineteen individuals (15.3%) heard of BV from farmers, eight (6.4%) heard from milkers, and only one searched about BV on the Internet.

A total of 91 individuals (from 124) reported being involved in milking practices (Table 2). Although most individuals performing dairy activities (93.4%) reported they had good hygiene practices, only 29.7% said they used adequate disinfecting products (such as chlorine solution) to clean their hands and very few of them (2.5%) disinfected cows' teats before and after milking. Iodine solution was reported to be used only to disinfect cows' teats but not to disinfect milkers' hands. Noteworthily, 46.7% of individuals reported having contact with other animals besides dairy cattle, such as horses (141; 58.7%), dogs (112; 46.7%), and cats (30; 12.5%).

Table 1. Sources of information regarding bovine vaccinia (BV) reported by participants in rural areas of Serro city, Minas Gerais State, Brazil.

Sources of Information	**Number of Participants (%)**
Participants Who Had Previously Heard of BV	124/240 (51.7%)
From a farmer	19/124 (15.3%)
From a milker	8/124 (6.4%)
From health care professional	7/124 (5.6%)
From a veterinarian	7/124 (5.6%)
From TV	7/124 (5.6%)
From radio	6/124 (4.8%)
From Internet	1/124 (0.8%)
During an outbreak	94/124 (75.8%)

Table 2. Hygiene measures adopted by 91 individuals who reported being involved in milking activities in rural areas of Serro city, Minas Gerais State, Brazil.

Hygiene Measure	***n***
Disinfection of hands (*n* = 85)	
With water and soap only	85/91 (93.4%)
With chlorine solution	27/91 (29.7%)
Time frame	
Before start milking only	1/85 (1.2%)
Between different cows	83/85 (97.6%)
Before start and after finish milking	1/85 (1.2%)
Disinfection of cow's teats (*n* = 78)	
Water and soap only	78/91 (85.7%)
Chlorine solution	24/91 (26.4%)
Iodine solution	36/91 (39.5%)
Time frame	
Before start milking only	9/78 (11.5%)

Between different cows		67/78 (85.9%)
Before start and after finish milking		2/78 (2.5%)
Disinfection of milking machine *		31/91 (34.1%)
Time frame *		
Before start milking only	0/31	
After start milking only		14/31 (45.2%)
Before start and after finish milking		17/31 (54.8%)

* For those who reported mechanical milking only.

Table 3 shows the characteristics of likely unvaccinated individuals (n = 126, age < 36 years old) that tested positive for IgG and all participants (n = 240) that tested positive for IgM antibodies. All individuals positive by IgM were older than 36 years and would have been vaccinated, but only one had the presence of a smallpox vaccine scar. Regarding sex, the majority (5 of 7) of IgG positive individuals and IgM positive (3 of 4) were male. Most individuals reported contact with bovines and equids. Involvement in milking activities were reported by 72.7% of individuals (among which 4 were IgG positive and 3 were IgM positive), while consumption of raw milk was reported only by two IgG positive individuals. On the other hand, only one IgM positive female reported consuming cheese made from raw milk, while 57.1% of IgG positive individuals reported doing so. Interestingly, three individuals that tested positive for IgG antibodies were affected by a BV outbreak in 2011. The main symptoms reported by all individuals were fever, headache, myalgia, and lymphadenopathy. They also reported the presence of lesions on their fingers and hands. Two individuals reported the illness lasted at least 15 days, while one lasted for 30 days.

Table 3. Characteristics of IgG and IgM positive individuals from rural areas in Serro city, Minas Gerais, Brazil.

Serology					**Contact with Animals**				***** Hygiene Practices**					
Gender	**Age**	**IgG OD-COV ***	**IgM OD-COV ***	**Presence of Smallpo × Vaccination Scar**	**Bovines**	**Equids**	**Dogs**	**Cats**	**Practice Milking**	**Raw Milk Consumption**	**** Cheese Consumption**	**Disinfection of Hands**	**Disinfection of Cow's Teats**	**Disinfection of Milking Machine**
M	32	+ (1.163)	-	No	Yes	Yes	Yes	No	Yes	No	NA	Yes	Yes	No
F	14	+ (1.063)	-	No	No	No	Yes	No	No	No	NA	No	No	No
M	31	+ (0.998)	-	No	Yes	Yes	NA	NA	Yes	No	NA	Yes	Yes	No
F	33	+ (0.991)	-	No	No	No	NA	NA	No	No	Yes	No	No	No
M	21	+ (0.791)	-	No	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	No
M	29	+ (0.568)	-	No	Yes	Yes	NA	NA	Yes	No	Yes	Yes	Yes	No
M	53	+ (0.275)	-	No	Yes	Yes	NA	NA	Yes	Yes	Yes	Yes	Yes	No
F	38	-	+ (0.101)	No	Yes	Yes	Yes	No	No	No	Yes	No	No	NA
M	70	-	+ (0.123)	Yes	No	Yes	Yes	No	Yes	No	NA	Yes	Yes	NA
M	67	-	+ (0.472)	No	Yes	Yes	Yes	No	Yes	No	NA	Yes	Yes	NA
M	39	-	+ (0.110)	No	Yes	Yes	NA	NA	Yes	No	NA	Yes	Yes	Yes

* OD-COV (cut-off value), which was determined by the mean of the optical density obtained from the negative controls plus three standard deviations

of the mean. ** Cheese made from raw milk. *** For those who reported practicing milking.

3. Discussion

In this study, half of the surveyed individuals knew about BV, and even though the disease is endemic in the region, most participants became aware during an outbreak. This finding highlights the need to spread knowledge about BV (and VACV) and its consequences to public health and local dairy economies. It is also important to emphasize that the study population seems to have limited access to Internet, which could restrict their ability to find information regarding BV and VACV infections. Furthermore, very few individuals received information from healthcare or veterinary professionals [20].

We also explored the knowledge regarding hygiene measures that could help prevent VACV infections from a likely source, cattle handling, among individuals who participate in milking. Most individuals reported practicing simple measures to disinfect their hands (water and soap), and few individuals used chlorine and/or iodine solution for disinfection of hands and cows' teats. A recent study showed that use of devices typically filled with iodine or chlorine solutions after milking may be effective in deactivating VACV particles, which could be protective against VACV infections [23]. Other studies have also shown that hygienic practices are important to reduce the risk of viral infections, highlighting the importance of frequent handwashing and use of different solutions such as water and soap and hypochlorite solutions to prevent Ebola transmission.

To better understand if the individuals included in this study were exposed to VACV or not, we decided to evaluate the presence of anti-OPV IgG and IgM antibodies; the latter would suggest a recent primary exposure to OPVs. As vaccination against smallpox in Brazil was discontinued in 1978, individuals less than 35 years old are unlikely to have been vaccinated against smallpox and have residual IgG antibodies. Our results also showed that 7 individuals less than 35 years old were IgG positive and four participants tested positive for IgM antibodies. In studies of IgM responses to OPXV in non-western populations, we have found between 3 and 5% of individuals produce positive IgM responses in the absence of recent exposure (M. Townsend, unpublished correspondence). Without clear epidemiological link and knowledge of potential higher background, we cannot confirm recent infection by positive IgM results. However, the finding of IgG responses in 6.1% of unvaccinated individuals clearly indicates prior VACV exposure. Indeed, Serro region is known for a high number of VACV outbreaks [2,24–26] and our results provide additional evidence of prior and possible recent

viral circulation. These findings thereby highlight the need for an active epidemiological surveillance of OPV in the area, especially due to the burden caused by BV in the local dairy economy.

One young individual (14 years old) that reported no milking activities and no contact with farming animals (dairy cattle and horses) tested positive for IgG antibodies. However, the 14-year-old female reported she had contact with domestic dogs, raising the hypothesis that these animals could act as an alternative source of VACV infection in rural areas. Costa and colleagues have described the presence of VACV DNA in urban domestic dogs from Belo Horizonte, Southeast region of Brazil [27]. Although in that study it was not possible to obtain swab from lesions or other clinical samples to attempt VACV isolation, the detection of VACV DNA through molecular assays suggested VACV infection in urban domestic dogs [27]. Moreover, Peres et al. have also detected VACV DNA in dogs from rural areas during a BV outbreak [28]. In this context, it is also worth mentioning that cats are a common vector for CPXV in Europe, human infections being acquired mainly through direct contact with infected domestic cats [8]. Although the 14-year-old female did not report she had contact with domestic cats, this fact may warrant special attention due to the possibility of domestic cats acting as vectors for VACV. Additional studies should investigate the role of domestic cats as vectors for VACV.

VACV has already been documented to spread within households, including through household fomites [29,30]. However, in some cases, the source of the infection is unknown, especially in individuals who did not participate in milking activities. Costa and co-workers have suggested alternative routes of zoonotic VACV infections in the study area, such as the consumption of cheese and raw milk [26]. In addition, the direct contact with other

potential VACV hosts such as cats, dogs, and rodents [27,31,32] could also pose a risk for viral transmission to humans.

During a Buffalopox virus (a close variant of VACV) outbreak in Western Maharashtra, India, Gurav and colleagues suggested that individuals with oral lesions could have been infected due to the consumption of raw milk [33]. Additionally, some studies in Brazil have detected viable VACV particles in milk and dairy products [34–36]. However, there's no evidence of oral infections caused by VACV directly associated with the consumption of raw milk, artisanal cheese, or other dairy products to date. Our findings also highlight the need to better investigate alternative routes of zoonotic VACV transmission, as well as the need to improve the knowledge of the population regarding the risks of potential VACV exposure through raw milk and dairy products.

We developed educational materials that target farmers and milkers and provide an overview and basic information about prevention measures against VACV infections that could enhance BV control and prevention efforts, especially for vulnerable populations located in endemic areas. The proposed materials present valuable information that is helpful for people living in rural areas. It is written in English, Portuguese, and Spanish, and was included in this article as supplementary material (Supplement Figures S1–S6). As the occurrence of BV and natural VACV infections have been documented in South American countries [2,13–15], the Portuguese and Spanish versions of the educational material will be helpful for healthcare professionals and policy-makers who are not fluent in English. Public health managers could download the educational material in Portuguese and Spanish and distribute to health departments and to dairy farms during field expeditions aiming to inform, educate, and raise awareness regarding BV and VACV infections.

We believe that educating the most affected population (mainly those at high risk) such as, veterinarians, healthcare professionals, and public health personnel about the different aspects of BV could help reduce the burden on public and veterinary health. The educational materials can also stimulate the rural community to participate in the surveillance activities and provide community members with basic knowledge to protect themselves against the VACV disease. Further studies could be planned to measure the impact of the educational materials presented here, including a comprehensive approach that

includes access to government services, improving access to PPE and other resources, educational campaigns, and barriers associated with access to information. The proposed educational materials could also help farmers and dairy workers to better understand which variables are the most important to decrease the burden of BV and VACV during day-by-day activities.

4. Materials and Methods

4.1. Study Area and Population

This study was performed in Serro city (18°36′17” S 43°22′46” W), located at 312 km North of Belo Horizonte city, the capital of Minas Gerais State [2,37,38]. According to information from Brazilian Institute of Geography and Statistics (IBGE), the total pop- ulation estimated for 2012–2013 in Serro is 20,833 inhabitants, distributed in an area of 1,217,813 km^2, with 7938 inhabitants located in rural areas [37]. BV is endemic in Serro region and different studies have already reported several outbreaks since 2005 [2,24–26]. Furthermore, other studies conducted in the absence of outbreaks have also reported VACV infections in humans, dairy cattle, horses, and wild animals through serological assays [23,32,37–39].

Serro has a tradition of cheese production, introduced by the Portuguese settlers from Serra da Estrela region more than two centuries ago, when the first cattle farms in the region were formed to support the gold and diamond mining industries [40]. With the decay of the gold cycle, the municipality of Serro intensified its agricultural activity and cheese was the product that guaranteed foreign exchange for the region and the whole state, due to the volume and quality that it represented for the market. Since then, Serro cheese has been featured as a symbol of cultural identity by the peculiar flavor and mode

of production (using raw milk) and is recognized as a culturally important and distinct product [41].

4.2. Dairy Practices and Exposure Assessment

During 2012–2013, a total of 240 individuals with permanent residence in rural areas were invited to participate in the survey and a structured questionnaire was applied as previously described [37]. The farms were selected based on a list of farms provided by the local health stations. We attempted to include all individuals in the household, and each individual was interviewed separately.

The questionnaire was developed to capture demographic data and daily activities in rural areas of Serro that could be potentially associated with VACV exposure [37]. The questionnaire was administered via face-to-face interviews. The questionnaire consisted of questions, divided into five sections: socio-demographic data, bovine vaccinia knowledge (informative questions about the occurrence of BV outbreaks), information on previous outbreaks, dairy practices, and preventive measures. In this study, we focused on questions related to bovine vaccinia knowledge, dairy practices, and preventive measures. The "knowledge questions" (better described as informative questions regarding BV outbreaks), were designed to assess awareness of the population regarding BV outbreaks or VACV infections. Questions about dairy practices were focused on milking practice and milkings per day, consumption of raw milk and cheese made from raw milk, and manipulation of raw milk for cheese production. Furthermore, the questions related to "hygiene measures" allowed us to assess if the population could somehow avoid viral exposure. We also collected serum samples from interviewees for serologic evidence of VACV exposure.

4.3. IgG and IgM Antibodies Detection

Serum samples were screened by an enzyme-linked immunosorbent assay (ELISA) for the presence of anti-OPXV IgG and IgM antibodies as previously described [42] but with a modification for positive identification described below. Individuals aged ≥36 years were considered as vaccinated during the smallpox vaccination campaign and a positive result would not be possible to interpret accurately (vaccination vs exposure). We decided to test only individuals younger than 36yo (n = 126) for the presence of IgG antibodies, while all 240

individuals were tested for the presence of IgM antibodies.

A purified and formaldehyde-inactivated VACV-Dryvax strain diluted in 0.01 M carbonate buffer (Sigma-Aldrich®®, Saint Louis, MO, USA) at pH 9.6 was used as antigen. All samples were tested in duplicate. Specific antibody binding was revealed by the addition of 3,3′,5,5′-tetramethylbenzidine (TMB, Sigma-Aldrich®®, Saint Louis, MO, USA) substrate with absorbance measured at 450 nm. Values were expressed as the optical density (OD). A cut-off value (COV) was determined by the mean of the ODs obtained from the negative controls plus three standard deviations of the mean. This COV was subtracted from OD values resulting in the final corrected OD (OD-COV). An additional
criterion of 0.1 above this OD-COV was added to account for additional variation in non-western populations.

4.4. Ethical Considerations

Ethical clearance was obtained from Research Ethics Committee of Universidade Federal de Minas Gerais under the registration protocol FR–413704. Prior to data collection, the objectives of the study were explained and an informed consent was obtained from all participants. For minors, consent was obtained from parents or guardians.

5. Conclusions

After smallpox eradication, the importance of poxviruses has decreased in human medicine, not being unusual. Our data show that even individuals living in endemic areas at high risk do not know about BV or VACV infections. This study points to the importance of knowledge about BV among farmers and rural workers, which could enhance the

efforts for BV control and prevention. Our study also provides valuable insights to health authorities and decision-makers regarding basic preventive measures for BV that may be useful for improving surveillance and response.

References

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/pathogens10050511/s1. Figure S1: Quick suggestions, in English, of guides and practices to reduce the burden of bovine vaccinia (BV) among farmers and milkers, Figure S2: Booklet with detailed suggestions, in English, on how to recognize lesions caused by VACV and hygiene measures that can be adopted during the milking process, Figure S3: Quick suggestions, in Spanish, of guides and practices to reduce the burden of bovine vaccinia (BV) among farmers and milkers, Figure S4: Booklet with detailed suggestions, in Spanish, on how to recognize lesions caused by VACV and hygiene measures that can be adopted during the milking process, Figure S5: Quick suggestions, in Portuguese, of guides and practices to reduce the burden of bovine vaccinia (BV) among farmers and milkers, Figure S6: Booklet with detailed suggestions, in Portuguese, on how to recognize lesions caused by VACV and hygiene measures that can be adopted during the milking process.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study. For minors, consent was obtained from parents or guardians.

Data Availability Statement: Data is contained within the article or supplementary material.

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Review

Twenty Years after Bovine Vaccinia in Brazil: Where We Are and Where Are We Going?

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Abstract: Orthopoxvirus (OPV) infections have been present in human life for hundreds of years. It is known that Variola virus (VARV) killed over 300 million people in the past; however, it had an end thanks to the physician Edward Jenner (who developed the first vaccine in history) and also thanks to a massive vaccination program in the 20th century all over the world. Although the first vaccine was created using the Cowpox virus (CPXV), it turned out later that the Vaccinia virus was the one used during the vaccination program. VACV is the etiological agent of bovine vaccinia (BV), a zoonotic disease that has emerged in Brazil and South America in the last 20 years. BV has a great impact on local dairy economies and is also a burden to public health. In this review, we described the main events related to VACV and BV emergence in Brazil and South America, the increase of related scientific studies, and the issues that science, human and animal medicine are going to face if we do not be on guard to this virus and its disease.

Keywords: *Vaccinia virus*; bovine vaccinia; public health; zoonosis; neglected disease; laboratory diagnosis

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1. Introduction

For centuries in world history, humanity has faced a fatal disease named smallpox, which seemed impossible to fight. Throughout its existence, smallpox was responsible for the death of hundreds of millions of people worldwide. Its eradication in 1980 is considered one of the most outstanding achievements of human medicine and public health, reaching its 40th anniversary in 2020 [1,2]. The elimination of smallpox was only possible thanks to an eradication program developed and adopted for decades by the World Health Organization (WHO), which consisted of an extensive vaccination of the entire world population in the 20th century. After smallpox was declared eradicated, there was no need to keep vaccinating people once the Variola virus (VARV) was no longer circulating among the human population. Therefore, the worldwide vaccination was discontinued [3].

Historically, vaccinology started with the smallpox vaccination process developed by the British physician Edward Jenner at the end of the 18th century [4]. Jenner curiously observed that the injuries caused by the Cowpox virus (CPXV) in humans, due to the direct contact with infected cows during the milking process, were similar to the lesions caused by VARV [1,4–6]. Hence, the observation of CPXV infections was the preamble to the first vaccine produced worldwide.

Jenner used samples of CPXV collected from infected dairy cows, inoculated in humans, and found that these individuals did not develop smallpox later, confirming the hypothesis of cross-immunization between viruses belonging to the genus *Orthopoxvirus* (OPV) [7]. From this discovery, the technique was improved over the following years and gave rise to the first vaccine in history, which was distributed around the world through arm-to-arm transport, and a few years later would be produced by experimentally infected calves [1,4,6].

After several decades of vaccination, the CPXV isolate used in this vaccine distributed worldwide was supplanted by Vaccinia virus (VACV) [8]. The exact moment in history that it occurred is still unknown, but since the studies about this virus kept increasing throughout the years, VACV was used instead of CPXV in the 20th century in the WHO eradication program of VARV [9]. One of the exciting and useful features of the OPV is sharing an immunological cross-reactivity due to the antigenic similarity among its species [7,10]. The use of VACV and its effectiveness in the eradication of smallpox has made this virus extremely important in the history of virology and immunology. However, even presenting a tremendous scientific relevance and diversity of research on VACV worldwide, its origin and natural reservoir are still unknown [1]. In Brazil, in the last 20 years, there are records of the emergence of a disease directly related to VACV, the bovine vaccinia (BV), which causes natural infections in cattle herds and humans [11–13]. BV is an emerging viral zoonosis characterized by ulcerative lesions on the skin and mucous membranes, affecting mainly dairy cattle and milkers [14]. In dairy cattle, the lesions occur mostly on the teats and udders, are often accompanied by mastitis that can progress to the temporary commitment of the mammary glands. Consequently, it decreases milk production, causing a significant impact on the dairy economy, reducing
profits [1,14–17].

In humans, the clinical manifestations of BV are mainly lesions located at the primary site of infection, usually on the fingers and hands of milkers who have a history of unpro- tected contact with infected animals [12,14,15,17]. However, additional lesions as a result of the self-inoculation process have also been described in other body sites such as the face, eyes, and genital region, and in humans, BV is associated with high morbidity [18–21]. The process between the

onset of the initial symptoms and the healing of the ulcerative skin lesions takes approximately 21 days. Systemic symptoms such as fever, headache, malaise, myalgia, inguinal, and cervical lymphadenopathy are also present about three days after the initial symptoms [16–20].

The classic form of VACV transmission is through direct contact between the milkers and infected dairy cows, which characterizes BV as an occupational zoonosis [16]. Further- more, the sick dairy workers are often removed from work, which results in disruption of the service in the affected rural properties, as well as additional expenses related to the replacement of human resources and decreases the familiar incomes of these affected milkers [13,20,22,23]. The following discussion of VACV and BV researches illustrates chronologically how they have become a very important part of virology and epidemiology history in Brazil during the past 20 years.

2. The Main Areas of BR-VACV Endemicity and the Epidemiological Profile

The first BV outbreaks were identified in the Southeastern region of Brazil, in the States of São Paulo and Rio de Janeiro in 1999, followed by Minas Gerais in 2000 [11,12]. After the initial records of BV outbreaks, VACV has quickly spread to other states and reported in all Brazilian territory. In the past few years, the occurrence of VACV has been reported for the first time in history in Brazil's neighboring countries. The current epidemiological scenario shows the circulation of VACV in an extensive area of the Brazilian territory and other South American countries such as Argentina, Uruguay, and Colombia (Figure 1) [17,24–28].

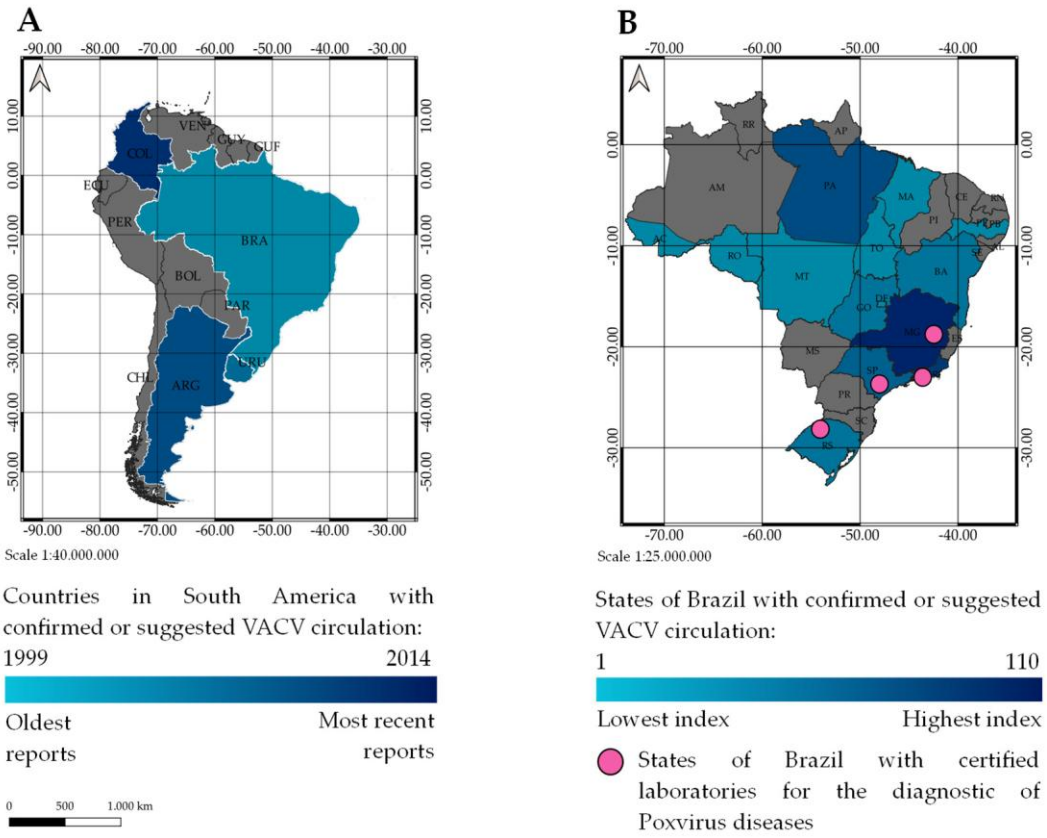

Figure 1. Characterization of vaccinia virus circulation in the South American continent. (**A**) Map of South America indicating the countries where VACV has been detected or suggested to be circulating. The countries in gray color have not recorded VACV detection or suggestion so far. The blue gradient in the left highlights the first records of VACV in Brazil starting in 1999 to the last one in 2014, in Colombia. (**B**) Map of Brazil indicating the States where VACV has been detected or suggested to be circulating. The states in gray color have not recorded VACV detection or suggestion so far. The blue gradient in the right highlights the States from the lowest to the highest index of records reported by scientific publications. Acre (AC), Maranhão (MA), Mato Grosso (MT), Pernambuco (PE), Rondônia (RO), Tocantins (TO), and the Federal District (DF) present only one record each. On the other hand, Minas Gerais (MG) is the state with the highest number of VACV cases (110 records). The pink circles indicate the States of Brazil where there are certified laboratories for the diagnosis of Poxvirus diseases. This map was made using the Free and Open Source QGIS program based on free shapefiles by Instituto Brasileiro de Geografia e Estatística (IBGE) available at https://www.ibge.gov.br/geociencias/downloads-geociencias.html (accessed on 16 October 2020).

Outbreaks and/or VACV occurrences have been recorded in 11 Brazilian states over these 20 years [29–43]. However, only in the States of Minas Gerais and Goiás (Midwest region) BV is mandatory reported by the public health

departments [44,45]. It is also interesting to note that there is a centralization of the diagnosis in the laboratory response network located in few research centers or governmental institutions. These reference cen- ters are distributed in the States of Minas Gerais, São Paulo, and Rio de Janeiro (Southeast region), and in the State of Rio Grande do Sul (South region). With the increasing number of BV cases over time, this scenario reflects how BV and VACV occurrences are still neglected in Brazil. Therefore, forming a cooperative network with strategic actions to communicate, monitor, prevent, and improve research is very important and recommended. This network would be essential to increase partnerships and investments in health, agriculture, and environment research, for a prompt national and global response to reduce the impacts caused by VACV. However, as suggested by Zanella and co-workers, the surveillance of VACV is very scarce, creating more space for the virus to spread and increasing the burden related to its occurrence [46].

The first signs of the emergence of BV in Brazil prompted numerous relevant questions regarding VACV epidemiology, natural history, its hosts, and transmission chain [12,15,29]. Figure 2 shows the number of scientific publications related to the emergence of VACV in Brazil over the past 20 years and the discoveries regarding its natural history and other related fields. It is possible to observe a gradual increase in scientific publications related to VACV and BV until 2017. From that year on, the number of scientific publications decreased drastically until 2020. It is also important to highlight a massive reduction in the budget destinated to science and public health in Brazil in the last four years [47,48]. Moreover, Brazil has experienced Dengue, Zika, Yellow Fever, and Chikungunya viruses threat, which have set up significant public health issues, compromising the financial support to study other viruses and pathogens of great public health interest.

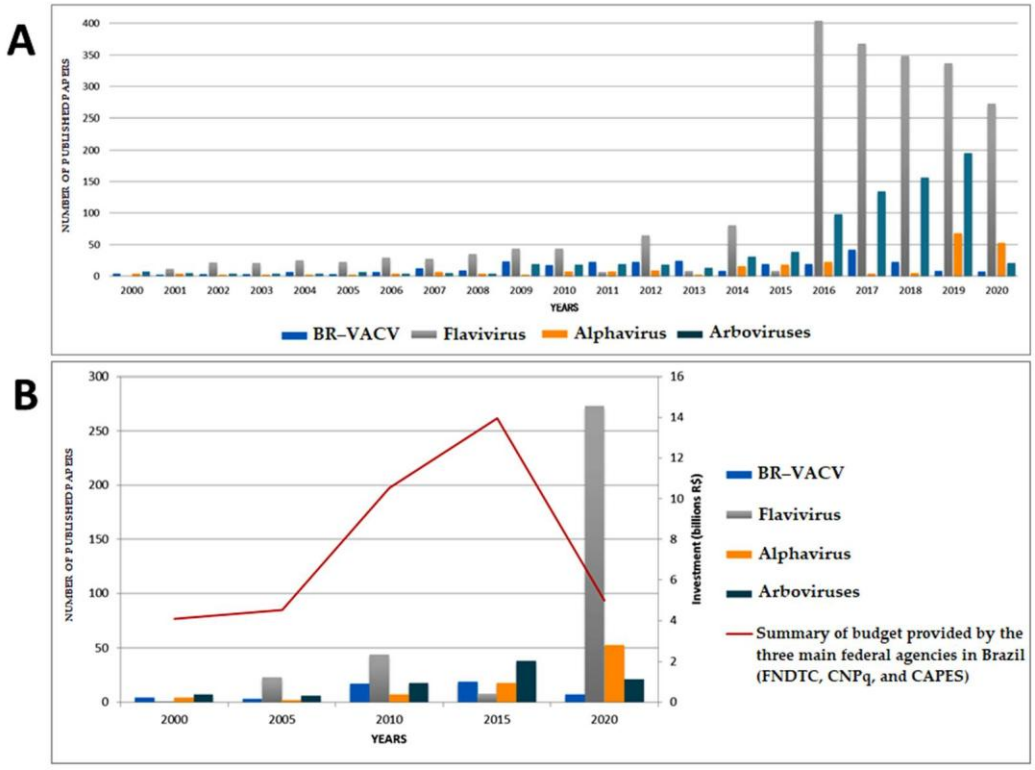

Figure 2. (**A**) Distribution of published scientific papers about Vaccinia virus and/or Bovine Vaccinia, Flavivirus, Alphavirus and Arboviruses during 2000–2020 in Brazil. We included a total of 3.511 publications identified by conducting electronic searches in PubMed platform, available at https://pubmed.ncbi.nlm.nih.gov/ (accessed on 15 March 2021). Published studies were identified using the keywords Vaccinia virus, Bovine Vaccinia, and Brazil (n = 281); Flavivirus

and Brazil (n = 2.184); Alphavirus and Brazil (n = 243); and Arboviruses and Brazil (n = 803). An average of 13.4 VACV publications per year were identified. (**B**) Analysis between the numbers of published papers in Brazil (according to the theme of interest) and the summary of scientific research investments over the past twenty years. Although the decrease in publications of BR-VACV is related to the reduction in funding for its research, the same is not valid for the research related to arboviruses.

After the emergence of VACV and the evolution of studies related to its natural circulation, several findings of great relevance have supported its epidemiology. The first studies associated with the seroprevalence of OPV in rural populations emerged in 2010. Mota and colleagues retrospectively analyzed individuals from Amazon rural villages in North Brazil, finding a seroprevalence of 27.9% [49]. Interestingly 23.4% of the individuals were not vaccinated against smallpox, suggesting they could be exposed to naturally circulating OPV [49]. In 2015, a report from Figueiredo et al. showed a seroprevalence of 9.8% in individuals from rural areas where BV cases have not been observed since the late 1990s [50]. The value of seroprevalence studies as surveillance tools for infectious diseases in the general population is significant. Hence, to better understand the burden of VACV

infections, to identify risk factors, target interventions, and monitor trends, Costa and colleagues designed the first epidemiological study in an endemic area of Brazil [43]. In that survey, almost 31% of studied individuals tested positive for neutralizing antibodies. Increasing of age and previous BV outbreak in the rural properties were independently associated with neutralizing antibodies [43].

Borges and colleagues described a seroprevalence of neutralizing antibodies against OPV in 75.7% of dairy cows sampled in rural communities of Minas Gerais State [22]. Furthermore, the presence of domestic felids on a property was significantly associated with diminished odds of a cow having OPV-neutralizing antibodies. Another study also conducted in Minas Gerais State revealed a seroprevalence of neutralizing antibodies against OPV in 20.6% was also described in equids, raising questions about the role of equids in the VACV epidemiological chain, as well as unrecognized infections and silent circulation [51].

3. Genetic Characterization of Brazilian Vaccinia Viruses (BR-VACV)

Over the past 20 years, the genetic characterization of the BR-VACV isolates is gener- ally carried out by analyses of specific genetic markers. However, the absence of whole- genome sequences from isolates represents a significant gap to better understand the origin and evolutionary history of the viruses circulating in Brazil. Despite that, another signifi- cant discovery is the demonstration that BR-VACV is grouped into at least two different clusters based on genetic diversity: group I and group II [52–54].

Through histopathological and immunohistochemistry analysis in experimentally infected BALB/c mice, it was possible to identify the virulence patterns presented by differ- ent VACV isolates [52–58]. The isolates belonging to group I are less virulent in vitro and in the murine model, while the isolates belonging to group II exhibit great virulence [53,54,57]. Subsequent findings demonstrated that these two groups could co-circulate in the same geographic area and co-infect the same host [34,52]. Despite the existence of two VACV groups circulating in Brazil, the group I viruses have been more frequently isolated when compared to viruses from group II. However, the available laboratory methodology for isolation could be a bias [55].

The first isolation of two different VACV strains in the same BV outbreak was

de- scribed among neighboring farms in the rural area of the state of Minas Gerais, in 2001. During this outbreak, the viruses named Guarani P1 virus (GP1V) and Guarani P2 virus (GP2V) were isolated and characterized [52]. It was observed that several conserved genes also present in other representatives of OPV genus were detected in both GP1V and GP2V. However, this study's main finding was to indicate that different isolates from different places could establish a natural circulation, demonstrating that VACV can have multiple origins and raise a new perspective to explain the genetic diversity observed in Brazil [14,53]. Future studies would reinforce the hypothesis of VACV coinfection in the same host during the same outbreak, showing the existence of a diversity of clones associated with viral infection [54,55,59]. Although these findings are essential in the VACV history, the knowledge regarding how the diversity of clones and genetic variety can interfere in BV outbreaks with different characteristics, different virulence patterns, and clinical presentation in animals and in humans still need to be better explored.

The approach of VACV genetic diversity in Brazil has expanded considerably. Some genes have been identified as useful markers to discriminate between the BR-VACV groups. Analysis of the A56R gene, that encodes for the viral hemagglutinin, revealed a molecular signature based into 18-nucleotide deletion in Group I of BR-VACV [11,12,14]. Another 18-nucleotide deletion in BR-VACV Group I was observed for the gene that encodes the A- type inclusion body protein (A26L), together with an additional 12-nucleotide deletion [60]. Furthermore, a 10-nucleotide deletion is also present for the gene that encodes the CC- chemokine-binding protein (C23L) [57] and a 15-nucleotide deletion for the serine protease inhibitor-3 gene (K2L) gene [54]. On the other hand, these molecular signatures are not detectable in group II, which is composed of several Brazilian isolates and the reference

sample VACV–Western Reserve [11,12,14,54,57,60]. These approaches are important, not only for sample identification but also to infer ancestry and to investigate hypothetical correlation of each sample or group with its unique epidemiological and biological features. Many studies focused on identifying molecular targets in the following years al- lowed a better characterization of viral diversity. These efforts were also significant for the evolution of the VACV diagnosis, and several protocols for laboratory identification became available [57–63]. However, the evolution of the viral isolation, identification, and characterization protocols was not followed by its implementation in the country's reference laboratories, showing a gap between the scientific discoveries and sanitary agen- cies and responsible organs positioning or animal defense facing outbreak emergencies in

Brazilian states.

The complete genome sequences of only two BR-VACV are available so far, Cantagalo and Serro viruses [56,64]. Phylogenetic analysis performed by Medaglia and colleagues showed evidence of a novel, independent cluster of VACV formed by the wild strains Cantagalo and Serro viruses, the Brazilian vaccinal strain IOC (VACV-IOC), and *Horsepox virus* (HSPV). These findings support the hypothesis that BR-VACV could be derived from an ancient smallpox vaccine sample related to *Horsepox virus* that escaped to nature, representing feral VACV that evolved independently of the Brazilian vaccinal strain used in the 1970s after splitting from a most recent common ancestor related to *Horsepox virus* [64]. However, the lack of additional genome sequences of wild BR-VACV samples hampers a conclusive statement about their origins.

Recently, the analysis of the complete genome of clinical isolates of the Cantagalo virus, collected in the early years and at the epicenter of the emergence of VACV in Brazil, revealed genetic characteristics not shared among the isolates. These data suggest different events of VACV spreading in the Southeastern region of Brazil, reflecting the complex genomic diversity of the isolates related to the first outbreaks [65]. The sequencing of more isolates would allow better identification of new genetic markers, elucidates the genetic diversity of BR-VACV that circulate throughout Brazil and probably in other South American countries. The whole-genome sequencing could also help shed light regarding the origins of BR-VACV.

4. Classic Transmission and Alternative Routes for Zoonotic BR-VACV Infections

The first studies related to VACV and BV in Brazil focused on describing outbreaks affecting bovines and humans to understand the transmission dynamic, identification, and biological and molecular characterization of the etiological agent [11,12,14].

After that, the identification of the host spectrum has also been one of the main themes investigated. The detection of VACV in wild rodents, non-human primates, marsupials, procyonids, and equids a few years after the registration of the first BV outbreak has contributed significantly to assessing the dynamics of virus circulation and maintenance in wild and rural environments [30–37,66]. Additionally, VACV circulation has also been detected among domestic animals (cats and dogs) in urban areas [67,68] (Supplementary Table S1).

Although many studies have been trying to elucidate the specific role of farming and wild animals in the VACV transmission chain, there are still many gaps to fill regarding the occurrence of BV outbreaks. However, once VACV was detected in a broad spectrum of hosts in rural and urban environments, it is possible to suggest that some of the particular species can contribute to the spread of VACV to new environments [32,67,68]. Some studies have shown that bovines have a crucial role as viral amplifiers due to the elimination of infectious viral particles through feces and milk in the environment [12,69,70]. These findings suggest that the infectious particles eliminated in the environment could be a source of contamination to other animals (e.g., rodents) in rural areas, spreading to wild areas and maintaining the viral dynamic in nature [33,68,70,71]. Thereby, further studies and the adoption of preventive measures are necessary to reduce the impact of VACV infections and BV outbreaks. Considering the clinical manifestations of BV, if there is no

knowledge for correct management during new and frequent outbreaks, it is not difficult to imagine the increasing burden of BV in the near future.

The establishment of protocols for the detection and characterization of circulating BR-VACV provided the expansion of studies related to the viral host spectrum knowledge. Thus, the detection and characterization of BR-VACV in wild and urban environments (besides rural) began to be conducted in Brazil around the decade of 2010. In the study conducted by Abrahão and colleagues, a VACV strain named Mariana virus (MARV) was isolated from a peridomestic rodent during a BV outbreak in a rural area of Minas Gerais State [33]. Additionally, the same virus was also isolated from humans and dairy cattle affected during that outbreak. This fact strengthens the hypothesis that other animals such as rodents, could be potentially related to the spread of VACV from the rural to wild environments and vice versa [33]. Further studies conducted in areas with history of BV outbreaks corroborated Abrahão's findings, demonstrating the circulation of VACV in wild and synanthropic rodents, reinforcing the hypothesis that wild rodents could be implicated as viral reservoirs [66,72,73].

The studies related to the VACV circulation in wild environments raised epidemi- ological questions regarding its transmission cycle. Hence, Abrahão et al. (2009) and Miranda et al. (2017) proposed hypothetical models for VACV transmission through dif- ferent environments [33], and interaction networks highlighting the wild animals as links between natural and anthropic environments [66]. Furthermore, Costa and colleagues have also proposed a hypothetical model to explain the circulation of VACV in domestic dogs and wild coatis from a transitioning area between urban and wild environments [68]. Using a serological and molecular approach, it was found that the animals were exposed to VACV, and they can potentially work as bridges promoting the circulation of VACV throughout wild and urban environments and posing a threat to human health [68]. Another study detected the presence of VACV in urban domestic cats in Brazil, reinforcing the possibility of transmission of VACV to humans and veterinary professionals, similar to the cases of CPXV in Europe [67].

Another study showed molecular evidence of OPV circulation in capybaras that inhabit the Lagoa da Pampulha (part of the famous architectural complex

of Pampulha in Belo Horizonte, Southeast region of Brazil) and surroundings, raising questions about these rodents in the VACV transmission cycle, as well as the presence of VACV in urban areas [73]. Further studies have also demonstrated VACV circulation in capybaras in the State of São Paulo, confirming the susceptibility of these large rodents to the virus [74]. Finally, the detection of VACV in other wild animals (rodents, marsupials, chiropterans, and cingulates) in the South and Southeast regions of Brazil, outside the context of BV outbreaks, revealed that these animals could potentially act as hosts in the epidemiological chain of VACV in urban environments, and possibly play a role in the transmission to humans [75].

The possibility of disseminating VACV in the urban environment and increasing BV cases should be better investigated to shed more light on the VACV emergence. It is estimated that, due to the discontinuation of smallpox vaccination, a large proportion of the population living in urban environments have never been exposed to OPV [3,76,77]. Moreover, there are more and more immunosuppressed individuals in a society due to chronic and acquired conditions, whose exposure to VACV could evolve into a severe form of infection. Furthermore, the potential establishment of the virus in urban domesticated animals can contribute significantly to the maintenance of an urban cycle [17,27,78,79]. Hence, further studies are necessary to better understand the potential entry of VACV into the urban environment.

As BV affects mainly dairy cattle and milkers, several queries regarding the transmis- sion of VACV through milk and dairy products have been raised. Even during scientific conferences in Brazil and worldwide, questions such as “is there any chance someone could get infected with VACV by drinking milk from infected cows?” have emerged. Indeed, studies focused on the relationship between VACV transmission and dairy production have

grown significantly over these 20 years of VACV history in Brazil [20–22]. Figure 3 displays a timeline with chronological events related to the detection of VACV in dairy products. The first records of the presence of VACV in milk samples from naturally infected cows occurred during 2005–2008. Abrahão and colleagues detected and isolated viral particles on chicken egg chorioallantoic membranes (CAM) and also observed cytopathic effects on embryo fibroblasts (CEFs), in addition to molecular and phylogenetic analysis [80]. Hence, this study became one of the main references to raise questions about milk's risks as a potential alternative route of VACV transmission.

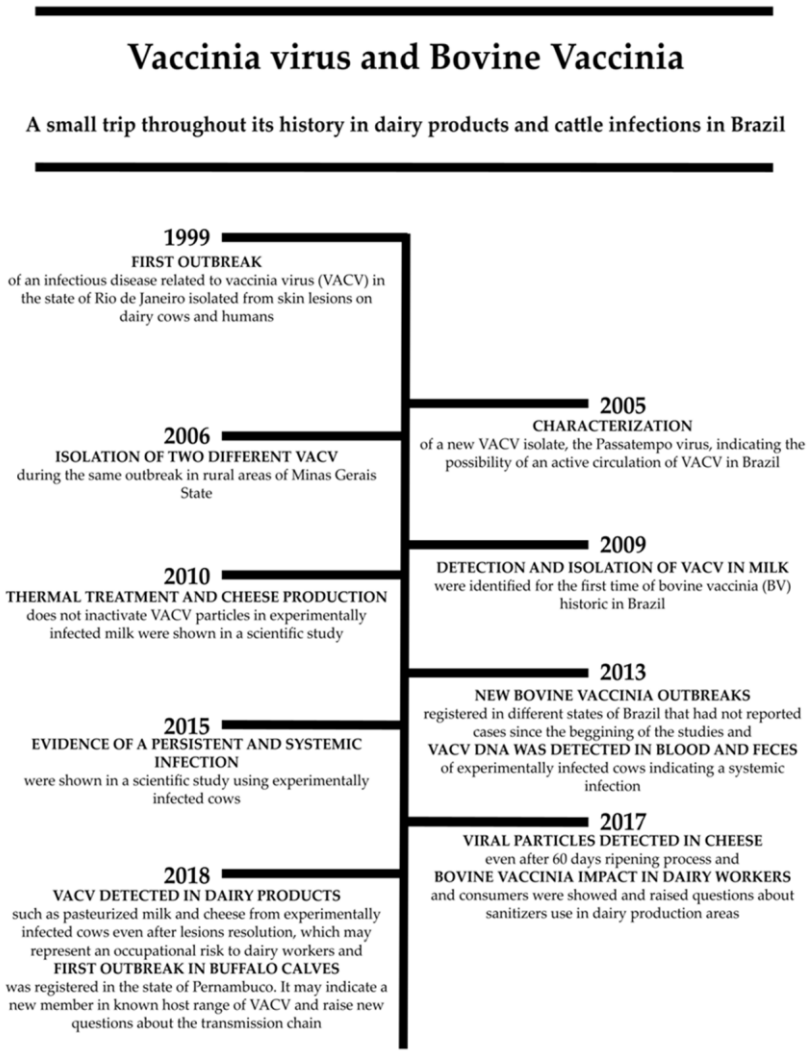

Figure 3. A vertical timeline of the main events regarding the Vaccinia virus occurrence dairy products and cattle in Brazil during 2000–2020.

In the following years, other correlated researches would appear, revealing that VACV viral particles could not be inactivated even after thermal treatments

of milk and during cheese processing, both in their production and in their maturation period. The detection of infectious viral particles (from both groups I and II) in milk samples and dairy products confirmed this hypothesis [80–84].

It is noteworthy that serological and molecular evidence was detected in naturally and experimentally infected cows, and dairy products produced from the milk of these animals, raising the discussion about the persistence of systemic infection in these animals [13,84]. In addition to this evidence, Borges et al. demonstrated that even in rural properties with no record of BV outbreaks, dairy cattle could present neutralizing antibodies against VACV [22]. Furthermore, the practice of milking without properly sanitizing cows' udders

can directly affect cattle exposure and milkers [22]. All the studies conducted over the past few years reinforce the possibility that dairy products could work as vehicles for VACV dissemination and potential risks generated to public health [78,80–84].

5. The Transversal Impact of BR-VACV

In the last 20 years, milk and dairy products' production and consumption have grown significantly in Brazil. Data from Brazilian Agricultural Research Corporation (EMBRAPA) [85] have shown that the milk production in Brazil increased 139% between 1990 and 2019. Brazil ranks as fourth among 20 countries regarding total milk production and annual growth rates, being behind India, the United States, and Pakistan [85,86]. The State of Minas Gerais leads the ranking of the milk production, followed by Paraná and Rio Grande do Sul [85,86].

The tradition in milk production and its derivatives is a striking feature of Minas Gerais State, significantly impacting the Brazilian economy [85]. Historically, Minas Gerais has a tradition in dairy production and has been primarily affected by BV outbreaks. In addition to dairy farming, the State of Minas Gerais is internationally known for its production of artisanal cheeses prepared by local farms in the countryside, being registered as intangible and cultural heritage, showing great relevance to the State economy, as well as to the country [85–87]. Given this traditional feature, dairy products can also be considered as essential assets for tourism purposes. According to Kamimura et al., the State of Minas Gerais has lots of tourist regions and many tourists feel attracted by the artisanal cheese production, learning about the process during scheduled tours [88]. This recognition directly impacts the economy of Minas Gerais. Thus, all issues affecting dairy production in the local farms greatly affect the local economy and national.

The demonstration that these products can act as alternative routes for zoonotic VACV transmission, the demand for monitoring and developing preventive strategies for quality control of both milk and its derivatives are necessary. Furthermore, proper training emphasizing the importance of hygiene measures to milkers focused on milking activities and artisanal cheese production would be essential. Therefore, the awareness and training of milkers and farmers to acceptable dairy practices, the identification of any problems during

production, and the supervision and orientation by trained professionals would be critical to result in excellent and safe products.

The quality control of milk and dairy products through the process of pasteurization is of great importance in preventing the transmission of VACV and other infectious agents, also preventing these products to potentially act as alternative routes of viral dissemination in the rural and also urban environments [17,22,25,87,88]. However, the impact of milk and dairy products as vehicles for VACV spreading is still poorly explored. In regions recognized by the production as artisanal cheese, the burden of VACV maybe even more significant since the artisanal cheeses are essentially made with crude milk (no thermal processes are applied) [89–91].

In Brazil, very few laboratories have the knowledge, tools, and capacity for BV di- agnostics, despite many outbreaks have been described in the country (especially in the Southeast region). In addition, the increasing danger of potential establishment of VACV urban cycles and consequent outbreaks makes it extremely necessary to invest in research that can elucidate questions about the eco-epidemiological cycle in different areas, the origin of the virus, its natural reservoirs, and new therapies to control the burden of VACV infections. Furthermore, the investment in new, efficient, and rapid tools for VACV and other OPV detection and increasing the diagnostic capacity for other laboratories through- out Brazil would be valuable for the surveillance and monitoring of VACV and future BV outbreaks.

The consolidation of VACV infections in large populations can lead to problems for public health services. The undergraduate courses in Medicine and Nursing at the main public universities that are considered high-quality educational institutions and where most research is conducted in Brazil, sometimes do not include or dedicate a small portion of

the teaching load regarding Poxviruses in disciplines related to Microbiology and Virology (Supplementary Table S2). Thus, newly trained health professionals are not being prepared to deal with the burden of VACV occurrence and even with BV outbreaks.

The exclusion of teaching about Poxviruses in many undergraduate courses can be partially explained by the emergence of new viruses and new diseases that affect humans and are more present in the population's daily lives after the eradication of smallpox, as well as how health authorities still neglect VACV and BV. As a result, professionals do not know about the clinical symptoms characteristic of poxviruses, including VACV infection and its epidemiology. It has not been uncommon for cases in which health professionals offer incorrect treatment, with the false conception that this is another type of infection, which makes the epidemiological mapping of cases of BV in Brazil even more complex [92]. In 20 years, one study was conducted to evaluate the healthcare professionals' knowl- edge and perceptions about bovine vaccinia in Brazil and the results showed that 43.1% of healthcare professionals that work in an endemic area have never heard about the disease, which could be attributed to not being well-informed enough to recognize and therefore report clinical cases accurately [92]. The lack of knowledge of health professionals also
reflects in the BV notification data across Brazil.

6. Where Are We Going with the BV and/or VACV Threat?

Despite the growing number of scientific publications and research on BV and VACV natural circulation in Brazil, many gaps in the knowledge of its epidemiology and natural history have not been filled yet. The sequencing and genetic characterization of new isolates are still needed to assess genetic relatedness and determine relationships among different BR-VACV strains and hosts. The reports of the expansion of BV in Brazil and in other South American countries have not been enough to establish epidemiological surveillance, being BV and VACV still neglected by health authorities. Because most of the research on VACV and BV was focused on describing the outbreaks and its consequences, there are still many gaps to explore in VACV epidemiology, such as its prevalence among milking cattle, other farms and domestic animals, dairy workers, and the general population.

The absence of a unified system and accurate records provided by the

healthcare agencies are out of date and do not demonstrate VACV and/or BV epidemiology's actual situation. It is necessary to develop continuing education practices for all professionals working with VACV and/or BV. Hence, all kind of professionals dealing with VACV emer- gence, such as medical doctors, nurses, epidemiologists, veterinarians, laboratory workers, as well as public health authorities, people involved in fieldwork, and administrative per- sonnel, could offer greater credibility in the registration of cases and data, thus providing better and efficient preventive actions.

Furthermore, future studies aiming to understand the prevalence of VACV and/or OPV infections in urban populations and monitor the levels of immunity against VACV are necessary. Additionally, routine disease surveillance through physicians and laboratories is also essential to better understand the presence of VACV and the emergence of outbreaks in vulnerable populations, monitoring the associated risk factors to guide better public health practices.

This review aimed to highlight the main findings in these 20 years of history of VACV in Brazil through studies relevant to the understanding of the situation that the virus and the disease are in the country. The lack of investment in research, the unpreparedness of health professionals and agencies to deal with the disease, and the dairy products showing aptitude as alternative routes of infection raise the question of how BV should be treated from this moment on. Thus, the need for further studies is clarified so that all gaps are filled to control and monitor the disease in Brazil.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/pathogens10040406/s1, Table S1: List of hosts and susceptible animals to BR-VACV, and the association to transmission to humans; Table S2: List of the main Universities in Brazil that mention the teaching of Virology and Poxviruses in their undergraduate courses.

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Review

Here, There, and Everywhere: The Wide Host Range and Geographic Distribution of Zoonotic Orthopoxviruses

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Abstract: The global emergence of zoonotic viruses, including poxviruses, poses one of the greatest threats to human and animal health. Forty years after the eradication of smallpox, emerging zoonotic orthopoxviruses, such as monkeypox, cowpox, and vaccinia viruses continue to infect humans as well as wild and domestic animals. Currently, the geographical distribution of poxviruses in a broad range of hosts worldwide raises concerns regarding the possibility of outbreaks or viral dissemination to new geographical regions. Here, we review the global host ranges and current epidemiological understanding of zoonotic orthopoxviruses while focusing on orthopoxviruses with epidemic potential, including monkeypox, cowpox, and vaccinia viruses.

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1. Poxvirus and Emerging Diseases

Zoonotic diseases, defined as diseases or infections that are naturally transmissible from vertebrate animals to humans, represent a significant threat to global health [1]. Among the species recognized as pathogenic to humans, more than half originated in animals, and some have been characterized as emerging or re-emerging pathogens [2,3]. Most zoonotic pathogens originated in wild and domesticated mammalian hosts such as bats, rodents, and primates [4]. The analysis of global trends indicates that new zoonotic threats will continue to emerge at an accelerating rate, and are mainly associated with an growthing population, changes in land use, climate changes, increased intercontinental travel, and expanded trade networks [4,5].

Poxviruses are of great veterinary and human importance and infect numerous vertebrate and invertebrate animals, including humans. The *Poxviridae* family is di- vided into two subfamilies, namely: *Chordopoxvirinae*, which infect vertebrates, and *Ento- mopoxvirinae* (A–C), which infect invertebrates. The *Chordopoxvirinae* subfamily is further divided into 18 genera (*Avipoxvirus, Capripoxvirus, Centapoxvirus, Cervidpoxvirus, Crocodylid- poxvirus, Leporipoxvirus, Macropopoxvirus, Molluscipoxvirus, Mustelpoxvirus, Orthopoxvirus, Oryzopoxvirus, Parapoxvirus, Pteropopoxvirus, Salmonpoxvirus, Sciuripoxvirus, Suipoxvirus, Vespertilionpoxvirus*, and *Yatapoxvirus*), distinguishable by their serological reactions [6,7].

The family *Poxviridae* comprises large, brick-shaped or ovoid enveloped viruses containing a linear, double-stranded DNA genome approximately 200 kilobase pairs in length [7,8]. Poxviruses are among mankind's longest and best-known viruses mainly because of their most feared and lethal representative, *Variola virus* (VARV), the causative agent of smallpox. Before its remarkable eradication in 1980, VARV represented a centuries- old threat to humans worldwide and killed approximately 300–500 million people during the 20th century [9]. The global eradication of smallpox marked the culmination of an

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intensive vaccination program and quarantine measures promoted by the World Health Organization (WHO) [8,10,11]. Although VARV was eradicated 40 years ago, many chal- lenges regarding poxvirus infections persist, including the worrisome possibility of VARV reintroduction by accidental release, its use as a biological weapon, or the emergence and re-emergence of zoonotic orthopoxviruses worldwide [12,13].

Orthopoxviruses are remarkable for their wide host spectrum, ranging from humans to domestic and wild animals (Figure 1). *Orthopoxvirus* is the most important and well- characterized poxvirus genus, mainly due to its impact on human and animal health [7,8]. Here, we review the major aspects related to the dynamics and emergence of zoonotic orthopoxvirus infections worldwide, focusing on the host range and current epidemio- logical situation relating to monkeypox (MPXV), cowpox (CPXV), vaccinia (VACV), and VACV-like viruses.

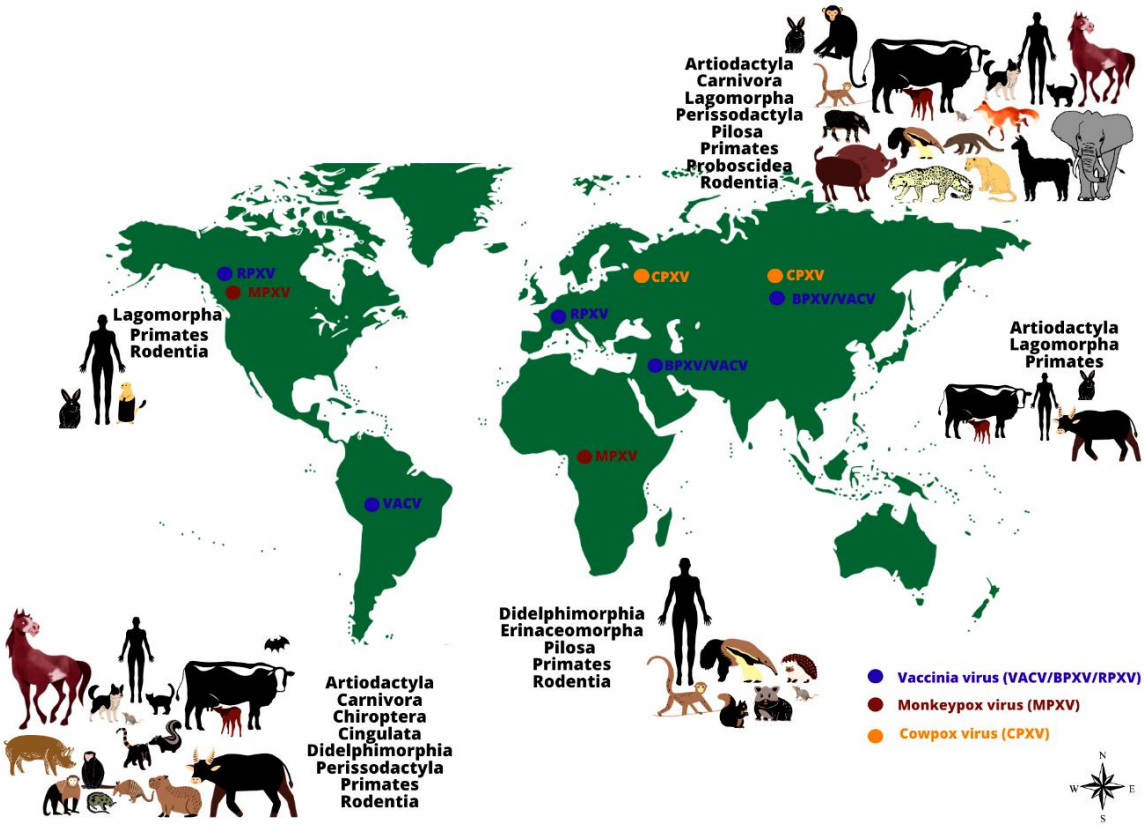

Figure 1. The worldwide distribution and host range of monkeypox, cowpox and vaccinia viruses.

The image shows the range of animal hosts (represented by orders) that have

been demonstrated to be naturally infected by some *Orthopoxvirus* species, according to different regions of the world (except by Monkeypox virus in the United States of America, repre- sented by imported cases). Orthopoxvirus infections have been demonstrated in animals belonging to different orders, using different methods (virus isolation, molecular detection of viral genomes or serological screening for antibodies against orthopoxviruses). The oc- currence of some zoonotic orthopoxviruses has already been confirmed (by virus isolation or molecular detection of the viral genome) in some geographical regions (indicated by colored dots: blue: vaccinia virus (including buffalopox and rabbitpox viruses) in South America, Europe, Asia, and the Middle East; brown: monkeypox virus in Africa and North America; orange: cowpox virus in Europe and Asia).

2. Orthopoxvirus

The *Orthopoxvirus* genus comprises VARV, VACV, CPXV, MPXV, *camelpox virus*, *Akhmeta virus*, and other species with zoonotic potential. All orthopoxviruses share significant DNA sequence similarity and are immunologically cross-reactive and cross-protective. Infection with any orthopoxvirus is considered to generate protection against exposure or re- exposure to any other member of the genus [14,15]. Orthopoxvirus species are named primarily according to the hosts from which they were first isolated and identified; however, the name does not necessarily represents its natural reservoir or complete host range [8,16–19]. Despite the large number of studies, little is known about the primary hosts and reservoirs of zoonotic orthopoxviruses in nature, or their transmission and maintenance cycles [20]. Regarding the host range, orthopoxviruses can be both highly specialized and host re- stricted or generalists with a broad host range. For instance, VARV is a highly specialized virus that infects only humans, whereas MPXV, CPXV, and VACV are examples of gener- alist zoonotic orthopoxviruses that can infect several mammalian host species and also spillover into humans [20].

The evolution of generalists pathogens requires the successful crossing of host trans- mission barriers [21]. These include geographical, ecological, and behavioral constraints that separate a virus from its possible recipient hosts; virus-host cell incompatibility, such as tissue tropism, differences in receptor binding, genome replication, production, and shedding of infectious particles; and host immunity evasion, which includes cellular bar- riers or responses that restrict the infection and/or evasion of a virus from the innate immune system of its host [22]. To overcome these barriers, orthopoxviruses have dif- ferent biological features that can synergistically contribute to the transmission to, and exploitation of, a broad range of new hosts species as observed for CXPV, MPXV, and VACV. Orthopoxviruses can cause both local lesions on the skin and systemic infections, resulting in direct and indirect transmission routes. When accompanied by viral particle stability in the environment, this can increase the likelihood of potential hosts being exposed to the virus independently of direct contact with infected hosts. In addition, orthopoxviruses can infect a variety of mammalian cells in a manner that is mostly independent of species- specific receptors and have large genomes that carry the information essential for viral

replication, thereby increasing the possibility of successful infection in a new cell/host. Although the double stranded DNA genomes of orthopoxviruses have low mutation rates when compared with other viruses, such as RNA viruses, orthopoxviruses possess a genetic arsenal comprising several immune-regulatory, virulence, and host range genes [20]. The variety of host-genes among poxviruses enables them to express different viral proteins with important roles in cell tropism, as well as in the modulation of host signaling pathways and immunomodulatory responses, thereby establishing optimal cellular conditions for viral replication [23]. Finally, many of the strategies employed by orthopoxviruses to evade host immune defenses target conserved elements of the immune system in different poten- tial hosts [20]. Combined, these features altogether are crucial for virus-cell and virus-host interactions and can contribute to the success of viral replication and transmission.

Despite the eradication of smallpox, the possibility of its re-emergence or the emer- gence of other orthopoxviruses in human and animal populations is a relevant global health issue. As smallpox vaccination is no longer mandatory, most of the world's popu- lation that is under 40 years of age lacks immunity against orthopoxviruses [24,25]. This scenario is highlighted by numerous reports in recent years of human diseases caused by zoonotic orthopoxviruses such as MPXV [26–33], CPXV [34–41], VACV-like [42–49], and Akhmeta virus [18]. To date, the circulation of orthopoxviruses among wild and domestic animals has been recorded in different regions of the world, including South America, Africa, Europe, the Middle East, and Asia [27,40,42,43,50–57]. These facts raise concerns regarding the host ranges and distribution of orthopoxviruses, as well as their potential to cause outbreaks in animals and human populations, thereby further impacting animal and public health.

2.1. Monkeypox Virus

Monkeypox virus isolates are subdivided into two clades, namely, the West African and the Congo Basin clades, based on genetic and phenotypic (virulence) differences [58]. Notably, several studies have indicated that the clinical signs are similar between infections caused by viruses from either clades [59]. The first observation of MPXV infection was reported in 1958 during an outbreak of pustular rash illness in cynomolgus macaques (*Macaca fascicularis*) arriving in Copenhagen, Denmark, from Singapore [60]. Despite being named after the first described host, non-human primates are accidental hosts for MPXV [61].

Further insights into the range of taxa susceptible to MPXV infection were obtained by laboratory studies and field surveys. MPXV infections have been reported in a broad range of rodents, such as mice (*Mus musculus*), rabbits (*Oryctolagus cuniculus*), hamsters, woodchucks (*Marmota monax*), jerboas (*Jaculus* sp.), and porcupines (*Atherurus africanus*) (Table 1). Similarly, based on methods such as viral isolation, molecular assay, or experimen- tal infection, susceptibility to MPXV infection was reported in ant-eaters (*Myrmecophaga tridactyla*), black-tailed prairie dogs (*Cynomys ludovicianus*), southern opossums (*Didelphis marsupialis*), short-tailed opossums (*Monodelphis domestica*), African hedgehogs (*Atelerix* sp.), and several non-human primate species. Additionally, serological surveys have implicated several African rodents, including giant pouched rats (*Cricetomys* spp.), African dormices (*Graphiurus* spp.), rope squirrels (*Funisciurus* spp.), and sun squirrels (*Heliosciurus* spp.) as primary orthopoxvirus hosts in Africa [61–63].

Among Old World non-human primates, cynomolgus monkeys (*Macaca fascicularis*), sooty mangabeys (*Cercocebus atys*), orangutans (*Pongo pygmaeus*), and chimpanzees (*Pan troglodytes*) can be infected with MPXV. Among New World non-human primates [60,64–75], the common marmosets (*Callithrix jacchus*) was shown to be susceptible to MPXV infection through intravenous inoculation [76] (Table 1).

In 2003, a MPXV outbreak occurred in the United States of America (USA). Human infection was associated with direct contact with ill pet prairie dogs that were kept near to infected exotic animals imported from Ghana, West Africa [77]. This episode, as well as the infection of rodents, heightened

concerns regarding the introduction of MPXV into the Americas. Meanwhile, the susceptibility of several African rodents to MPXV raised worries about the transmission of the virus to humans, as these animals are sometimes kept as pets [78,79].

Although humans are also accidental hosts [61], MPXV became the most significant pathogenic zoonotic orthopoxvirus for humans since the eradication of smallpox, given its associated morbidity (systemic infection) and lethality. The first human MPXV infection was described in 1970 for a 9-month old child in the Democratic Republic of Congo who had presented smallpox-like skin eruptions [70,80]. Several other human cases were reported in the following years. From 1970 to 1999, the WHO reported at least 404 confirmed and approximately 500 suspected human cases of monkeypox in different African countries (Central African Republic, Cameroon, Nigeria, Côte d'Ivoire, Liberia, Sierra Leone, and Gabon), but mainly in the Democratic Republic of Congo [52,81,82]. From the 2000s, alongside outbreaks in the Democratic Republic of Congo, the Republic of Congo, and South Sudan, the first human cases outside the Africa continent were also described. During May and June of 2003, cases of people with febrile illness and skin eruptions were notified to the Wisconsin Division of Public Health, but no deaths were reported and no person-to-person transmission was proven [78]. The source of this outbreak was traced back to imported infected exotic animals from Ghana [52,62,78]. Fortunately, the multi-state episode of captive rodent infection in the USA was short-lived, and the transmission cycle in the country was broken [83].

Table 1. Hosts and susceptible animals to monkeypox virus infection.

Order/Family of	Species	Method Investigation *	Association to Human Infection **
Primates/ *Hominidae*	Humans (*Homo sapiens*)	viral isolation	yes
	Orangutans (*Pongo pygmaeus*)	viral isolation	yes
	Chimpanzees (*Pan troglodytes*)	viral isolation	no
Primates/ *Cercopithecidae*	Sooty mangabeys (*Cercocebus atys*)	PCR/ viral isolation	no
	Cynomolgus monkeys (*Macaca fascicularis*)	viral isolation	yes
Primates/ *Callithrichidae*	White-tufted marmosets (*Callithrix jacchus*)	Lab. Infec.	no
Rodentia/*Chinchillidae*	Rabbits (Oryctolagus cuniculus)	Lab. Infec.	no
Rodentia/*Muridae*	Inbred mouses (*Mus musculus*)	Lab. Infec.	no
Rodentia/*Cricetidae*	hamsters	Lab. Infec.	no
Rodentia/*Nesomyidae*	Giant-pouched rats (*Cricetomys* sp.)	PCR/ viral isolation	no
Rodentia/*Gliridae*	*African dormices* (*Graphiurus* sp.)	PCR/ viral isolation	no
	Rope squirrels (*Funisciurus* sp.)	PCR/ viral isolation	yes

Rodentia/*Sciuridae*	*Black-tailed prairie dogs* (*Cynomys ludovicianus*)	PCR	yes
	Woodchucks (*Marmota monax*)	PCR/ viral isolation	no
	Jerboas (*Jaculus* sp.)	PCR/ viral isolation	no
Rodentia/ *Dipodidae*			
Rodentia/*Hystricidae*	Porcupines (*Atherurus africanus*)	PCR/ viral isolation	no
Pilosa/*Macroscelididae*	Ant-eaters (*Myrmecophaga tridactyla*)	viral Isolation	no
Didelphimorphia/ *Didelphidae*	Southern opossums (*Didelphis marsupialis*)	PCR/ viral isolation	no
	Shot-tailed opossums (*Monodelphis domestica*)	PCR/ viral isolation	no
Erinaceomorpha/ *Erinaceidae*	African hedgehogs (*Atelerix* sp.)	PCR/ viral isolation	no

* Method of investigation: viral infection demonstrated by molecular assay (PCR) or viral isolation using samples obtained from naturally infected animals; Lab. Infec.: MPXV infection susceptibility was observed during experimental studies in laboratory. ** Transmission to humans already reported in the literature.

Alarmingly, several outbreaks of monkeypox in humans have been reported in African regions in the last decade. In 2010, two confirmed and eight suspected cases were described in the Republic of Congo related to the migration of refugees, regional inter-ethnic conflicts, or autochthonous cases. No deaths were reported among the confirmed cases, although one individual with suspected infection died [84]. In the same year, two cases of MPXV infection associated with hunting and the consumption of wild rodent meat were reported in the Central

African Republic, with no deaths [85]. Numerous suspected and confirmed cases were reported in the Democratic Republic of Congo, from 2010 to 2016 [86,87], and in Serra Leone in 2014 [88]. Several suspected and 12 confirmed cases, as well as three deaths were reported in different provinces in the Central African Republic (Mbomou, Basse-Kotto, and Haute-Kotto) [52,89,90]. In 2017, the Republic of Congo reported its largest MPXV outbreak (88 suspected and seven confirmed cases, with six deaths), which affected 18 villages in five districts. This outbreak presented risks of MPXV spreading to neighboring countries given the extent of population mobility and refugee presence in the region [30].

Some African regions have continuously reported human cases of MPXV infection in recent years (2017 to 2020), including the Central African Republican (27 confirmed cases and two deaths) [91,92], Nigeria (181 confirmed cases and seven deaths) [31,93], Sierra Leone (one confirmed case) [94], Liberia (two confirmed cases and two deaths) [95], Cameroon (one confirmed case) [96], and the Democratic Republic of Congo (numerous confirmed cases and 321 deaths) [33,97]. Recently (2018), three cases of monkeypox were reported in the United Kingdom. Two were of people who had traveled to Nigeria, while the third concerned a health care worker who had had contact with one infected patient. One of the patients who had traveled to Nigeria reported having contact with a person with a rash and the possible consumption of bushmeat, raising the possibility that this may have been a case of secondary or even tertiary human-to-human transmission. Meanwhile, the infection contracted by the British care worker confirms human-to-human MPXV transmission [96]. Other cases of MPXV infections outside of Africa were reported in Israel (2018) and Singapore (2019), for travelers who imported the disease from Nigeria [98,99].

The natural source of MPXV and its maintenance cycle in nature remains unknown as the virus has only been isolated twice in nature (wild animals): once from the rope squirrel (*Funisciurus anerythrus*), Zaire, in 1985 [62], and once from the sooty mangabey (*Cercocebus atys*), Côte d'Ivoire, in 2012 [100]. To date, naturally occurring MPXV infections remain confined to the forest regions of West and Central Africa [101,102]. Consequently, a higher proportion of human MPXV cases are reported in regions (mainly African villages) where humans and non-human primates live in close proximity. The consumption, hunting, and handling of meat derived from non-human primates, rodents, and other small mammals have also been associated with human cases of MPXV infection [71,85,86,103–105]. Close contact with rodents has also been implicated as a source of human infection [67,106].

Human cases of monkeypox have been increasing even though they may have been underreported. Notably, diagnostic capabilities in the affected countries are most often lim- ited, while health care workers worldwide are generally not aware of monkeypox disease. A lack of understanding about monkeypox disease associated with factors such as the in- creasing encroachment of humans into wild habitats, the inter-continental travel of people from endemic areas to

MPXV-free regions, and the importation of animals both as pets and for laboratory studies raises concern regarding MPXV emergence, surveillance, prevention, and control [15]. Additionally, vaccination against smallpox was ceased decades ago, resulting in an increasingly larger number of people that are vulnerable to infection by MPXV or other orthopoxviruses. Although some animal species have been described as being susceptible to MPXV infection, most of what is known about its pathogenesis and clinical characteristics is derived from descriptions of animals in captivity or laboratory facilities. As monkeypox is an emerging zoonotic disease with epidemic potential and much of its host range and maintenance cycle in nature remains obscure, advances are urgently needed to better understand natural cycle of MPXV.

2.2. Cowpox Virus

Edward Jenner was the first to document CPXV infection after observing local le- sions on the teats of cows, which he called "*cow-pox*". Then, in 1798, Jenner demonstrated the efficacy of "*true cow-pox*" scarification in inducing immunity against smallpox [8,107]. There were frequent reports of bovine cowpox cases until the early 1970s in Europe, with sporadic transmission to humans, mainly milkers, occurring via contact with infected cows [108]. However, the number of bovine cowpox cases decreased, while reports of "cowpox-like" infections in several animal species, such as cats and elephants, [109] in- creased. "Cowpox-like" infections were described in a broad range of captive and domes- tic animals like non-human primates [110–112], felines [108,111,113–117], dogs [118], ro- dents [39,50,111,119–125], foxes [126,127], rhinoceroses [15,114,128], tapirs [129], okapis [130],
horses [131], anteaters [114], mongooses [129], stone martens [132], bearcats [133], and farmed llamas [134,135] (Table 2). The viruses responsible for these infections induced clinical signs similar to those of CPXV infection such as hemorrhagic pocks on the chorioal-

lantoic membrane and A-type inclusions bodies, and were thus considered to be "true cowpox" [136,137]. Most of these animals are thought to be accidental hosts for CPXV, and not reservoirs. Rodents, particularly voles (*Microtus* spp. and *Myodes* spp.), are known to be the primary CPXV reservoirs in nature [136,138].

Table 2. Hosts and susceptible animals to cowpox virus infection.

Order/Family	Species	Method of Investigation *	Association to Human Infection **
Primates/*Hominidae*	Humans (*Homo sapiens*)	virus isolation	no
Primates/*Callithrichidae*	White-tufted marmosets (*Callithrix jacchus*)	virus isolation	no
Primates/*Cercopithecidae*	Barbary macaques (*Macaca sylvanus*) Cynomolgus macaques (*Macaca fascicularis*) Rhesus macaques (*Macaca mulata*)	virus isolation Lab. Infec. Lab. Infec.	no no no no
Carnivora/*Felidae*	Domestic cats (*Felis catus*) Cheetahs (*Acinonyx jubatus*) Lions (*Panthera leo*) Pumas (*Felis concolor*) Black panthers (*Panthera padus*) Jaguarundis (*Herpailurus yaguarondi*) Jaguares (*Felis onca*)	virus isolation virus isolation virus isolation virus isolation virus isolation virus	yes yes yes no no no no

		isolation	
Carnivora/*Canidae*	Dogs (*Canis lupus familiaris*) Foxes (*Vulpes vulpes*)	virus isolation Lab. Infec.	no no
Carnivora/*Herpestidae*	Banded mongooses (*Mungos mungo*)	virus isolation	no
Carnivora/*Ailuridae*	Bearcats (*Aiulurus fulgens*)	virus isolation	no
Perissodactyla/ *Rhinocerotidae*	Black rhinoceros (*Diceros bicornis*)	virus isolation	no
	White rhinoceros (*Ceratotherium s. simum*)	virus isolation	no
Perissodactyla/*Equidae*	Horses (*Equus caballus*)	virus isolation	no
Artiodactyla/*Bovidae*	Cows (*Bos taurus*)	virus isolation	yes
Artiodactyla/*Giraffidae*	Okapis (*Okapia johnstoni*)	virus isolation	no
Artiodactyla/*Camelidae*	Lamas (*Lama glama* sp.)	virus isolation	no
Rodentia/*Arvicolidae*	Field voles (*Microtus agrestis.*)	virus isolation	no
Rodentia/*Muridae*	Brown rats (*Rattus norvegicus*) Giant gerbils (*Rhombomys opimus*)	virus isolation virus isolation	yes no
Rodentia/*Cricetidae*	Root voles (*Microtus oeconomus*)	virus isolation	no
Rodentia/*Caviidae*	Patagonian cavys (*Dolichotis patagonum*)	PCR	no
Rodentia/*Castoridae*	Beavers (*Castor fibor canadensis*)	virus isolation	no
Rodentia/*Sciuridae*	Ground squirrels (*Citellus fulvus*)	virus isolation	no
Proboscidea/ *Elephantidae*	Asian elephants (*Elephas maximus*)	virus isolation	yes
	African elephants (*Loxodonta africana*)	virus isolation	no

* Method of investigation: virus infection demonstrated by molecular assay (PCR) or viral isolation using samples obtained fromnaturally infected animals; Lab. Infec.: MPXV infection susceptibility was observed during experimental studies in laboratory. ** Transmission to

humans already reported in the literature.

CPXV is currently mostly found in Europe and northern and central Asia where cases of infections in rodents, cats, and humans continue to be reported. In Great Britain, CPXV is endemic in rodents such as bank voles (*Myodes glareolus*) and wood mice (*Apodemus sylvaticus*), while in Turkmenistan and Russia CPXV was isolated in the laboratory as well as in wild rodents [119]. Furthermore, serological surveys have also detected orthopoxvirus infections in France, Austria, and Norway in voles and wood mice [119]. Antibodies against orthopoxviruses were also detected in red foxes (*Vulpes vulpes*) in Western Europe being possibly related to CPXV infection, halthough red foxes are also known to be susceptible

to ectromelia virus [119,139]. These reports of CPXV infection have occurred alongside an increasing number of reported infections in different animal species, leading to the designation of CPXV as an emerging health threat [140]. The first reported case of CPXV in a domestic cat occurred in 1977 in the Netherlands, and the number of CPXV infections in cats has since increased. According to Essbauer and collaborators (2010), more than 400 cases of CPXV infections in domestic cats were described in Western Eurasia until 2004 [15]. In cats, CPXV infection causes multiple skin lesions on the head, neck, forelimb, paws, and eyes (conjunctivitis), and the appearance of vesicles in the oral cavity and tongue. In the most severe cases, the disease can be systemic, affecting inner organs (mainly the lungs), with fatal outcomes being mostly associated with secondary bacterial infection [141]. Cats are the most affected domestic animals, mainly due to their predatory behavior against rodents, which are the CPXV reservoir in domestic and peridomestic environments [15,141–144]. However, the exact prevalence of feline cowpox is uncertain. CPXV infections in cats are mostly observed after increases in the rat population density [15,144].

The infection of pet rats and domestic cats by CPXV brings a higher risk of exposure to humans in the domestic environment, but rural or wild areas may be important as the source of infection [36]. Cowpox in humans is mainly caused by contact with infected domestic cats or rodents (such as *Rattus norvegicus*) that are kept as pets [15,34,37,38,121]. Even though human cowpox cases are usually self-limiting and not lethal, most people are susceptible to the disease, particularly children who are more often in close contact with pet animals [37,121]. The zoonotic potential of CPXV and its capacity to cause infection in wild and domestic environments are well established; however, many aspects of its natural maintenance cycle remains unknown. Besides the domestic animals, CPXV has a vast range of hosts and the increase in the breeding and commercialization of exotic animals raises concerns among health authorities regarding the emergence of cowpox, including in new geographical regions.

2.3. Vaccinia Virus and Related Viruses

Although VACV is the most extensively studied orthopoxvirus, its origin remains un- known [145]. *Vaccinia virus*, the prototype species of the *Orthopoxvirus* genus, is best known as the live attenuated virus used worldwide by the WHO in the smallpox vaccine [146–148]. Despite the successful use of

VACV as a vaccine, several vaccine strain-dependent compli- cations have been reported, including progressive vaccinia, eczema vaccinatum, vaccinia gangraenosum, and neurological complications [145,149]. During smallpox eradication campaigns, various VACV strains with different degrees of virulence were used. The highly attenuated and modified VACV Ankara is a well-stablished third generation smallpox vaccine [150,151]. For a long time, VACV vaccine strains were assumed to be incapable of establishing a natural cycle due to their attenuation in the laboratory. However, several VACVs have been isolated from different host species, and in different locations around the world [42,152–154]. Similarly, sub-lineages of VACV (as buffalopox virus (BPXV) and rabbitpox virus (RPXV)) have been consistently isolated in different countries and from a wide range of hosts [14–16,43,53,155].

In India, BPXV was first described in 1934, and was responsible for infections that mainly affected domestic buffaloes (*Bubalus bubalis*), but also cows and humans [155]. BPXV resembles VACV in terms of its properties (size, shape, structure, and physicochemical properties) [156], pathogenesis, and pathology. Phylogenetic analyses confirmed the monophyly of the BPXV and its likely origin from the VACV Lister vaccine [155–159]. Since its first description, outbreaks of BPXV have been reported in India, Pakistan, Nepal, Egypt, Bangladesh, Indonesia, Russia, and Italy [15,43,53,160–162].

Humans become infected with BPXV through close contact with infected animals and no human-to-human transmission has been reported to date. In 2004–2005, a nosocomial outbreak in humans occurred in Pakistan, and the source of infection was traced to buffalo fat used as a first-aid medication for covering skin burns. This unusual source of infection was indicative of indirect BPXV transmission [14,163]. Additionally, a variety of animal

species, such guinea pigs, BALB and white Swiss mice, cows, buffalo calves, rabbits, and chickens have been experimentally demonstrated to be susceptible to BPXV. Neverthe- less, the role of these species in BPXV transmission and maintenance in nature remains unknown [44,164] and requires clarification.

RPXV is another VACV described as affecting different animal species worldwide (Table 3). RPXV was first described between 1930–1933 after outbreaks in laboratory rabbits in the USA. Additional outbreaks were later reported in 1941 in the Netherlands, while several other cases were also reported in Europe and the USA [165,166]. To date, no human transmission has been described for RPXV [165,167].

Table 3. Hosts and susceptible animals to vaccinia and vaccinia-like viruses infection.

Order/Family	Species	Method Investigation *	Association to Human Infection **
Artiodactyla/*Bovidae*	domestic buffaloes (*Bubalus bubalis*)	PCR/ Viral isolation	yes
Artiodactyla/*Bovidae*	cattle/cows (*Bos taurus*)	PCR/ Viral isolation	yes
Primates/*Hominidae*	Humans (*Homo sapiens*)	PCR/ Viral isolation	yes
Primates/*Cebidae*	Capuchin monkeys (*Sapajus apella*)	PCR	no
Primates/*Atelidae*	Black-howler monkeys (*Alouatta caraya*)	PCR	no
Didelphimorphia/*Didelphidae*	Black-eared possums (*Didelphis aurita*)	PCR	no
	White-eared possums (*Didelphis albiventris*)	PCR	no
	Wooly-cuycas (*Caluromys philander*)	PCR	no
Carnivora/*Procyonidae*	Ring-tailed coatis (*Nasua nasua*)	PCR	no
Carnivora/*Felidae*	Domestic cats (*Felis catus*)	PCR	no
Carnivora/*Canidae*	Domestic dogs (*Canis familiaris*)	PCR	no
Cingulata/*Chlamyphoridae*	Armadillos (*Euphractus sexcintus*)	PCR	no
Perissodactyla/*Equidae*	Horses (*Equus ferus caballus*)		

			yes
		PCR/	
Donkeys (*Equus africanus* sp.)		PCR	yes
Mules (*Equus mulus*)		PCR	yes
Chiroptera/*Molossidae*	Black-molossus bats (*Molossus rufus*)	PCR	no
Broad-eared bats (*Eumops perotis*)		PCR	no
Lagomorpha/*Leporidae*	Rabbits [+]	PCR	yes
	(*Oryzomys* spp.)	PCR/ Viral isolation	no
	Black-footed colilargos (*Oligoryzomys nigripes*)	PCR	no
	Yellow pygmy rice rats (*Oligoryzomys flavenscens*)	PCR	no
Rodentia/*Cricetidae*	Rat-headed rice rats (*Sooretamys angouya*)	PCR	no
	Vesper mouses (*Calomys* spp.)	PCR	no
	Grass mouses (*Akodon* spp.)	Viral isolation PCR	no
	Hairy-tailed Bolo Mouses (*Necromys Lasiurus*)	PCR	no
	Bush mouses (*Cerradomys subflavus*)	PCR	no
Rodentia/*Echimyidae*	Hairy Atlantic spiny rats (*Trinomys setosus*)	PCR	no
Rodentia/*Muridae*	Inbred-mouses (*Mus musculus*)	PCR/	yes
	Black-mouses (*Rattus rattus*)	Viral isolation PCR	no
Rodentia/*Caviidae*	Capybaras (*Hydrochoerus hydrochaeris*)	PCR	no

* Method of investigation: viral infection demonstrated by molecular assay (PCR) or viral isolation using samples obtained from naturally infected animals; Lab. Infec.: VACV infection

susceptibility was observed during experimental studies in laboratory. ** Transmission to humans already reported in the literature. $^{+}$ Human infection from occupational exposure to rabbit skins inoculated with VACV.

Different VACV isolates also circulate in South American countries, including Uruguay, Argentina, Colombia, and Brazil [54–56,168]. In the last few decades, several outbreaks of VACV infection have occurred in Brazil where the disease caused by VACV is popularly known as "*bovine vaccinia*", due to its association with dairy cattle [42,168]. Bovine vaccinia is characterized by vesiculopustular exanthematous disease in cattle, anddairy workers who have direct contact with infected animals [169–171].

Since the detection of VACV in rural areas in Southeast Brazil, in 1999, several Brazilian- VACV (Br-VACV) isolates have been identified in the country (Araçatuba virus, Belo Hori- zonte virus, Cantagalo virus, Carangola eye virus 1, Carangola eye virus 2, Guarani P1virus, Guarani P2 virus, Mariana virus, Passatempo virus, Pelotas 1 virus, Pelotas 2 virus, and Serro virus) [148,169,172–175]. One hypothesis for the origin of the Br-VACVs assumes that they are derived from the spillback of a vaccine strain to the sylvatic environment [154,172], while another postulates that they may represent natural genetically and phenotypically diverse VACV populations, circulating in an unknown natural reservoir [148,152,173]. In particular, the presence or absence of an 18 nucleotide sequence within gene *A56R* gene (viral hemagglutinin) was proposed to be a molecular marker that can separate Br-VACVs into two distinct clades (group 1 and group 2) [176–178]. The existence of at least two clades was further confirmed through genetic and evolutionary analyses, of Br-VACVs, causing in- fection or co-infections in diversity of hosts in Brazil. [47,48,54–56,153,169,174,175,179–188]. In addition to the genetic diversity, some studies have also shown distinct biological profiles between the two Br-VACV groups [189,190]. The biological implications of this diversity in the context of the epidemiology and clinical evolution of the disease in humans should be further investigated.

Initially, VACV outbreaks were described as affecting dairy cattle and humans in rural environments. Consequently, the epidemiology of bovine vaccine in Brazil is associated with economic losses resulting from compromised milking herds [42,171,191,192]. In Brazil, bovine vaccinia have been mainly reported in the Southeast (Minas Gerais State), which has the largest dairy cattle herds in the country [42,148]. Nevertheless, VACV circulation in Brazil has already been documented for all the regions, affecting farm animals other than cattle, as well as wild animals [42]. Consistent with its wide geographical occurrence in

Brazil, VACV has been detected in different biomes and related fauna. VACV genomes and antibodies against orthopoxviruses have been detected in a broad range of animalsincluding non-human primates (*Sapajus apella* and *Alouatta caraya*) [193]; procynoides (*Didelphis aurita, Didelphis albiventris,* and, *Nasua nasua*) [188,194]; cingulates (*Euphractus sexcintus*) [185]; marsupials (*Didelphis* sp. and *Caluromys philander*) [153,194]; bats (*Molossus rufus* and *Eumops perotis*) [185]; and wild rodents (*Oligoryzomys nigripes*, *Oligoryzomys flavescens*, *Sooretamys angouya*, *Calomys* sp., *Akodon* sp., *Necromys lasiurus*, *Necromys squamipes*, *Trinomys setosu*, *Cerradomys subflavus*, *Mus musculus*, *Rattus rattus,* and *Hydrochoerus hydrochaeris*) [153,180,185,195,196]. Furthermore, VACV has been detected in diverse peridomestic and domestic animals, including buffaloes [183,197,198], horses, donkeys [174,181,182,195], pigs [195], cows [195], dogs [188], cats [179], and mice [184]
(Table 2).

Although direct VACV transmission between wild and domestic animals and between wild animals and humans has not been documented to date, these possibilities cannot be excluded. Several studies have indicated that cattle have a role as amplifiers in the bovine vaccinia cycle and have also demonstrated that VACV excretion in feces may favor viral transmission and its maintenance in the environment [170,199–201]. Subsequently, it was proposed that other farm animals could also be implicated in the VACV transmission chain, although direct transmission to humans has yet to be documented. Lastly, wild rodents could be VACV reservoirs, while peridomestic rodents could act as the link for VACV spread between wild and rural environments, promoting the transmission among wild mammals and farm animals [183,184].

Although VACV is known to have a broad range of hosts, many aspects of its natural history remain unknown. Bovine vaccinia is mainly caused by contact with infected cattle

and is associated with economic losses to the dairy industry in Asia and South Amer- ica [42,43,45,53,148]; however, the epidemic potential of VACV is a reality. Although VACV infection is usually self-limiting and not lethal, the disease profile in immunocompromised individuals may be differentially affected, presenting with severe and generalized manifes- tation [202], similar to that observed for cowpox. As currently documented for VACV, until the 1970s, CPXV mainly infected cattle and milkers. However, when cattle were replaced by cats and other animals as the primary hosts of CPXV infection, the number of human cases of CPXV infection increased. Given the similarities with CPXV, its plausible that VACV could follow similar path. Although farm animals are important sources of infection, the commercialization and consumption of dairy products could be alternative routes of zoonotic VACV transmission. In addition, VACV circulation in domestic animals such as cats and dogs bring the risk of viral transmission to humans in the domestic environment. The urban emergence of VACV could be an important health burden due to the unpre- paredness of healthcare professionals to correctly identify and handle emerging cases [203]. Moreover, VACV infection presents a high attack rate, and VACV emerging cases in an urban area, where agglomeration of people is more frequent, could favor transmission, and trigger a public health emergency [148,204].

3. What Is Next for Monkeypox, Cowpox, and Vaccinia Viruses?

The history of poxviruses and orthopoxviruses has frequently been related to hu- man cultural behavior. The establishment of agricultural settlements is considered one of the factors that favored the emergence of smallpox approximately 10,000 years ago. Orthopoxviruses continue to emerge and re-emerge due to the increasing proximity of humans to wild and rural habitats. Following smallpox eradication, the global scenario is marked by a vast naïve human population and the wide circulation of different or- thopoxviruses. These facts raise concerns on the possible epidemic potential of these viruses in animals and humans. In fact, zoonotic orthopoxviruses already represent an important issue for animal health and economics. An example is the case of VACV and BPXV that have been associated with significant economic losses resulting from dairy cattle and livestock infection in several Asian and South American countries [42,43,53,191,192].

Currently, MPXV is mostly observed in Africa; CPXV in Europe and

Asia; BPXV in Asia and the Middle East; and VACV in South America [53–56,119,140,160,162,168,205]. Al- though the factors that restrict the geographic distribution of some zoonotic orthopoxviruses are still unknown, their distribution range has been increasing as MPXV has been exported to parts of the USA [77], United Kingdom [96], Israel [98], and Singapore [99]. Legal or illegal trade of animals or animal-derived products, migration of animal populations, and traveling and migration of people are some factors that can contribute to the geographic dissemination of orthopoxviruses on local or global scales. Indeed, animal trade leading to the MPXV importation into the USA illustrated how globalization can favor the spatial spread of viruses [77]. On a local scale, migration of refugees within Africa is another example related to MPXV dissemination [84].

Because orthopoxviruses such as VACV, CPXV, and MPXV have genetic and pheno- typic traits that allow them to possess a variety of mammals hosts [23], one cannot exclude the possibility of the virus infecting susceptible hosts in new geographical areas. These viruses are more prevalent in certain animal species, such as VACV in cattle and BPXV in buffalos. Molecular and immunological factors may be associated with productive infections in these animals while ecological factors may be linked to transmission between individuals of the same species. On the other hand, to cross host barriers and infect a new host, a virus must be able to infect and replicate in the new host, evade the immune system, and be efficiently transmitted [22]. Regardless of the remote possibility of an orthopoxvirus infecting new hosts, the current host plasticity is already notable, especially for MPXV, CPXV, and VACV. In addition, the emergence of a virus in a new host does not necessarily require evolutionary changes (mutations, rearrangements, etc.). One example

of this process is the canine distemper virus, which has a very wide host range in mammals and its emergence in these species appears to be limited primarily by contact [22].

Orthopoxvirus outbreaks are usually related to populations living in rural areas or small villages. However, factors such as a high population density, increased urbanization, agriculture activities, deforestation, approximation to wild habitats, and inter-continental travel of people from endemic to pox-free regions may introduce poxviruses into different zones including urban environments [3,206]. A primary concern related to infected ani- mals in periurban and urbanized environments is associated with a possible increase in orthopoxvirus transmission in a naïve population and even human-to-human transmission. The epidemic potential of a virus is related to several factors, including geographical distribution, route of transmission, pathogenicity, and host range, among others. The epidemic potential may be lower for orthopoxviruses than for other RNA viruses or viruses transmitted by airway routes. Nevertheless, orthopoxviruses are remarkable regarding their transmission and dissemination among several hosts and environments. Wild and domestic animals could act as intermediate hosts for the emergence or re-emergence of or- thopoxviruses in the human population. For instance, CPXV is transmitted from wild to do- mestic animals and then to humans, MPXV can be transmitted directly from wild animals to humans, and VACV is transmitted from domestic animals to humans [15,42,85,86,104,144]. Zoonotic orthopoxviruses may be transmitted either directly or indirectly and new forms of viral transmission have been described, which is a concern for public health. Milk and dairy products might be a potential source of VACV exposure or transmission [191]. Even under a low transmission rate, human-to-human transmission has already been demonstrated for zoonotic orthopoxviruses (MPXV and VACV) [96,207]. These are significant findings that should be further evaluated and closely monitored.

The cessation of routine vaccination against smallpox decades ago has resulted in a large contingent of people that are susceptible to orthopoxvirus infections, which have high morbidity rates. Moreover, in immunosuppressed individuals, exposure to orthopoxvirus infection can result in severe forms of the disease, or even death [208]. To date, there have been no reports of fatalities resulting from CXPV or VACV infection; however, MPXV infection in humans

can progress to a lethality of up to 10% [209]. These facts indicate that VACV, MPXV, and CPXV pose a potential threat not only for humans, but for animals in different regions of the world. Together, these factors highlight the need for continuous epidemiological surveillance and the need to better understand the natural cycles and evolution of orthopoxviruses, their host range and reservoirs, the burden of outbreaks and dissemination of orthopoxvirus-associated diseases. This information is crucial for the development and application of control measures such as sanitary barriers and public policies aimed at controlling these viruses.

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Short Communication

Detection and Molecular Characterization of *Yellow Fever Virus*, 2017, Brazil

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Abstract: At the end of 2016, Brazil experienced an unprecedented yellow fever (YF) outbreak. Clinical, molecular and ecological aspects of human and non-human primate (NHP) samples collected at the beginning of the outbreak are described in this study. Spatial distribution analyses demonstrated a strong overlap between human and NHP cases. Through molecular analyses, we showed that the outbreak had a sylvatic origin, caused by the South American genotype 1 YFV, which has already been shown to circulate in Brazil. As expected, the clusters of cases were identified in regions with a low vaccination coverage. Our findings highlight the importance of the synchronization of animal surveillance and health services to identify

emerging YF cases, thereby promoting a better response to the vulnerable population.

Keywords: *Yellow fever virus*, Non-human primates, Emerging infectious diseases, Zoonotic virus, Arboviruses, Outbreak

Yellow fever virus (YFV), together with *Dengue virus*, is responsible for major emerging diseases in South America (WHO 2017). YFV (Family *Flaviviridae,* genus *Flavivirus*) is maintained through a sylvatic cycle, in which mosquitoes from genera *Haemagogus* and *Sabethes* are, so far, identified as the main vectors. Furthermore, non-human primates (NHP) act as hosts, viral amplifiers, and also play an important role as sentinel animals (Monath 2001; Costa et al. 2011; Kuno et al. 2017; Vasilakis and Weaver 2017). Humans participate in this cycle as accidental hosts, usually by coming into contact with a forested environment (Monath 2001; Barrett and Higgs 2007). In Brazil, the urban circulation of YFV has not been recorded since 1942 (Carrington and Auguste 2013; Monath and Vasconcelos 2015). Brazil is experiencing an unprecedented outbreak of YF, with a lethality rate of 32%, which is higher than ob-

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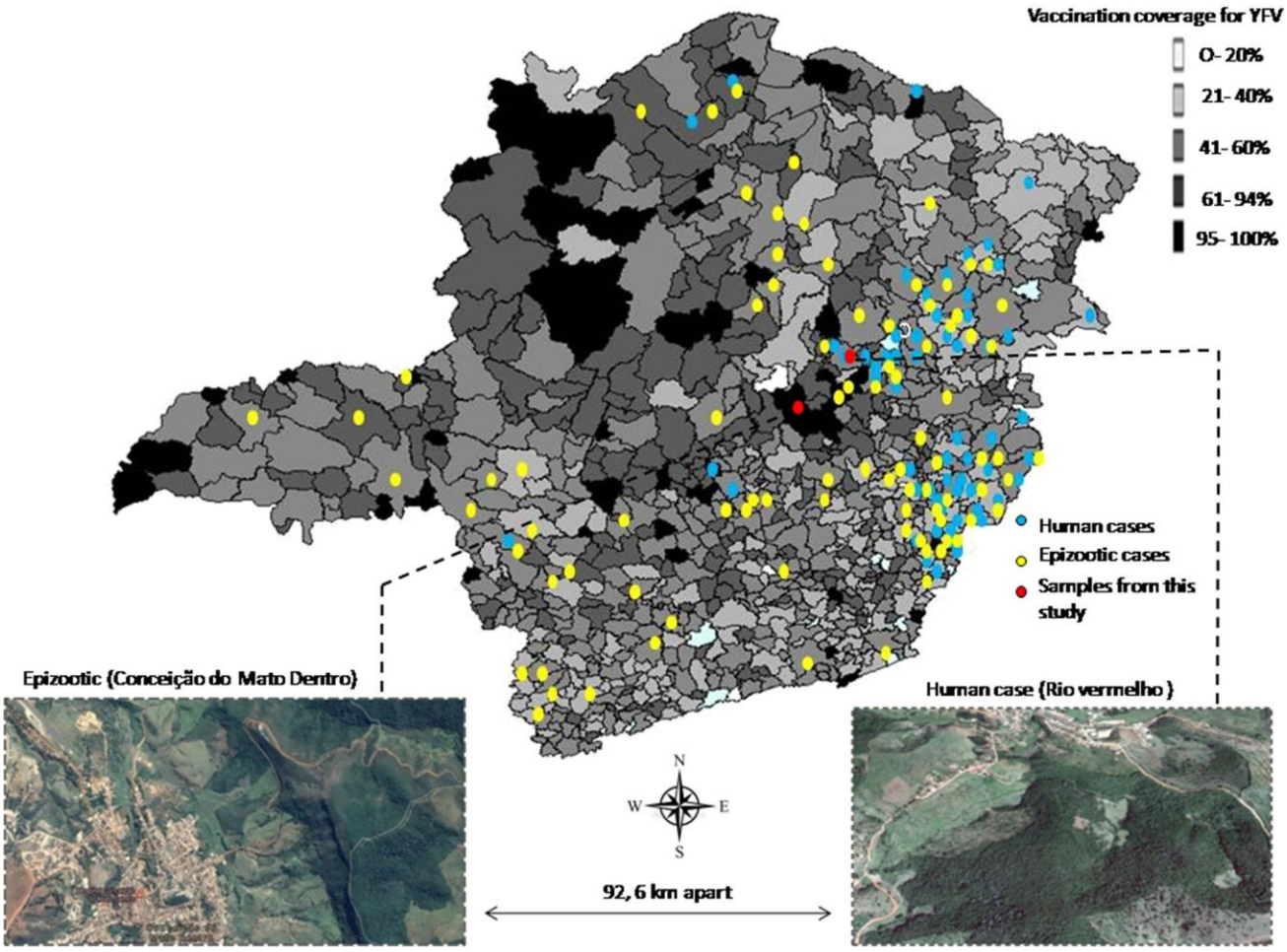

Figure 1. Geographical distribution between human cases and confirmed epizootics of YFV in Minas Gerais in 2017. Map of Minas Gerais evidencing the vaccination coverage by the municipality (Brasil 2017b). Vaccination coverage is shown in grayscale. Yellow and blue dots indicate, respectively, human cases and confirmed epizootics (Brasil 2017a). Red dots indicate the collection area of the samples used in this study.

served in previous outbreaks (10–15%). Most cases were concentrated in the southeastern region of Brazil, with notification of epizootics and fatal human cases in regions previously considered free of the disease (WHO 2017). The 2016/2017 outbreak was mainly concentrated in the southeastern region of the country, with the epicenter and onset of the outbreak in Minas Gerais (MG) state, where about 60% of human cases (465 cases) and deaths (152 cases) were confirmed, in addition to the majority of epi- zootics (Brasil 2017a, b). Furthermore, other affected states in the southeastern region of Brazil include Espı´rito Santo, Rio de Janeiro and Sa˜o Paulo (Bonaldo et

al. 2017; Brasil 2017a; Barbosa et al. 2018; Moreira-Soto et al. 2018). De- spite the decline in the number of cases, the outbreak continues to progress in 2018 (Saúde 2018). In this study, we analyzed samples from one patient who lived in a rural area of Rio Vermelho, MG state ($18°17^{0}37^{00}S$; $43°00^{0}33^{00}W$) (Fig. 1). Ethical clearance was obtained from the Ethics Committee on Human Research of Universidade Federal de Juiz de Fora under the protocol number 1.431.969. The patient, a 34-year-old male, was admitted to the hospital on January 23, 2017, the beginning of the yellow fever out- breaks in Brazil, presenting the following symptoms: fever, oliguria, myalgia, intermittent diarrhea, and severe thrombocytopenia. The patient received YF vaccination 1 day before onset of symptoms and was hospitalized 1 day after. On day 8 after onset of symptoms, he was moved to an intensive care unit (ICU) due to severity of clinical conditions (liver failure, gingival bleeding and encephali- tis). Despite intensive support therapy, the patient died

10 days after onset of symptoms (Supplementary Fig. 1). For clinical confirmation of YFV infection, serum and ur- ine samples were collected on days three and six after hospitalization. RNA virus was extracted by using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany). The viral RNA was used for cDNA synthesis using random primers and MMLV enzyme (Promega, USA). cDNA was used as the template in a pan-flavivirus qPCR targeting the NS5 region and in a conventional PCR for CprM region (Patel et al. 2013; Jorge et al. 2017). The pan-flavivirus reactions were tested in duplicate. The serum sample collected on day 3 post-onset of symptoms tested positive for pan-flavivirus; however, urine sample on day three tested negative. These results corroborate previous findings that during viremia, the virus had not been shed, differently from what had already been reported in the convalescent phase (Barbosa et al. 2018). To rule out infection with dengue, chikungunya, and enterovirus, additional qPCR assays were also performed (De Morais Bronzoni et al. 2005; Dierssen et al. 2008; Leo et al. 2009); however, no positive results were detected. Amplified cDNA fragments from NS5 and CprM were directly sequenced in both directions by Sanger methodology on the ABI3130 platform (Life Technologies, Foster City, USA). Sequence quality was analyzed by Ap- plied Biosystems Sequence Scanner Software v1.0 (Applied Biosystems, 2012). For the optimal alignment of the NS5 and CprM regions, ClustalW (www.ncbi.nlm.nih.gov/pmc/articles/PMC308517) and MEGA version 7 (www.mega software.net) were used. BLAST analyses revealed that the sequences (GenBank access numbers: MF580491; MF580493) showed high identity with other YFV sequences deposited in GenBank. Phylogenetic analysis demonstrated that our sample grouped with the South American geno- type 1 already circulating in Brazil (Fig. 2 and supple- mentary Fig. 2).

In addition to the human case, we analyzed two NHP carcasses found in Conceic¸a˜o do Mato Dentro city in January and February 2017 (beginning of the report of outbreaks of yellow fever in Brazil). Conceic¸a˜o do Mato Dentro ($19°02^{0}14^{00}$S; $43°25^{0}30^{00}$W) is located 92 km from Rio Vermelho (Fig. 1). NHP carcasses were obtained by field veterinarians working in an area affected by the out-
break. Field veterinarians were authorized by the Brazilian Environment Ministry SISBIO license number 111.019/ 2016 to work in the area collecting animal samples. The NHP investigated in this study belonged to the species *Callicebus personatus* and were both females. During the autopsy, a large amount of petechiae in the gastric mucosa,

blood clots in the stomach contents, small yellowish areas in the hepatic parenchyma, edema and hyperemia of the eyelid, and mild pulmonary edema were observed (Fig. 3). Samples of liver, heart, spleen, lung, stomach, and kidney were tested. For YFV detection, RNA was extracted from 30 mg of NHP tissues by using RNAesy Mini Kit (Qiagen, Hilden, Germany). Additional tests were performed as described above for the human sample. All tissue samples analyzed by PCR tested positive for YFV except heart. Sequencing of the NS5 region confirmed YF infection. CprM sequencing was also obtained from one NHP liver sample (GenBank access numbers: MF580492; MF580494; MF580495). BLAST analyses showed a high identity with jungle YFV sequences from samples collected in previous sylvatic circulation deposited in GenBank. Phylogenetic analysis demonstrated that these samples also grouped with the human sample from Rio Vermelho. All analyzed sam- ples (human and NHP) grouped with South American genotype I (Fig. 2 and Supplementary Fig. 2), which has been already recognized as circulating in Brazil and being responsible for current outbreaks described in north and central-west regions (Bonaldo et al. 2017; Mir et al. 2017a, b; Moreira-Soto et al. 2018). The samples from this study showed a greater similarity to each other than to other YFV sequences detected in current outbreaks around the coun- try. In addition, the comparison of our sample of *Callicebus* with another sample of *Alouatta* (Bonaldo et al. 2017) from this same outbreak showed high similarity to each other (Fig. 2).

Many factors may have contributed to the emergence of YF in MG state that are not fully understood. Low vaccination coverage rate for YF (Fig. 1), in addition, could have contributed to the large amount of human cases ob- served in this outbreak. Rio Vermelho had a low vaccina- tion coverage (around 43%) despite the Brazilian Ministry of Health's recommended coverage above 95% and was included in a cluster region for YF (Fig. 1) (Brasil 2017c). This could indicate potential failures in the state vaccina- tion policy (Pessanha 2009). Furthermore, it is important to note that Rio Vermelho is located in a geographical region of MG state with recurrent histories of YF outbreaks. The patient in this study was a resident from a rural area of Rio Vermelho and presented an ongoing yellow fever infection after receiving the yellow fever vaccine. The vac- cination was 1 day before onset of symptoms, ruling out the possibility of an adverse event following immunization (WHO 2008). Our data highlight the importance of strengthening the mass vaccination campaign, especially

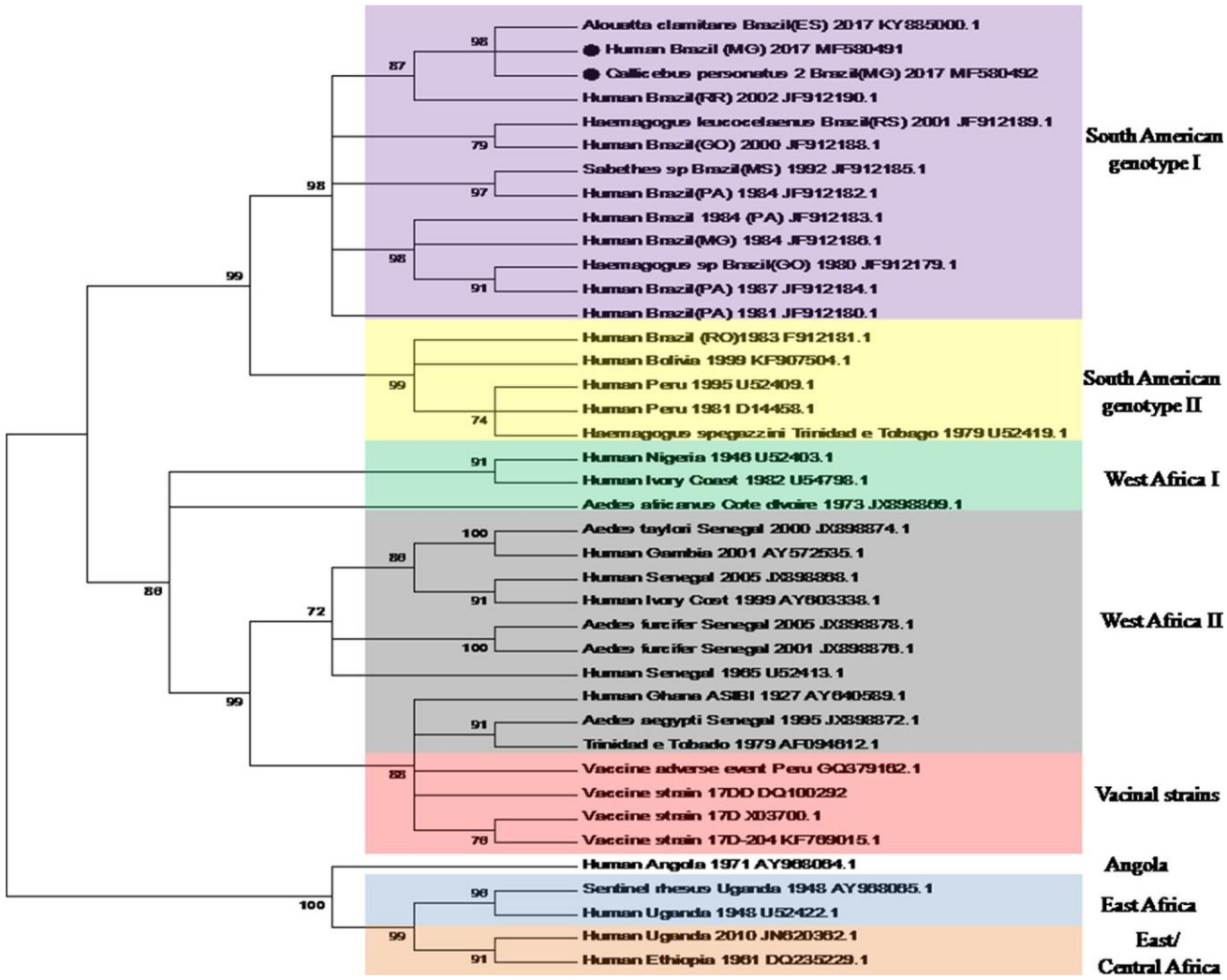

Figure 2. Phylogenetic tree constructed based on nucleotide sequences of 945 bp of *Yellow fiver virus* CprM region. The selection of the best-fit nucleotide substitution model was performed using jModelTest (Posada 2008). Phylogenetic tree reconstruction using maximum likelihood methods (ML) was performed using MEGA 7 (http://www.megasoftware.net) with 1000 bootstrap replicates by use of the Akaike information criterion (AIC) (Guindon et al. 2010). Based on the AIC, the GTR + G was the best-fit model. These parameters were used for phylogenetic tree reconstruction. Black circles indicate samples in this study, and numbers along branches are bootstrap values. For each sequence, the host, place of origin, and date were reported. For the Brazilian samples, the state was also reported in parentheses: Espı´rito Santo (ES); Goia´s (GO), Mato Grosso do Sul (MS); Minas Gerais (MG); Para (PA); Rio Grande do Sul (RS); Roraima (RR).

among populations living on the fringes of wild environ- ments.

Several factors may also have contributed to this great emergence of YF in MG state. For the

zoonotic spillover of this magnitude to occur, several factors related to reservoir host distribution, reservoir host density, prevalence of infection, and intensity of infection must be in line with other factors that relate to pathogen release from reservoir host, vector survival and movement, and human exposure to pathogen (Plowright et al. 2017). In the last instance, anthropogenic alterations may be contributing to the change in the dynamics of these factors and thus con- tributing to the magnitude of this outbreak. Future studies should be conducted to clarify some of these issues. In Brazil, the primate genera mostly associated with YF epi- zootics are *Alouatta, Cebus,* and *Callithrix,* although all NHP of New World monkeys are considered susceptible to the virus and therefore may act as hosts (Brasil 2005; De Almeida et al. 2012; Moreno et al. 2013; Saúde 2017). In NHP, the viremia is about 3–4 days, with death occurring within three to 7 days. The main clinical signs registered are fever, jaundice, apathy, dehydration, anorexia, oral and

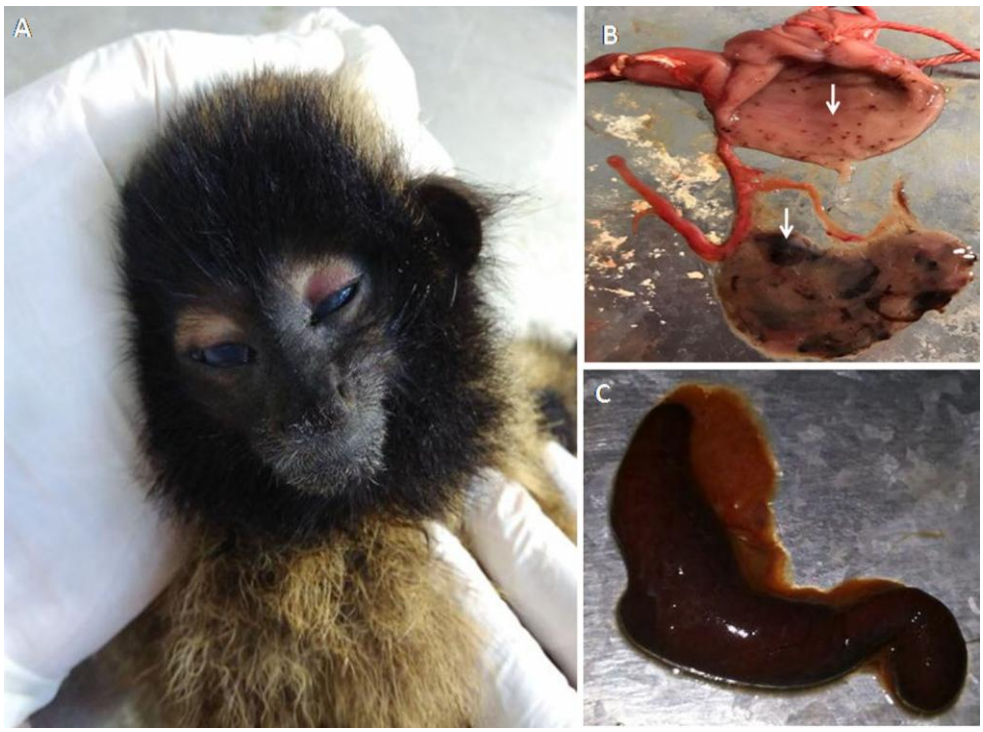

Figure 3. Photographs of the NHP necropsy. Edema and hyperemia of the eyelid (a); presence of petechiae in gastric mucosa and clots in stomach contents (b); hemorrhagic and friable spleen (possible *postmortem* alteration) (c).

intestinal hemorrhage, liver and kidney failure, fatty degeneration of the liver with extensive necrosis, and accumulation of lipids (Brasil 2005; Engelmann et al. 2014; Leal et al. 2016; Saúde 2017). A previous study described classic histopathologic commonalities with yellow fever associated disease in humans, such as midzonal lytic necrosis, apoptotic bodies, steatosis, and scarce paucicel- lular inflammation (Engelmann et al. 2014; Fernandes et al. 2017). Some of the NHP *postmortem* findings observed in this study corroborate clinical findings described in previ- ous studies. Our results on necropsy of NHPs identified the presence of petechiae in gastric mucosa, clots in stomach contents, and hemorrhagic and friable spleen (Fig. 3). Viral RNA detected in lung, liver, kidney, bladder, stomach, and intestine suggests that the pathological changes were probably caused by severe YFV multiplication process in these tissues. To date, there are a record of about 7000 animals that have been affected during this outbreak, showing the severity of YFV spread into the NHP popu- lation, which can be a threat to the species conservation (Brasil 2017d). There is an enormous lack of data regarding the clinical evolution of the disease in

NHP, and this is one of the few recent studies that includes, in addition to the molecular data, the clinical findings in these animals.

The data presented here highlight the importance in the synchronization of animal surveillance actions and also in health services to identify cases of emerging YF during the beginning of outbreak to promote a better response to the vulnerable population. On the other hand, the impor- tance of genetic monitoring to better understand and anticipate the viral emergence patterns is also necessary. Moreover, there is a gap regarding the knowledge of the YFV sylvatic cycle. Additional field studies that seek to evaluate NHP and other mammals that could act as hosts/ reservoirs in forest fragments from regions with recently reported outbreaks are urgent.

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viruses

Review

Re-Emergence of Yellow Fever in Brazil during 2016–2019: Challenges, Lessons Learned, and Perspectives

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Abstract: Yellow fever (YF) is a re-emerging viral zoonosis caused by the *Yellow Fever virus* (YFV), affecting humans and non-human primates (NHP). YF is endemic in South America and Africa, being considered a burden for public health worldwide despite the availability of an effective vaccine. Acute infectious disease can progress to severe hemorrhagic conditions and has high rates of morbidity and mortality in endemic countries. In 2016, Brazil started experiencing one of the most significant YF epidemics in its history, with lots of deaths being reported in regions that were previously considered free of the disease. Here, we reviewed the historical aspects of YF in Brazil, the epidemiology of the disease, the challenges that remain in Brazil's public health context, the main lessons learned from the recent outbreaks, and our perspective for facing future YF epidemics.

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Keywords: yellow fever; epidemic; epidemiology; ecology; public health; Brazil

1. Introduction

Yellow fever virus (YFV) is a positive-strand RNA virus that is the prototype of *Flavivirus* genus (*Flaviviridae* family) and is recognized as the etiological agent of Yellow Fever (YF) [1,2]. YF disease is characterized by an acute, febrile, hemorrhagic infectious disease, transmitted by mosquito vectors to human populations and non-human primates (NHP) in South America and Africa [2,3]. In Brazil, YF is considered a disease of compulsory notification, where all suspected cases must be immediately reported to the health authorities [2,4].

YF was responsible for hundreds of thousands of deaths between the 18th century and the beginning of the 20th century, and for recurrent epidemics in endemic regions of Africa and South America [2,5]. YF still remains a public health threat, leading to significant morbidity and mortality rates in the human populations of Africa and South America. A high case fatality rate (CFR) is observed, especially in South America, ranging from 40% to 60% [3,5,6]. The occurrence of rural (savannah cycles) and urban cycles is frequently reported in the old world, in addition to sylvatic cycles [7]. Large YF outbreaks occurred in Angola and the Democratic Republic of Congo during 2015–2016 [5,8], which placed YFV on the top list of arboviral threats by the Centers for Disease Control and Prevention (CDC) Global Disease Detection Operations Center [9]. According to the Pan American Health Organization (PAHO), the South American countries that reported the highest numbers of cases of YF during 1960–2019 were Brazil (3829 cases), Peru (3189 cases), Bolivia (1546 cases) and Colombia (701 cases) [10].

In Brazil, YFV is maintained in nature through enzootic/sylvatic cycles involving non-human primates (NHP) and mosquitoes of the genera *Haemagogus* and *Sabethes* [2,11]. YF has a seasonal pattern of occurrence, with most cases recorded from December to May. However, the occurrence of outbreaks is irregular, and viral transmission can change according to factors such as temperature, rainfall, high density of vectors, amplifying hosts, and low vaccination coverage of the human population [2]. Some factors have contributed to the elimination of the YF urban cycle, transmitted by urban vector *Aedes aegypti*, which include the introduction of vaccination since 1937, the mass immunization in the following decade, along with intense campaigns to eradicate the vector [2]. In this

scenario, the last registered urban YF case in the country occurred in 1942, followed by epidemic records related to sylvatic cycles, especially in the Amazon basin [2,11].

However, in 2016, one of the most significant epidemics of sylvatic YF occurred in Brazil, with most cases reported in regions considered free of the disease, or with little YFV circulation [5,11,12]. The YF cases exponentially increased during the 2016–2019 epidemic, highlighting concerns about the risks of YF reurbanization once the YFV outbreak reached the southeast region, the most populous region in the country [5,11]. The risk of reurbanization is sustained by vector plasticity in the Brazilian territory and by large susceptible populations that had no routine vaccination until the recent re-emergence [11,13–15]. In this review, we revisited the history of YF in Brazil and the substantial impact for public health since its introduction during the colonization period until the emergence observed in the 2016–2019 epidemic. Eco-epidemiological aspects of the disease, as well as the lessons and challenges from last
epidemic period, are also discussed.

2. A Brief History of Yellow Fever in Brazil

The YFV and its urban vector *Aedes aegypti* arrived in the Americas, including Brazil, through the slave-trading ships from West Africa during the period of colonization [5,8,16,17]. The first YF epidemic in Brazil was recorded in 1685, in the Northeast region, specifically in Recife and Pernambuco states [16,18] (Figure 1).

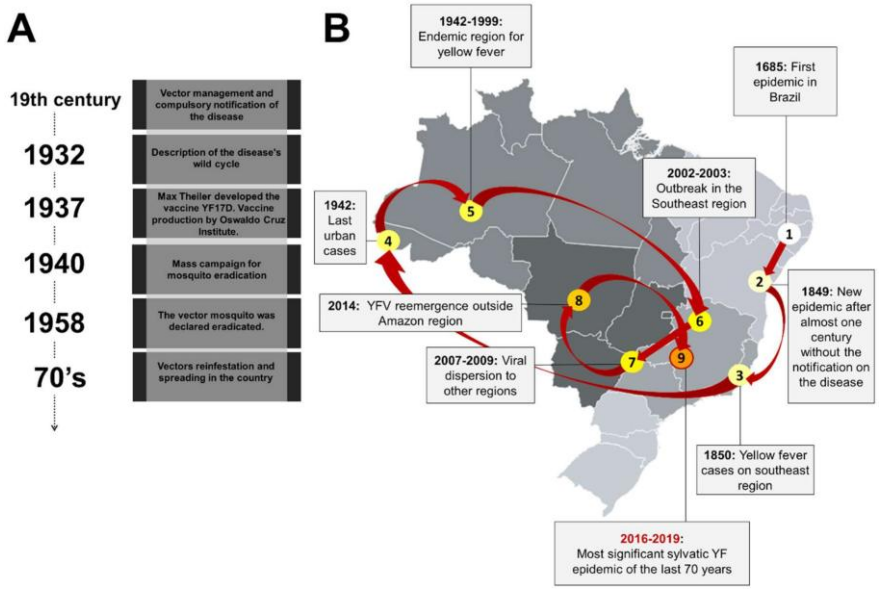

Figure 1. Map of the natural history of Yellow Fever in Brazil. (**A**) The timeline highlights sanitary measures adopted to fight against Yellow Fever in Brazil. (**B**) A map of Brazil showing the detection and distribution of *Yellow Fever virus* (YFV). The red lines connect the events of viral emergence in different regions of the country. The grey scale indicates the regions of Brazil from the lightest to the darkest as follows South, Northeast, Southeast, North, and Midwest.

In the subsequent years, YFV would hit other port cities in the Northeastern region, causing outbreaks. In the middle of 1850, after almost a century without notifications, YFV reached Rio de Janeiro (Southeastern region), causing more than 4000 deaths. Although the etiology of YF was still unknown and there was no proof of any transmission form, at the beginning of the 19th century several surveillance measures were adopted to fight against YF, including the mandatory notification of the disease and the hygienic and sanitary measures that indirectly contributed to the reduction in *Aedes* populations [16,19] (Figure 1). At the end of the 19th century, Carlos Finlay, a Cuban epidemiologist, proposed that YF was transmitted through mosquito bites [20–22]. However, it was not until 1900 that Walter Reed, a pathologist and bacteriologist, and his colleagues proved that YF was caused by a filterable agent and transmitted by the vector *Aedes aegypti* [23–26].

In the following years, because of a decrease in YF cases, the resources to fight against the disease were decreased, contributing to an urban YF epidemic in Rio de Janeiro in 1928–1929 [19]. In addition, in the 1930s, the sylvatic cycle was documented in the country [23–25] together with the discovery of the importance of NHP in the viral maintenance cycle [27].

Max Theiler, a South African doctor, developed a mouse model of YF infection to

demonstrate the potential of protection of serum antibodies against YFV. In the subsequent years, other research groups investigated the possibility of attenuating a wild type YFV, aiming to induce protective immunity in humans without causing any disease. As a result, the used wild type YFV was attenuated due to the development of an immunogenic and safe Yellow Fever-17D (YF-17D) vaccine strain [28]. In 1937, the vaccine against YF was produced by the Oswaldo Cruz Institute, today called Bio Manguinhos/Fiocruz [16–19,25,29]. In addition, by 1940 mass campaigns to eradicate the urban vector *Aedes aegypti* had begun; however, sporadic cases still occurred in several states, with the last urban case reported in Acre in 1942 [16,25]. In 1958, the PAHO officially declared that *A. aegypti* was eradicated in Brazil [16]; nevertheless, in the 1970s, the collapse of the continental program to combat vector mosquitoes led by PAHO culminated with the urban reinfestation of *A. aegypti*, and its spread to several Brazilian regions by the end of this decade [30–33].

The last two decades have witnessed the expansion of YFV circulation area in the country, where human cases and NHP epizootics were registered beyond the endemic Amazon region [34]. YFV spread to the East and South regions could be seen during 2002–2003, with cases registered in Minas Gerais and Rio Grande do Sul, and between 2007 and 2009, with confirmed cases in the North and Midwest regions, in addition to São Paulo, Paraná, and Rio Grande do Sul states [34–36]. The change in the spatial distribution of YF cases was even more evident during 2007–2009, with the confirmation of more than 100 YF cases in the South and Southeast regions, with a lethality rate of 51% [32,34,35] (Figure 1).

In 2014, YFV re-emerged in the Midwest region, in areas of Cerrado biome [11,34,37]. In the monitoring period of 2014–2015, based on the seasonality pattern, the occurrence of cases was mainly concentrated in Goiás and Mato Grosso do Sul states, and during 2015–2016 the cases were mostly concentrated in the Midwest region [12,37]. In a historical analysis, from 1980 to 2015, the period that precedes the most recent sylvatic epidemics, 789 YF human cases were registered in Brazil. In this 36-year interval, the outbreaks had an irregular pattern of annual incidence, with some periods of viral re-emergence [37]. At the end of 2016, the most significant sylvatic YF epidemic of the last 70 years began, affecting mainly the Southeastern region of Brazil [11,12,34,38].

3. Challenges and Lessons Learned

The extensive re-emergence of YF in Brazil started in late 2016, and, according to data from the Ministry of Health, 2237 human cases of YF and 759 deaths were recorded between December 2016 and June 2019 [12,39] (Figure 2).

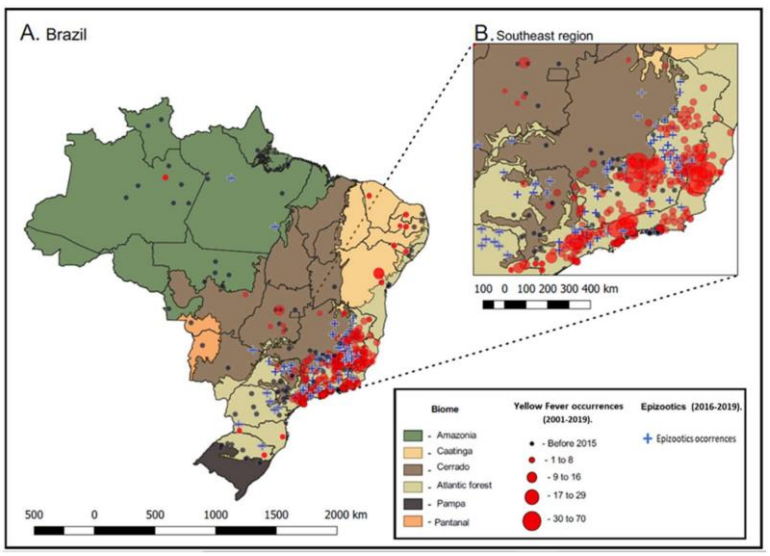

Figure 2. Spatial distribution of Yellow Fever (YF) cases in Brazil during 2001–2019. The map shows a correlation between human confirmed cases of YF and biomes (**A**), and confirmed epizootics in the Southeast region (**B**) during the 2016–2019 outbreak. The map was created using the Quantum GIS (QGIS) software.

The epicenter of epidemic was the Southeastern region of Brazil, specifically Minas Gerais and São Paulo states [11,12,40–42]. This outbreak was 2.8 times greater than what was recorded in the past 36 years [12,38]. In contrast to previous outbreaks concentrated in the Amazon and Central West regions, this outbreak was centered in the Southeastern region of Brazil, covering the Cerrado biome towards the region originally covered by the Atlantic Forest [11].

The recent re-emergence of YFV showed that the majority of the population affected by YF (82.8% during 2017–2018) were male. This population is in an economically active age range [12] and is

composed of residents of rural areas, probably due to work activities and proximity to forest sites, factors that contribute to the exposure of these individuals to YFV vectors [3,40,41]. Considering the data from Sistema de Informação de Agravos de Notificação (SINAN), an increase in the number of YF cases affecting male individuals has been observed since 2001 (Figure 3). The higher prevalence in males brings economic losses to their families and the region, as men are more likely to perform most of the activities in the field. Moreover, it is important to highlight that deforestation is a factor that can increase the risk of YFV spread to urban environments, raising opportunities for human exposure to fragmented forest areas with the occurrence of YFV sylvatic cycles [11,43].

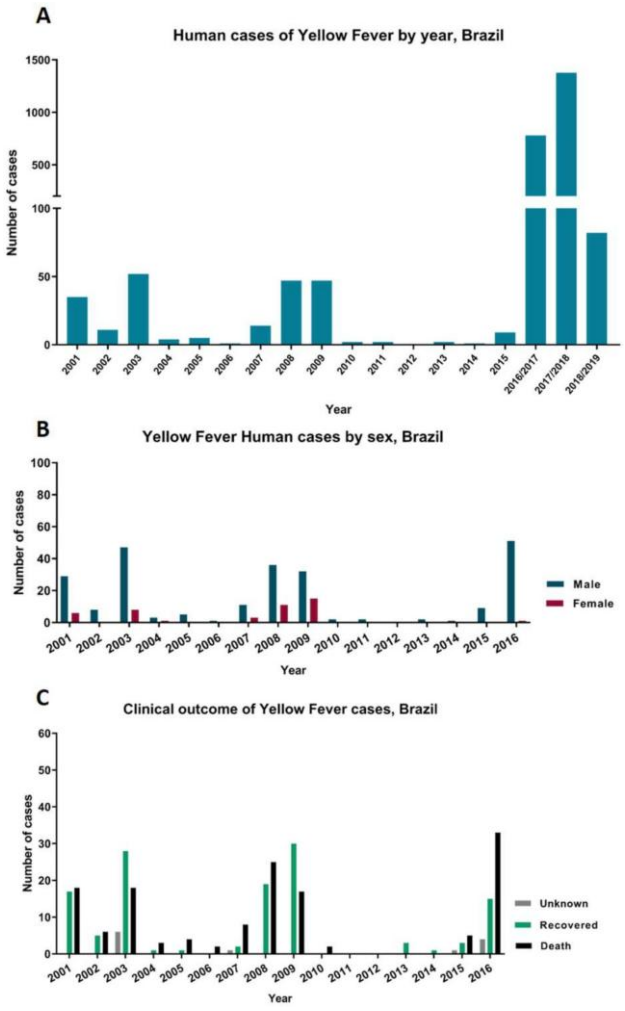

Figure 3. Distribution of human cases of YF in Brazil. (**A**) Distribution of confirmed human cases per year in Brazil for the monitoring period of (July to June) 2001–2019. (**B**) Distribution of YF human cases according to gender in

Brazil during 2001–2016. (**C**) Clinical outcome of human cases of YF in Brazil during 2001–2016. All data was obtained from Sistema de Informação de Agravos de Notificação (SINAN), and epidemiological reports of the Brazilian Ministry of Health.

The 2016–2019 YF epidemic also brought additional economic impacts for health authorities and for the public health in general. It is already known that the emerging infectious diseases (EID) cause significant impacts such as high costs associated with response plans, surveillance, and preventive actions [44]. Certainly, the re-emergence of YFV in Brazil caused a great burden for the public health services, since hundreds of Intensive Care Unit (ICU) beds were needed, in addition to expenses related

to the monitoring of patients in the different clinical stages of the disease, and the laboratory diagnosis of human and epizootics cases.

In this sense, one of the major challenges faced during the 2016–2019 epidemic was the establishment of standard protocols for clinical management of patients, which culminated in the implementation of the national catastrophe plan implemented by the government for the 2014 World Cup, at least by Minas Gerais state (the epicenter of outbreaks). In many hospitals, there was a change in patient management areas, an increase in the number of ICU beds and the hiring of healthcare professionals to serve the affected population. In addition, there was a need to create a transportation system for patients from rural areas to large urban centers, where reference hospitals and higher acuity care were located (Serviço de Infectologia do Hospital Eduardo de Menezes, reference of Minas Gerais state for Yellow Fever, personal communication). This decision was taken on an urgent basis, taking into account the rapid spread of YF and the worsening of the patients' clinical conditions. In fact, YF can present a broad clinical spectrum in humans, including asymptomatic infection, mild illness and severe disease; however, much is still unknown about the pathogenesis of this disease [3,5]. Considering this last outbreak in Brazil, a great advance in the disease's understanding has been reported and new clinical findings and outcomes have been described in the literature. Recent studies have reported the occurrence of late-relapsing and persistent hepatitis after YF [45,46] and other clinical findings were the occurrence of pancreatitis and progressive severe metabolic acidosis in severe cases of YF and manifestations in the central nervous system [47,48].

Certainly, the clinical management of severe forms constituted a major challenge since YF leads to liver failure with rapid evolution, compromises other vital organs and leads to a cytokine storm that culminates in plasma leakage and shock, which implies the need for intensive supportive therapies and treatments. Regarding the clinical management of patients with severe YF, Ho and colleagues showed that some measures such as the use of anticonvulsant drugs, routine use of intravenous proton pump inhibitors, aggressive early haemodialysis and plasma exchange were beneficial during YF treatment [47]. Additionally, studies related to understanding the predictors of mortality in patients with severe forms of YF affected during previous epidemic periods have been shown to be valuable. Hence, factors such as increasing age, male gender, higher neutrophil and leukocyte count, higher aspartate transaminase (AST) and alanine aminotransferase (ALT), bilirubin, and creatinine, prolonged prothrombin time, and higher viral load are significantly used as predictors of mortality of YF disease [49].

Furthermore, the recent YF epidemic also raised possibilities for increasing the

knowledge related to the clinical field and antiviral research, with recent studies showing that the repurposing of clinically approved drugs can represent a quick alternative to discover new antivirals for public health emergencies. Freitas et al. [50] demonstrated that YFV is susceptible, in vitro and in vivo, to sofosbuvir, a clinically approved drug against Hepatitis C virus (HCV). In addition, Mendes et al. [51] demonstrated a reduction in blood viremia and an improvement in clinical course with sofosbuvir treatment.

The management of vaccination in the affected population can also be considered a complex challenge in the YF epidemic scenario. In order to protect the largest possible portion of population against the disease, the Sistema Único de Saúde (SUS) distributed 45.1 million doses of the YFV vaccine in 2017 and 23.8 million doses in 2018 [52]. Currently, the North, Midwest, Southeast, and South regions of Brazil are considered areas with vaccination recommendation (ACRV), however, in the Northeast region, the vaccination has been recommended only for Bahia and Maranhão states, and in some municipalities of Piauí, Sergipe, and Alagoas states [39]. Vaccination campaigns against YF also aim to prevent expansion of viral circulation, which is also associated with the movement of people [40]. The 17DD vaccine produced by the Institute of Technology in Immunobiologicals (Bio-Manguinhos) of the Oswaldo Cruz Foundation (Fiocruz) leads to 98% protection [2,11].

The target public for which the vaccine is indicated are individuals from 9 months to 59 years of age who reside or travel to areas with vaccination recommendation [2,34]. In the current campaign, the vaccine is being used in a standard dose (0.5 mL), but during the YF epidemic period, the dose

fractionation strategy was adopted for some states, with the fractional dose (0.1 mL) corresponding to a fifth (1/5) of a standard dose [11,34,52,53]. This type of strategy is approved during emergency situations and has already been recommended by the World Health Organization (WHO) and used in outbreaks such as during the one that occurred in 2015–2016 in the African continent [52–56]. As the viral dissemination was favored by the vaccine shortage, with insufficient doses for the entire population, the vaccine fractionation strategy was an effective tool. In addition, strategies to control the *Ae aegypti* vector, which contribute to preventing viral spread and the resurgence of YFV, have little emphasis on the continent [57]. However, after emergency situations it is necessary to re-vaccinate the population with the full dose; the long-term protection provided by fractional doses in varied populations and epidemiological contexts is unknown [58]. In this sense, the study by Costa-Rocha and colleagues which evaluated the duration of humoral and cellular immunity after administration of reduced doses of the 17DD-Yellow Fever vaccine provided evidence to support the regular use of dose sparing strategies for YF vaccine in adults [59].

Currently, the WHO recommends one life-time dose of the YF vaccine; however, this is controversial for two reasons: the level of neutralizing antibodies drops years after vaccination and cases of YF infection have occurred in previously vaccinated individuals [40,60–63]. Even though YF vaccine is highly immunogenic and able to induce a robust antibody response and a strong and polyfunctional cellular immune response, recent studies demonstrated the importance of booster doses to ensure a long-term persistence of memory components in response to 17DD YF vaccine [60–62,64]. These recent findings suggest that in YF endemic areas, at least an additional dose of the vaccine should be administered after the first immunization, in order to avoid the reduction in neutralizing antibodies titers below the protective levels [60–62]. During the recent epidemic in Brazil, the priority was to vaccinate the largest portion of the population possible; however, it is recommended that after this emergency period the population receives a full dose of 17DD vaccine, reinforcing the idea that at least two doses are necessary [58,60,62,65].

With the re-emergence of YFV significantly affecting the Southeastern region of Brazil, and the consequent concern about the beginning of an urban cycle, there was an intensification of vaccination campaigns mainly in large urban centers [34,52]. The dislocation of cases, previously restricted to rural areas, to metropolitan regions, as recorded mostly in 2018, was of great concern and alert to epidemiological surveillance systems. However, the low vaccination coverage against YF in Brazil is a problem that

has persisted for decades [66]. In view of this, the current challenge is to achieve vaccination coverage of at least 95% in all Brazilian territory.

A recent study conducted by Stoffella-Dutra and colleagues revealed that, respectively, 25.8% and 26.5% of the rural and urban populations living in Serro region (state of Minas Gerais) did not present neutralizing antibodies against YFV [67]. Furthermore, 10 individuals from the same area that presented their vaccination card with proven vaccination against YFV tested negative for the presence of neutralizing antibodies. Considering the recent epidemic and the risks of YF re-urbanization, this finding raises questions about the real burden of YFV infections, in which the disease could be underestimated. Although the vaccination coverage in that region has improved [68], there are still a high number of individuals lacking any neutralizing antibodies response against YFV, which can increase the vulnerability of the populations, as well as the to the occurrence of new outbreaks or even epidemics [67].

Other studies performed in Brazil also highlighted the absence of neutralizing antibodies against YFV in individuals from rural and urban areas [69,70]. However, different from the results reported by Stoffella-Dutra and colleagues, few individuals tested negative. These findings draw attention to the fact that some areas in the state of Minas Gerais can present a high proportion of individuals with absence of neutralizing antibodies against YFV, which could potentially be a factor related to the recent 2016–2019 YF epidemic, reinforcing this state as the epicenter of the outbreaks [40].

The presence of individuals lacking a neutralizing antibodies response against YFV in endemic areas for YF disease also reinforces the importance of active epidemiological surveillance and continued

vaccination campaigns aiming to reach at least ≥95% coverage. This coverage is necessary because according to the WHO, a vaccine coverage of at least 80% would be important to prevent and control new outbreaks [71]. Indeed, learning about the current seroprevalence in regions under risk of YF disease can add valuable information that could help to assist national and international health authorities in the development of future vaccination strategies.

The 2016–2019 YF epidemic in Brazil was the most impactful in the past decades and several factors, including the failure of entomological and epizootic surveillance systems, low vaccination coverage in several regions of the country, and population migration, have potentially contributed to the recent epidemic scenario. In this context, issues related to vector control and NHP surveillance are also challenges to be covered.

Effective surveillance of the vector population is essential for implementing control strategies, as well as contributing to highlighting potential sources of transmission and potential new outbreaks, not only for YF, but also of other recurrent arboviruses in Brazil such as Zika, Dengue and Chikungunya [72]. Considering the plasticity of vectors involved in the YF transmission cycle and the existence of an urban vector widely domesticated in the country, it would be important that the PAHO Entomology and Vector Control Action Plan [73] become implemented, maintaining an active surveillance not only in Brazil but for all affected countries in America.

Similar to vector control strategies, NHP epizootics surveillance still need to be better established and optimized in all regions of Brazil for a greater control effectiveness, especially in those areas with higher risks of enzootic cycles. Hence, epizootics surveillance needs to be carried out in a sufficient time frame, capable of providing effective control for viral dispersion. Furthermore, information about illness and death in the NHP, obtained by the Epizootics Surveillance Program of Brazil and local health authorities, also fails to report and disseminate data.

The effective diagnosis of YF human cases is also another critical point. The delay in reporting the laboratory test results slowed down the processes of clinical intervention and surveillance. Apart from this, another issue that compromises better understanding of epidemics is the classification of cases into “cases under investigation”, which makes it difficult to understand the true dimension of the epidemic event due to the delay observed for resolution in cases discarded or confirmed as YFV infection or YF disease. This delay directly influences the possible actions to control and fight against the disease.

Considering the old classification of yellow fever’s endemic area in the country, the Brazilian coast and the Southeastern region were considered YF free areas, however, with

the re-emergence of YFV in 2016, this scenario can no longer be considered (Figure 2). There are factors that may be related to viral dislocation for the East and South regions of the country, among them the ecological changes (fragmentation of habitats, climate changes) and the patterns of human behavior, which may have contributed to the increased densities of vectors and NHP, and their consequent proximity to humans [5,11]. Political and social factors are also relevant in the complex recent re-emergence scenario of YF in Brazil, such as failure of political commitment and strategies to achieve satisfactory vaccine coverage and monitor population immunity in areas at risk for YF transmission. Besides poor basic sanitation in several regions of the country, which can contribute to the proliferation of vectors, anthropic environmental changes, such as the advancement of agriculture and peri-urban growth and insufficient health and surveillance policies, can contribute to the poor detection and control of outbreak situations [11,71]. Furthermore, Faria and colleagues estimated that virus lineages moved, on average, 4.25 km/day during the last outbreak. This velocity on vectors movement also reflected YFV lineage movement within the enzootic cycle and not the movement of asymptomatic infected humans. These findings also corroborate the fact that NHPs are not likely to carry the virus over long distances [11,40], different from infected humans and vector species that can help with viral dislocation, reaching greater distances [11,35,74].

Recent phylogenetic studies have shown that YFV strains circulating during the 2016–2019 epidemic presented a high identity with the South American genotype I, previously described circulating in the Amazon region [40,41,74–76]. Further analysis revealed that the strain 1E were

responsible for the recent epidemic, which was not associated with previous outbreaks already described in the Southeast region in 2000 and in 2008 [40,74–80]. Genomic analyses of YFV samples from this latest epidemic showed that the YFV lineage responsible for the 2016–2019 outbreak originated from Midwest region, spreading to Minas Gerais state at least two times, and reaching two distinct routes in the Southeastern region of Brazil [80]. The most affected states until 2018 were Minas Gerais, São Paulo, Rio de Janeiro and Espírito Santo. During 2019, São Paulo continued to report cases, with others being also reported in the states of Paraná and Santa Catarina [12,39].

4. Progress and Perspectives

The surveillance and control of vector populations are actions that must be constant in the YF context, especially in outbreak situations. The prevalence and distribution of vectors species are important key indicators of the risk of the occurrence of an urban cycle in certain regions and the necessity of direct control measures towards areas that need to be prioritized [71,81]. In this sense, it is important to think about a combination of mosquito control strategies that should include policies to improve basic sanitation in large cities and other strategies for direct control of the mosquito population, in addition to strategies such as the introduction of genetically modified or biologically manipulated mosquitoes. An example of this is the global initiative World Mosquito Program (WMP), which uses the symbiotic bacterium *Wolbachia* as a biocontrol tool to reduce the transmission of mosquito-borne diseases [82]. In Brazil, the *Wolbachia* method is coordinated by the Oswaldo Cruz Foundation (Fiocruz), under the guidance of the Ministry of Health, and the first releases of *Aedes aegypti* mosquitoes carrying *Wolbachia* began in 2014 in Rio de Janeiro, and the project is expected to reach 2.38 million people by 2023 [82]. Scientific evidence has demonstrated the ability of *Wolbachia* to reduce the transmission of Dengue, Zika, Chikungunya, and Yellow Fever viruses by the *Aedes aegypti* mosquitoes [83–86]. For YF control, this approach helps to prevent the onset of urban cycles of the disease [86]. In addition to all the challenges here exposed, Figure 4 represents an overview in terms of progress and remaining challenges related to the latest YF outbreak in Brazil.

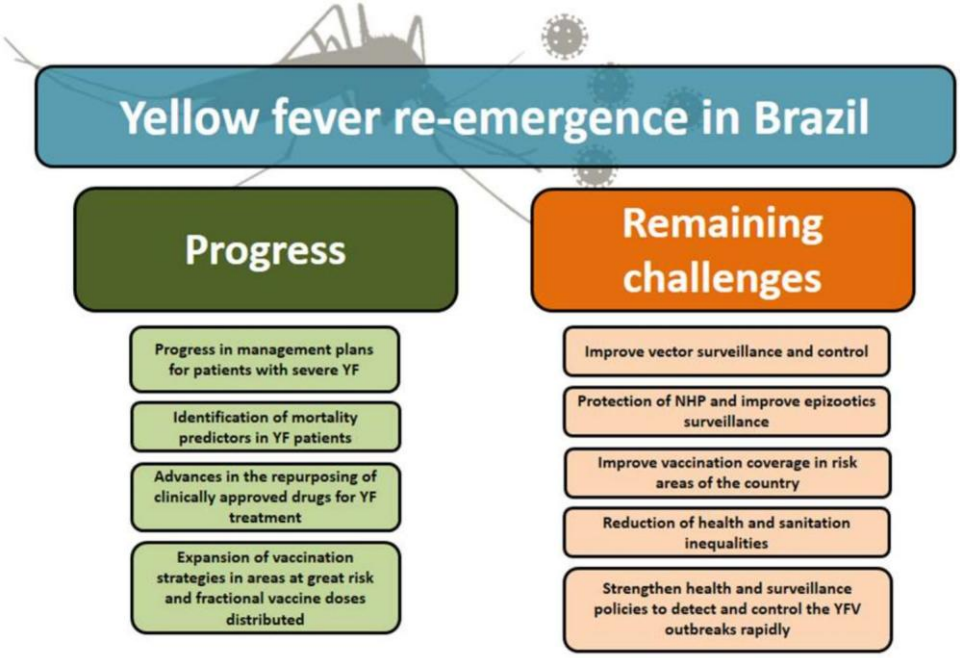

Figure 4. An overview of the YF re-emergence in Brazil. The flowchart highlights some progress that can be recognized during the 2016–2019 epidemic, and the challenges that still need to be covered to contribute to the surveillance and control of YF in Brazil.

Finally, and equally important is to know that the identification of epizootics provides an early warning of viral circulation and helps prevent YF human cases. Thus, the adoption of strategies for the protection of NHPs is fundamental not only for the conservation of species, but also due to the importance of these animals as sentinel hosts of the disease. In the context of sylvatic YF re-emergence experienced in Brazil, all confirmed human cases had as a probable site of infection (LPI) areas with previous occurrence of epizootics in NHP (Figure 2) [12,40,87].

Furthermore, it is important to note that the vector species distribution is related to YFV epidemiology, and, in this context, even more expressive epidemics can be caused when infected individuals are inserted in densely populated areas with the urban vector *A. aegypti* [9,14]. This species is the main vector for many flaviviruses, and it is widely distributed in the urban centers of the Southeastern region, and, due to the active circulation of YFV in this region, the threat of an urban cycle has proved to be concrete [13,15,74].

Vector competence analyses showed that the anthropophilic mosquitoes *A. aegypti* and

A. albopictus are highly susceptible to be infected and transmit YFV, in addition to the wild vectors

Haemagogus leucocelaenus and *Sabethes albiprivus* [13]. It is important to highlight the presence of

A. albopictus in Brazil, as it has spread throughout the country since 1980. Experimental studies suggested that YFV has the potential to adapt to *A. albopictus*, which is an opportunistic species, since it can move between urban and peri-urban habitats [13,88]. In addition, little is known about other vectors (especially the *Culicidae*) and their potential role in YFV transmission. Most of the work related to vector competence to sylvatic virus was performed a long time ago, and their results may not reflect the real-world data since the strain of virus and mosquitoes have been evolving and changing over the years. Taking into account the proximity of YFV circulation to the Atlantic coast and to large urban centers, it is worth noting that even poorly competent vectors can pose a threat in the transmission of pathogens if some factors such as high vector density, high human-biting rate and high survival rates are present. Thus, factors that may have favored the re-emergence of YFV in Brazil such as the presence of susceptible human populations, favorable climatic conditions, and the circulation of infected humans and NHPs are undeniable [11,13,35].

5. Conclusions

The emergence of zoonotic viruses is an important challenge for global public health, now more than ever in our connected world due of international travel and trade in which highly contagious diseases can quickly spread. Understanding the ecological gaps related to the EID and the impact of social changes on the control and prevention of possible epidemics is a challenge that requires international cooperation. The complex YF eco-epidemiology in Brazil is a case study to demonstrate the challenge for public health agencies and policy makers to effectively control and monitor disease. The fact that YF is a zoonosis and cannot be eradicated is an aggravating factor, especially considering the increased proximity of human populations to viral circulation areas. However, for a disease that presents high mortality in severe cases, the large population of unvaccinated and susceptible individuals may favor viral spread and re-emergence events in densely populated areas. Moreover, with viral circulation now present in the Atlantic Forest, close to large metropolitan areas in the Southeastern region, the risk of YF re-urbanization is highly concerning for human health and potential international viral spread. The occurrence of enzootic cycles makes arboviruses, especially YFV, a recurrent issue. From this point of view, it is important to emphasize the need to use efficient tools to prevent introductions of YFV into the urban cycle, such as the combination of efficient vector control strategies and large-scale vaccination campaigns. Hence, there is an urgent need to strengthen the Brazilian health systems in order to improve decision-making for control, response and prevention of future YF and other EID outbreaks.

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Absence of YF-neutralizing antibodies in vulnerable populations of Brazil: A warning for epidemiological surveillance and the potential risks for future outbreaks

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Yellow Fever (YF) is an acute febrile illness caused by yellow fever virus (YFV), a mosquito-borne fla- vivirus transmitted to humans and non-human primates. In Brazil, YF is a public health threat and may cause recurrent epidemics, even with the availability of a vaccine. We evaluated the sero-status for YFV in 581 individuals living in a risk area for YF in Brazil. The area presents history of cases and is located in the southeast region of country where outbreaks of YF have been reported since 2016. Through, a PRNT assay, we found 25.8% of individuals lacking YF-neutralizing antibodies. Furthermore, neutralizing antibodies were not detected in 10 individuals with proven vaccination. Our findings rein- force the importance of surveillance systems and the need of an urgent intensification of immunization programs in regions with YFV circulation. Monitoring susceptible individuals that could act as potential disseminators for YFV in risk areas should also be considered.

1. Introduction

Yellow fever (YF) is a hemorrhagic fever caused by the yellow fever virus (YFV), an arbovirus (arthropod-borne virus) that belongs to the family Flaviviridae, genus Flavivirus [1,2]. YFV is endemic in tropical regions of African and South American coun- tries, where the virus is maintained in wild transmission cycles involving arboreal vector mosquitoes and non-human primates (NHP) [1,3,4]. In South America, the enzootic cycle involves the vectors of genera Haemagogus and Sabethes, that accidentally may transmit the virus to humans [1,3,5].

Besides the sylvatic cycle, an urban transmission cycle also occurs, characterized by YFV transmission to humans via the Aedes

aegypti vector [1,6]. In the urban cycle, humans are the primary host and factors such as immunological susceptibility, high popu- lation density in urban centers, high vector density, and climate conditions may favor the transmission of YFV [6,7,8]. The occur- rence of urban cycles highlight the potential for viral spread, and the risk of re-urbanization of the disease [7]. Therefore, the vacci- nation against YFV is the most effective preventive measure, so the development and distribution of the 17D vaccine in the 1930s has been correlated with a reduction of the disease in endemic areas [4,6,9]. In Brazil, the 17DD vaccine sub- lineage is used since 1937, which may have contributed to the elimination of the YF urban cycle in the country (no urban YF cases have been reported since 1942) [4,10,11]. To reduce the risk of outbreaks and the re- urbanization of YF, as well as guarantee the protection of the pop- ulation, vaccination coverage must reach the ≥95% level recom- mended by the Brazilian Ministry of Health [12].

In 2016, one of the most significant reported epidemics of syl- vatic YF began in Brazil and according to the Ministry of Health, 2,115 human cases were confirmed from July 2016 to June 2018 [13]. The outbreaks were mainly concentrated in the states of Minas Gerais, São Paulo, Rio de Janeiro and Espírito Santo, regions of southeastern Brazil that historically had low circulation of YFV

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in recent years [8,13]. During YFV re-emergence, an expansion in the viral circulation area has been observed. YFV has moved from the Brazilian Amazon, spreading throughout the Cerrado biome, and reaching areas of the Atlantic Forest, which has a high diversity of NHP species and sylvatic vectors [7,11]. Additionally, the south- east region is located within the Atlantic Forest biome, concentrating the most populous urban centers in the country, and several municipalities from this region had low medium vaccination cov- erage by the time the 2016 outbreak started [7,13,14,15].

Epidemics of YF are a burden for public health and economy, and moreover YFV presents a high potential for dispersion. Therefore, this disease should be considered a potential threat and be actively monitored [8,16,17]. In Brazil, there are few seroprevalence data for YF, and this lack of knowledge represents a gap in understanding the burden of at-risk populations in the country. Seroepidemiological studies contribute to enhance public health actions and strategies that aim to control viral dispersion and to reduce the occurrence of new epidemics in risk areas. Taking into account the re-emergence of YF in the Southeast Brazil and the need of vaccination in risk areas, this study aimed to evaluate the sero-status for YFV in a population located in an area with pro- ven viral circulation.

2. Material and methods

2.1. Study area

This study was carried out in Serro city (18° 36 ’17" S 43° 22 ’46" W), north-central region of Minas Gerais State, Brazil. Serro city has a population of 20,835 inhabitants, of which 12,895 are urban residents [18]. The local economy is mainly based on agricultural and dairy activities. The area has vegetation covered by Cerrado and Atlantic Forest, with extensive fragmented or mosaic areas due to anthropic action [19–21] (Supplementary Fig. 1).

2.2. Population and study measures

This cross-sectional study was conducted on a random sample of individuals from the urban area of Serro during 2015, 2017, and 2018. Individuals from all neighborhoods were enrolled. Participation in the study was voluntary, no exclusion criteria were used, and individuals of all age groups were enrolled. We addition- ally included the rural population of Serro sampled during 2012– 2013 [22].

For both populations, urban and rural, a semi-structured and pre-coded questionnaire was applied to elicit demographic data. Demographic data included age, gender, self-reported race/ethnic- ity, occupation, income, and

educational level. For samples col- lected during the YF outbreaks (2017 and 2018), YF exposure/risk factors for the presence of YF-neutralizing antibodies questions were also collected. These factors focused on behavioral habits such as access to forest areas and rural properties, the presence of NHP near to the household, history of vaccination against YF, and past infection with YFV, dengue virus, and Zika virus.

The sample size for the urban population was calculated by using Open-Epi version 3.01 (https://www.openepi.com), using an expected prevalence of 50%, alpha error of 5%, accuracy of 10% around the estimate and design effect of 1.0.

2.3. *Plaque reduction neutralization test*

Serum samples were collected from each participant and stored at —20 °C. To assess the presence of YF-neutralizing antibodies we used a plaque reduction seroneutralization test (PRNT), considered the gold standard for the differential diagnosis of flavivirus infections [23–26]. Initially, the sera were heated at 56 °C for 30 min to denature the complement system proteins. Samples were then diluted in Eagle's Minimum Essential Medium (MEM) (GIBCO®, USA) free of fetal bovine sera (FBS) in a proportion of 1:20. Diluted samples were added to a solution (1:1) containing approximately 150 plaque-forming units (PFU) of YFV 17DD (lot 980FPO662). The final solution was homogenized and incubated for 1 h at 37 °C, in a 5% CO_2 atmosphere. Six-well plates containing VERO cells (ATCC no. CCL-81) monolayers (80% confluence) were inoculated with virus/serum solutions, and incubated at 37 °C for 1 h in 5% of CO_2 atmosphere. Two ml of semi-solid medium (Carboxymethyl cellu- lose 1% (SYNTH®, Brazil) and 199 2X Medium (CULTILAB, Brazil) with 2% FBS was added to each well, cell monolayers were incubated for 5 days at 37 °C in 5% of CO_2 atmosphere. Vero monolayers were fixed with formalin (10%) (SYNTH®, Brazil) and stained with crystal violet solution (1%) (SYNTH®, Brazil). Each six-well plate included one well reserved for cell control, and all samples were tested in duplicate. A sample was considered positive when the average num- ber of PFUs was lower than half of the PFUs counted in the virus control (at least a 50% reduction in PFUs). The virus control, also known as the negative serum control, was made by using only FBS instead of sera; and submitted to the same protocol.

2.4. Statistical analysis

A descriptive analysis of the results was carried out and com- parisons between those participants with and without neutralizing antibodies against YFV using a 2-tailed Fisher's exact test with sig- nificance level of 5% ($p \leq 0.05$) by using EPI-INFO software version 7.2 (http://www.cdc.gov/epiinfo/). Odds relative and confidence intervals of 95% were also calculated. All those variables that showed a significance level of 5% in the univariate analysis were tested again in a multiple logistic regression model.

2.5. Ethical considerations

This study was approved by the Research Ethics Committee of Universidade Federal de Minas Gerais under the registration proto- cols FR–413704 and 1.974.249, CAAE–65332216.9. 0000.5149.

Informed consent was obtained from all participants. In the case of minors, consent was signed by parents or guardians.

3. Results

3.1. Demographic profile of the study population

A total of 581 individuals were enrolled in this study. Women represented 51.5% of total participants whereas men were 48.5%. The average age of respondents were 38.4 years (ranging from 5 to 94 years), and the majority of them (62.0%) self-reported mixed skin color.

In the urban area, 363 individuals were included, being 54.0% women, the average age of participants was 40.9 years (ranging from 9 to 94 years). Most individuals 63.0% self-reported mixed skin color, 52.0% had completed elementary school, and 38.8% reported received between one and two minimum wages. The most common occupations were general service providers (25.0%) and rural workers (8.8%) (Table 1).

In the rural area, 218 participants were included, being most of them men (52.8%). The median age was 38 years (ranging from 5 to 90 years). Of these, 60.0% self-reported mixed skin color. The majority of participants (74.3%) reported a monthly family income of minimum wage or less, and completed only elementary school (63.7%). The most common reported occupations were rural work- ers (52.3%) and housewives (25.6%) (Table 2).

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Table 1

Demographic characteristics of the 363 surveyed individuals from urban area of Serro city, 2015–2018.

Demographics	N (%)*	$PRNT_{50}$ pos. (%)	$PRNT_{50}$ neg. (%)	P value	OR (95% CI)
Gender					
Female	196 (54,0)	141 (52,8)	55 (57,3)	0.5	1.2 (0.7–1.9)
Male	167 (46,0)	126 (47,2)	41 (42,7)		
Age (years)					
5–11	13 (3,6)	4 (31,0)	9 (69,0)	Reference	
12–18	43 (11,8)	32 (74,4)	11 (25,6)	0.007	6.5 (1.7–25.5)
≥19	307 (84,6)	231 (75,2)	76 (24,8)	0.001	6.8 (2.1–22.8)
Self-reported skin color					
Mixed	232 (64,1)	169 (63,5)	63 (65,6)	Reference	
Black	63 (17,4)	48 (18,0)	15 (15,6)	0.6	0.8 (0.4–1.6)
White	67 (18,5)	49 (18,4)	18 (18,8)	1.0	1.0 (0.5–1.9)
Education					
Have never gone to school	17 (4,7)	12 (4,5)	5 (5,2)	Reference	
Elementary school or less	188 (52,1)	137 (51,5)	51 (53,7)	0.8	1.1 (0.3–3.2)
High school	127 (35,2)	95 (35,7)	32 (33,7)	0.7	1.2 (0.3–3.7)
Higher (college)	29 (8,0)	22 (8,3)	7 (7,4)	0.7	1.3 (0.3–5.1)
Income					

≤ 1 min wage	90 (30,8)	63 (70,0)	27 (30,0)	0.4	0.7 (0.4–1.3)
> 1 min wage	202 (69,2)	152 (75,3)	50 (24,7)		
Occupation					
Rural worker	30 (8,5)	26 (86,7)	4 (13,3)	Reference	
Housewives	67 (18,9)	47 (70,1)	20 (29,9)	0.08	2.7 (0.9–10.2)
Students	56 (15,8)	36 (64,3)	20 (35,7)	0.02	0.3 (0.07–0.9)
Healthcare	20 (5,6)	17 (85,0)	3 (15,0)	<0.001	0.03 (0.005–0.14)
Others[y]	181 (51,1)	113 (73,5)	48 (26,5)	0.06	2.7 (0.9–9.6)

Income value in Brazilian currency in 2018 = R$ 954.00 (US$ 1.00 = R$ 4.09 approximately).

[**]Calculated by using T-test

[*] Totals may not add up to 100% due to missing data

[y] Others: Lawyer, Announcer, Banking, Notary, Engineer, Businesswoman, Civil Servant, Social Worker, Musician, Teacher and Artist.

Table 2

Characteristics of the 218 surveyed individuals from rural areas of Serro city, 2012–2013.

Variables	N (%)*	$PRNT_{50}$ pos. (%)	$PRNT_{50}$ neg. (%)	P value	OR (95% CI)
Gender					
Female	103 (47,2)	74 (45,1)	29 (53,7)	0.3	1.4 (0.7–2.6)
Male	115 (52,8)	90 (54,9)	25 (46,3)		
Age (years)					

5–11	17 (7,8)	1 (5,9)	16 (94,1)	Reference	
12–18	17 (7,8)	17 (100,0)	0	1	1 (0.06–17.4)
≥19	184 (84,4)	146 (79,3)	38 (20,7)	0.1	0.2 (0.03–1.9)
Self-reported skin color					
Mixed	131 (60,1)	101 (61,6)	30 (55,5)	Reference	
Black	52 (23,8)	43 (26,2)	9 (16,7)	0.4	0.7 (0.3–1.6)
White	35 (16,1)	20 (12,2)	15 (27,8)	0.02	0.4 (0.2–0.9)
Education					
Have never gone to school	22 (10,1)	20 (12,2)	2 (3,7)	Reference	
Elementary school or less	139 (63,8)	103 (62,8)	36 (66,7)	0.08	3.4 (0.9–23.0)
High school or more	57 (26,1)	41 (25,0)	16 (29,6)	0.07	3.8 (0.9–26.9)
Income					
≤ 1 min wage	162 (74,3)	129 (79,6)	33 (20,4)	1.0	0.8 (0.2–2.9)
> 1 min wage	17 (7,8)	14 (82,3)	3 (17,7)		
Occupation					
Rural worker	114 (52,3)	19 (16,7)	95 (83,3)	Reference	
Housewives	56 (25,7)	14 (25,0)	42 (75,0)	0.2	0.6 (0.2–1.3)
Students	32 (14,7)	16 (50,0)	16 (50,0)	0.0002	0.2 (0.08–0.4)
Others[y]	16 (7,3)	5 (31,2)	11 (68,8)	0.2	0.4 (0.1–1.5)
Contact with					

wildlife	107	34 (63,0)	73 (44,5)	0.02	2.1 (1.1–
Yes	(49,1)				4.0)
No	111	20 (37,0)	91 (55,5)		
	(50,9)				

Income value in Brazilian currency in 2013 = R$ 678.00 (US$ 1.00 = R$ 4.09 approximately).

** Calculated by using T-test

* Totals may not add up to 100% due to missing data

y Others: Bricklayer, driver, civil servant and zootechnist.

3.2. *Prevalence of YF-neutralizing antibodies*

YF-neutralizing antibodies were detected in 431 individuals, representing an overall prevalence rate of 74.2% (CI95%=67.4–81.4). In the rural population, 164 individuals had YF- neutralizing antibodies, prevalence rate of 75.2% (CI95%=64.4– 87.4), while the overall prevalence rate in the urban population was 73.5% (CI95%=65.0–82.9). The prevalence varied by year

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among the urban population, 76.9% in 2015, 68.5% in 2017, and
83.16% in 2018 (Supplementary figure 2).

3.3. *Individuals susceptible to YFV infection according to the absence of neutralizing antibodies*

Fig. 1 displays the distribution of individuals presenting or not YF-neutralizing antibodies. Considering both rural and urban pop- ulations, a total of 25.8% individuals had no detectable neutralizing antibodies against YFV (n = 150/581). A total of 54 individuals from rural area (24.8%) were seronegative for YF-specific neutralizing antibodies. The average age of seronegative individuals was
27.9 years, and 66% of these individuals (n = 36) fell into the risk group for YFV infection (19–59 years old). In the urban population, 26.5% of the individuals did not show neutralizing antibodies (n = 96). The average age of the seronegative individuals was 39 years, and 58% (n = 56) of these individuals belonged to the risk group for YFV infection (19–59 years old).

For the urban population inserted in the YF epidemic period (2017–2018), no individual reported a proven history of YF or Zika, although 0,96% (n = 3) reported prior infection with DENV. At the time of collection, none of the participants from the two popula- tions declared clinical alterations.

Considering the two populations, 30 children (5–11 years old) were included in the study, in which 13 from the urban area and 17 were from the rural area. Anti-YF neutralizing antibodies were detected in four children from the urban area, while only one from the rural area had positive serology.

A total of 297 of participants (95.6%) sampled during 2017– 2018 reported a history of vaccination against YF. However, only 52 (16.8%) participants validated this information through a

vaccination card, and 59.6% (n = 31) has received one dose of YFV-17DD vaccine, 38.5% (n = 20) two doses and 1.9% (n = 1) three doses. Of those with validated vaccination cards, 10 individuals (19.2%) did not have detectable YF-neutralizing antibodies. This group consisted of four men and six women, of which the age range varied from 9 years to 62 years. It should be noted, a 18 years old male that tested negative for the presence of neutralizing anti- bodies and without history of comorbidities had three vaccine doses registered on his vaccination card. Additional information is presented in Table 3. A study population flowchart by collection area and by year is provided in the Supplementary Material.

3.4. *Potential risk factors significantly linked to YFV seroprevalence*

Potential risk factors related to the YFV exposure are shown in Table 4. For the urban population, only occupation was associated with the presence of YF-neutralizing antibodies (Table 1). Students and healthcare workers were less likely to present antibodies com- pared to rural workers ($p = 0.02$, OR = 0.3, 95%CI = 0.07–0.9, and $p <0.001$, OR = 0.03, 95%CI = 0.005–0.14, respectively).

For the urban population, teenagers (12–18 years) and adults (≥19 years) were more likely to present YF-neutralizing antibodies compared to children (5–11 years) ($p = 0.007$, OR = 6.5, 95%CI = 1.7–25.5 and $p = 0.001$, OR = 6.8, 95%CI = 2.1–22.8, respectively).

Individuals who self-declared white skin color were less likely to present antibodies than individuals who self-reported mixed skin color ($p = 0.02$, OR = 0.4, 95%CI = 0.2–0.9). Moreover, students from rural areas were also less likely to present antibodies compared to rural workers ($p = 0.0002$, OR = 0.2, 95%CI = 0.08–0.4). On the other hand, individuals who reported having contact with wild environ- ment were more likely to present YF-neutralizing antibodies

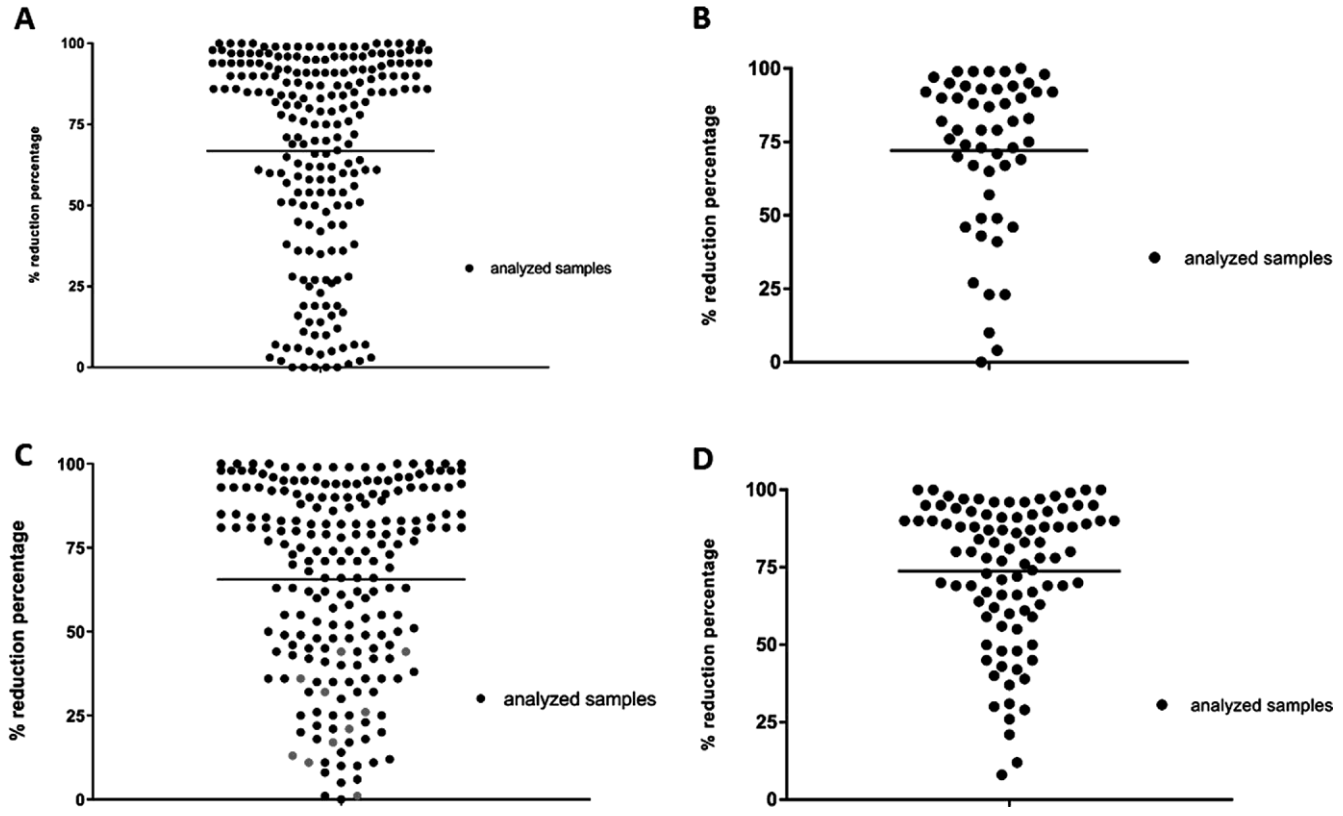

Fig. 1. Scatter plot of percentage reduction of serum samples in relation to virus control. Yellow Fever neutralizing antibodies measured by 50% Plate Reduction Neutralization Test (PRNT). The highlighted line represents an average reduction percentage of all samples. A: Rural Population (2012–2013, n = 218); B: Urban Population (2015, n = 52); and C: Urban Population (2017, n = 216). The red dots are the 10 individuals with validated vaccination cards that did not have detectable neutralizing antibodies. D: Urban Population, (2018, n = 95). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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Table 3

Participants that had proven YFV vaccination history through vaccination cards and did not show detectable YF-neutralizing antibodies.

Serro area	Collection year	Sample number	Sex	Age	Chronic disease	YF vaccination record dates	$PRNT_{50}$
Urban	2017	9	F	51	diabetes and bronchitis	2003–2013	negative
Urban	2017	38	M	9	no	2009–2015	negative
Urban	2017	65	M	62	no	2001	negative
Urban	2017	87	F	15	no	2003	negative
Urban	2017	90	F	12	no	2006	negative
Urban	2017	91	M	10	no	2009–2016	negative
Urban	2017	92	M	18	no	2001 – 2003 – 2011	negative
Urban	2017	108	F	10	no	2008	negative
Urban	2017	113	F	29	no	2003–2015	negative
Urban	2017	117	F	17	no	2001	negative

Table 4

Exposure factors to Yellow Fever virus assessed in 363 surveyed individuals from urban areas of Serro city, 2015–2018.

Exposure factors	N (%)*	$PRNT_{50}$ pos. (%)	$PRNT_{50}$ neg. (%)	P value	OR (95% CI)
Have you visited forest areas?					
Yes	178 (57.2)	134 (43.1)	44 (14.2)	0.3	1.3 (0.8–2.1)
No	131 (42.2)	92 (29.6)	39 (12.5)		
Have you visited rural areas?					
Yes	197 (63.4)	146 (47.0)	51 (16.4)	0.6	1.1 (075–1.9)
No	113 (36.3)	81 (26.0)	32 (10.3)		
There were any primates near to the locations you visited?					
Yes	101 (32.5)	75 (24.1)	26 (8.3)	0.7	1.1 (0.6–1.9)
No	200 (64.3)	145(46.6)	55 (17.7)		
Have you received YF vaccine?					
Yes	297 (95.6)	220 (70.7)	77 (24.7)	0.3	2.1 (0.4–10.5)
No	7 (2.2)	4 (1.3)	3 (1.0)		
How many doses of the YF vaccine did you receive?					
1	62 (20.9)	44 (14.8)	18 (6.1)	Reference	
2	125 (42.1)	91 (30.6)	34 (11.4)	0.8	0.9 (0.4–1.8)
3	14 (4.7)	11 (3.7)	3 (1.0)	0.6	0.7 (0.13–2.6)

* Totals may not add up to 100% due to missing data.

compared to those individuals that had no contact with wild envi- ronments (p = 0.02, OR = 2.1, 95%CI = 1.1–4.0).

In the multivariate logistic regression model,

variables indepen- dently associated with neutralizing antibodies were age and con- tact with wild environment. Older individuals were 5% more likely to present neutralizing antibodies compared to younger indi- viduals (OR = 1.05; CI 95% = 1.03–1.07). Furthermore, those indi- viduals who reported to have contact with the wild environment were 2.5 more likely to present neutralizing antibodies (OR = 2.5, 95%CI 1.2–5.2) than those who did not have contact with wild environment.

4. Discussion

Significant events of YF re-emergence in southeastern Brazil during 2016–2018 signal that YFV is no longer limited to forest areas and/or Amazon region, but instead that transmission in the enzootic cycle is occurring near and within large urban centers [7,13,27]. The viral spread close to large unvaccinated populations and also individuals who failed to seroconvert shows that the YF burden could be underestimated. The current scenario of YF epi- demics reflects the growing concern about re- urbanization of YF [4,7,8].

Here, we demonstrated that a quarter of the population from both the rural and urban areas of Serro city, located at the epicen- ter of the recent YF epidemic period, showed no protective immu- nity against YFV. Until 2002, no evidence of YFV circulation was reported in Serro micro-region [20]. However, from December 2002 to march 2003 an YF outbreak was reported, in which Serro recorded 40% of confirmed cases [20,28].

Since the beginning of YF outbreaks in Brazil, vaccination campaigns were intensified, mainly in the southeast region, the

epicenter of the outbreaks, that reported thousands of human cases [13,27]. However, the estimated number of unvaccinated individuals remains high in Minas Gerais state (2,726,228 million) and the recent overall vaccination coverage is only 92.7% [29]. In recent years (during 2007–2019), accumulated vaccination cover- age in Serro has improved from 84.3% to 98.7% [30]. Our data showed that similar proportions of urban and rural population are protected from YFV. Importantly, the seroprevalence rates fall far below the accumulated vaccination coverage for Serro during 2007–2019 and below the target of 95% established by the Min- istry of Health.

Our findings also demonstrated that older individuals were more likely to present YF-neutralizing antibodies. This result could be linked to the fact that these individuals may have received more doses of the 17DD vaccine throughout their lives compared to younger individuals. These data could in part explain the wild out- break patterns, where the vast majority of cases are observed in male patients, young adults of economically active age who may not have been vaccinated [13].

Rural workers were more likely to have antibodies than other categories related to occupation, which could be explained by the fact that they were more exposed in the rural and probably wild environments where wild viruses circulate naturally [7,8]. It's important to note that the estimated percentage of YF asymp- tomatic cases is around 40–65% [31]. However, this fact can also be related to well established vaccination campaigns to this popu- lation that historically is known to be at risk of YF disease [7,32]. Considering what we discussed for the rural workers, as expected, individuals that reported contact with the wild environment were also more likely to present YF-neutralizing antibodies.

We have detected a high proportion of seronegative individuals from both urban and rural areas. These data may indicate that

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there may be a deficit in vaccination in the municipality, which could result in a large percentage of the population vulnerable to YFV infection. Indeed, in this study we detected a high percentage of individuals (27%) from urban area that did not show YF- neutralizing antibodies during 2017 and 2018. This data suggests that individuals in urban area were susceptible to YFV infection during an ongoing outbreak in Minas Gerais, which could favor the viral spreading in different urban areas and the emergence of new cases.

In summary, we expected a higher seroprevalence of YFV for both rural and urban populations in Serro, which is located in an area at risk for YF and has intensified the vaccination campaigns against YF during the 2017 epidemic. Although accumulated vacci- nation coverage in Serro has improved since 2007, the current cov- erage is still not enough, which warns of the need for better surveillance and vaccination campaigns [14,30].

Teenagers and adults were more likely to present YF- neutralizing antibodies compared to children in urban area. We can attribute this correlation to a bias related to the small size of the children (5–11 years) population sampled (n = 13). However, the seroprevalence of 16.7% in children for both populations, can be correlated with the differences in immune response observed with age, where seroconversion rates are generally lower in children.

Previous studies have already shown that the seroconversion rate is generally lower in children, with the 17D and 17DD vaccines varying from 67 to 94%, and 89.7–98.2% for adults. This difference may be related to the immaturity of the immune system, which can contribute to less robust immune responses, the presence of maternal antibodies, and simultaneous infections that can inter- fere or decrease the immune response [33–36].

Our results highlight the need for booster doses of the 17DD vaccine in Brazilian endemic areas to guarantee long lasting pro- tecting immunity, especially for children living in areas at risk for YF transmission. However, it is important to emphasize that the methods used in this study do not allow us to assess the differ- ences in seroprevalence observed regarding age.

Interestingly, we also found that almost 20% of seronegative individuals had proven YFV vaccination history (10/52) which almost two times more than compared to findings reported by Miyaji and colleagues [37]. The rate of seroconversion for the vac- cines 17D and 17DD range from 89.7% to 98.2% in adults and from 67% to 94% in children [33]. Other studies have also reported cases of previously vaccinated individuals who developed natural infec- tion [27,38].

According to the literature, some comorbidities can affect the immune response induced by vaccines [39]. So, in our study, there was only one report of comorbidity, being a 51-year-old woman with diabetes and bronchitis, but this result cannot

be conclusive. Nevertheless, these data highlight the susceptibility of a group of previously vaccinated individuals to a disease that exhibits a high mortality rate and has no treatment, and reinforces the recommen- dation for the adoption of vaccine booster doses in Brazil to avoid risk [40]. In the light of these results, the recommendation of the World Health Organization (WHO) for the adoption of only one dose of the vaccine, especially in endemic areas should be recon- sidered [40–45].

The 17DD attenuated vaccine has an efficacy of 90%–98%, but

not all vaccinated individuals maintain lifelong protection [4,40]. So, the decline of protective immunity by specific neutralizing anti- bodies over time is a concern. After primary YFV vaccination, the neutralizing antibody levels may decrease significantly in subse- quent years [40,44,45]. Unfortunately, further investigation of this hypothesis could not be performed in this study due to the fact that the individuals included in the study did not provide details for their vaccination history.

Outside of epidemic periods, the adoption of at least three doses of 17DD vaccine at a 10-year interval provide a better induction of long-term immunity [40]. Therefore, our findings reinforce the need for re-vaccination of individuals residing in YF endemic areas, since the absence of YF-neutralizing antibod- ies can indicate waning immunity over time or even failure of vaccination.

Serro is located in a transition area between the biomes of Cer- rado and Atlantic Forest, with considerable anthropic actions mainly due to mining, agricultural, and livestock activities [19,20,46]. Thus, the presence of fragmented forest areas and a large part of the population living in rural areas are risk factors that may be related to the emergence of YF cases [7,21]. However, the risk of urban YFV transmission by the Aedes aegypti vector in Serro is currently low (0.4% rate of vector infestation), which is consid- ered satisfactory [47].

Few studies are focused on assessing the seroprevalence of Brazilian populations against YF [37,48]. Besides that, vaccine cov- erage estimates do not always reflect the reality of immune protec- tion of a population; therefore, seroprevalence studies are useful tools to validate these estimates, especially in poorly epidemiolog- ically monitored regions [49,50]. Through the tracking of popula- tions immunity for vaccination against various diseases, these studies help to alert to the existence of immunization failures and susceptible

populations [50].

The YF vaccine is able to induce both a strong activation of the humoral response, with the production of neutralizing antibodies, and the immune cellular response [33,51]. Thus, in addition to antibodies, the cellular response also plays an important role in the protection triggered by the YF vaccine, and both types of immune responses can be used as protective markers [40,45]. However, neutralizing antibodies still remain the main correlate of protection against YFV, being the titer of neutralizing antibodies considered the standard to check for postvaccination immunity. Furthermore, studies with animal model also suggest that neutral- izing antibodies are efficient against challenge with virulent strains of YFV [51,40].

In addition, our study highlights a gap between calculated vac- cine coverage and immune protection. Failure to report possible YF cases to health agencies may contribute to underestimating the magnitude of epidemics and, consequently, the extent of suscepti- ble individuals residing in areas at high risk for viral transmission. Our study has limitations. We were unable to establish a longi- tudinal study with the participants, which could provide more information and help to better monitoring immunity to YF in this population. We were also unable to evaluate cross-reactivity between other flaviviruses. However, there was a low incidence of dengue infections during 2007–2012 in the study area, and only nine cases of dengue fever were reported in Serro according to DATASUS (Departamento de Informática do Sistema Único de Saúde do Brasil) [52,53]. Furthermore, data and timing of vaccina- tion for YF were not collected from all participants and, therefore, our data does not allow a more robust analysis of the conditions that lead to the absence of detectable antibodies after a

vaccination.

5. Conclusions

In conclusion, we demonstrated that one-quarter of individuals located in areas of high risk for YF in Brazil do not have immunity against YFV and a high proportion of individuals with prior vacci- nation history still remain at risk with contraction of disease. This data concerns for the possibility of an increasing number of YF human cases in this area at high risk for both the sylvatic and urban cycles of the disease. Our findings emphasize the importance of an active epidemiological surveillance and draws attention for

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continued vaccination campaigns aiming ≥ 95% of coverage. Our data also provide useful information that could assist local, national, and international health authorities to develop future vaccination strategies, especially in areas at great risk of viral transmission and with a considerable number of non-vaccinated individuals. Considering that in different regions of Brazil sero- prevalence studies aiming to assess whether YFV vaccination coverage really reached levels ≥ 95% are scarce, and we found a seronegativity of 25.8% for rural and urban populations, future YF vaccination campaigns and further specific tests to evaluate immu- nity in the populations are needed. In summary, we reinforce the importance of seroepidemiological studies which can help direct public health policies to decrease the occurrence of YF epidemics in areas at risk and contain viral dispersion.

Declaration of Competing Interest

The authors declare that they have no known competing finan- cial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2020.07.077.

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