UNIVERSIDADE FEDERAL DE MINAS GERAIS

ESCOLA DE VETERINÁRIA

Programa de Pós-graduação em Ciência Animal

DEVELOPMENT, POTENCY AND EFFICACY TEST OF AN INACTIVATED VACCINE USING THE VACCINIA VIRUS STRAIN GP2

Ana Carolina Diniz Matos

Belo Horizonte

2016

Ana Carolina Diniz Matos

DEVELOPMENT, POTENCY AND EFFICACY TEST OF AN INACTIVATED VACCINE USING THE VACCINIA VIRUS STRAIN GP2

Thesis submitted to the Post Graduation Program from Escola de Veterinária, Univesidade Federal de Minas Gerais, in partial fulfillment of the requirement of Doctor in Veterinary Science

Major: Preventive Veterinary Medicine
Adviser: Prof. Dr. Zélia Inês Portela Lobato
Co-adviser: Prof. Dr. Andrey Pereira Lage
Prof. Dr. Maria Isabel Maldonado Coelho Guedes

Belo Horizonte

Escola de Veterinária – UFMG

2016

Matos, Ana Carolina Diniz, 1986-
M433d Development, potency and efficacy test of an inactivated vaccine using the vaccinia virus strain GP2 / Ana Carolina Diniz Matos. – 2016.
78 p. : il.

Orientador: Zélia Inês Portela Lobato
Co-orientadores: Andrey Pereira Lage, Maria Isabel Maldonado Coelho Guedes
Tese (doutorado) – Universidade Federal de Minas Gerais. Escola de Veterinária
Inclui bibliografia

1. Bovino de leite – Doenças – Teses. 2. Tetas – Ferimentos e lesões – Teses. 3. Vacinia – Teses. 4. Resposta imune – Teses. 5. Vacina veterinária – Teses. I. Lobato, Zélia Inês Portela. II. Lage, Andrey Pereira. III. Guedes, Maria Isabel Maldonado Coelho. IV. Universidade Federal de Minas Gerais. Escola de Veterinária. V. Título.

CDD – 636.214 089 69

FOLHA DE APROVAÇÃO

ANA CARLOLINA DINIZ MATOS

Tese submetida à banca examinadora designada pelo Colegiado do Programa de Pós-Graduação em CIÊNCIA ANIMAL, como requisito para obtenção do grau de DOUTORA em CIÊNCIA ANIMAL, área de concentração em MEDICINA VETERINÁRIA PREVENTIVA.

Aprovada em 30 de Maio de 2016, pela banca constituída pelos membros:

Profª. Zélia Inês Portela Lobato
Presidente – Orientador

Dr. Anselmo Vasconcelos Rivetti Júnior
Laboratório Nacional Agropecuário - LANAGRO

Profª. Elaine Maria Seles Dorneles
Universidade Federal de Lavras - UFLA

Prof. Flávio Guimarães da Fonseca
Instituto de Ciências Biológicas - UFMG

Prof. Nelson Rodrigo da Silva Martins
Escola de Veterinária - UFMG

Universidade Federal de Minas Gerais
Escola de Veterinária
Av. Antônio Carlos, 6627 -
Caixa Postal 567 – CEP 30123-970
Belo Horizonte – Minas Gerais
Telefone: (31) 3409-2057/2059(fax)
www.vet.ufmg.br
E-mail cap@vet.ufmg.br

Dedico esta tese aos meus pais, pelo apoio sempre incondicional para a realização dos meus sonhos.

"Quando penso que alcancei o meu limite, vejo que sou capaz de ir muito mais longe"

ACKNOWLEDGEMENTS/ AGRADECIMENTOS

Meu agradecimento inicial é aos meus pais, pelo exemplo de vida e todo o incentivo para que eu possa alcançar meus objetivos e realizar sonhos.

À minha irmã, Ju, muito obrigada pelo apoio e incentivo de sempre.

Ao Diego, agradeço o amor, paciência, companheirismo e incentivo ao meu crescimento pessoal e profissional.

Aos meus amigos e minha família muito obrigada por entenderem por muitas vezes a minha ausência e incentivarem minha busca pelo conhecimento.

Um agradecimento especial à Prof. Zélia, minha orientadora, pela confiança e exemplo profissional.

À Prof. Maria Isabel, minha mais do que co-orientadora, querida Bel, muito obrigada por todo auxílio, exemplo e conhecimento.

Ao Prof. Andrey, meu co-orientador, agradeço os ensinamentos.

Aos meus amigos e companheiros do LPVA, em especial à Iza, Prof. Érica, Júlio, Grazi,Ana Fraiha, Aristóteles, Tiago e Natália, pela ajuda em todas as etapas deste trabalho e tantos outros que realizamos.

À Fê Villani, muito obrigada por toda a ajuda na citometria, por me acompanhar nos longos dias de leitura no citômetro, por me auxiliar em todos os imprevisíveis problemas da citometria. Sobrevivemos, com muita emoção! E muito obrigada à Prof. Walderez Dutra, por possibilitar a realização de leituras no citômetro do laboratório e à Luisa pela paciência e grande ajuda.

Aos professores, técnicos e demais funcionários do Departamento de Medicina Veterinária Preventiva e do Centro de citometria do ICB obrigada pela colaboração.

Ao Seu Geraldo por toda a ajuda nos experimentos com as novilhas. Ao LANAGRO pela disponibilização do espaço e ao Alexandre (Baixinho) pelo suporte dado nesta fase experimental.

Ao Prof. Peter Mertens, muito obrigada pela oportunidade, confiança e ensinamentos. À sua equipe um agradecimento especial pela excelente recepção nos laboratórios do Instituto Pirbright. À Dra. Kyriaki (Kiki) agradeço a paciência, amizade, apoio e todo ensinamento. Ao Dr. Simon Carpenter e sua equipe, obrigada por me receberem sempre tão bem seu laboratório e pela paciência e confiança em ensinar conhecimentos sobre Culicoides. – *I would like to acknowledge Prof. Peter Mertens for the oportunity, trust and teaching. To Peter Mertens team I acknowledge the warm welcome at the Pirbright Institute. Dr. Kyriaki, my dear Kiki, thank you for the patience, help and teaching. Dr. Simon Carpenter and team, thank you for the patience, welcome and teaching about Culicoides*

A CAPES, pela bolsa de doutorado e doutorado sanduíche. Obrigada também ao CNPq e a FAPEMIG pelo apoio financeiro a pesquisa.

E como sempre, o meu imenso agradecimento, respeito e admiração aos animais...

E a Deus agradeço por me iluminar e proporcionar momentos e conquistas como esta.

TABLE OF CONTENTS

TABLE OF FIGURES

TABLES

ABREVIATIONS

AH – aluminum hydroxide
ARAV – Araçatuba virus
ATCC – American type culture collection
BAV – BeAn 58058 virus
BPL – beta-propiolactone
BRA-VACV – Brazilian *Vaccinia virus* isolates
BV – bovine vaccinia
COTV – Cotia virus
CTGV – Cantagalo virus
DPC – day post challenge
DPI – day post infection
ECE – embrionated chicken egg
EV – enveloped virus
FCS – fetal calf serum
GP1 – Guarani Property 1 virus
GP2 – Guarani Property 2 virus
HA – hemagglutinin
iGP2 – inactivated GP2
ME – microemulsion
MEM – minimum essential Eagle medium
MIF – mean intensity of fluorescence
MOI – multiplicity of infection
MV – mature virus
NA – neutralizing antibodies
OW – oil-in-water
PBMC – peripheral blood mononuclear cells
PBS – phosphate buffered saline
PFU – plaque forming units
PSTV – Passatempo virus
PV1 – Pelotas virus 1
PV2 – Pelotas virus 2
SAV – SPAn232
VACV – *Vaccinia virus*
WHO – World Health Organization

ABSTRACT

Bovine vaccinia (BV), caused by *Vaccinia virus* (VACV), is a zoonosis characterized by exanthematic lesions in the teats of dairy cows and milkers hands. Due to the occurrence of many BV outbreaks in dairy farms in all Brazilian regions, since 1999, there is a need to improve the control and prevention measures of the disease. Vaccination is one of the major tools to prevent viral diseases, and it could be an alternative for BV prevention. The main objective of the research was the development of vaccine formulations against BV using the inactivated VACV strain GP2 as antigen combined with different adjuvants. Then, potency and efficacy tests were performed in mice and cattle. VACV strain GP2, inactivated by BPL in association with adjuvants was effective to induce a humoral immune response against infection by the homologous VACV in Balb/c murine model. The induction of the humoral immune response, as measured by neutralizing antibody (NA) titration was variable according to the adjuvant used with the viral antigen. The vaccine formulation using aluminum hydroxide associated with saponin as adjuvants induced high titers of NA in all vaccinated mice, giving 100% protection in Balb/c murine model after the challenge. This formulation was selected and tested in cattle. In the target species, bovine, this vaccine formulation was able to induce the production of NA and activate cellular immune responses after challenge, conferring 100% protection against clinical signs in vaccinated heifers submitted to the challenge by VACV-GP2.

Key words: Bovine vaccinia, immune response, vaccine, *Vaccinia virus*.

RESUMO

A vaccinia bovina (VB), causada pelo *Vaccinia virus* (VACV), é uma zoonose caracterizada por lesões exantemáticas nos tetos de vacas em lactação e nas mãos de ordenhadores. A emergência de surtos de BV em todas as regiões brasileiras, desde 1999, sinaliza para a necessidade de medidas de controle e prevenção. Vacinação é um dos principais instrumentos para a prevenção de doenças virais, e pode ser uma alternativa para a prevenção da VB. O principal objetivo deste trabalho foi o desenvolvimento de formulações vacinais contra a VB utilizando como antígeno a amostra VACV GP2 inativada formulada com diferentes adjuvantes e realizar testes de potência e eficácia em modelo murino e na espécie alvo, bovino, respectivamente. A amostra VACV GP2 inativada por BPL em associação à adjuvantes foi eficaz na indução de resposta imune humoral e proteção contra infecção pelo VACV homólogo em modelo murino. A resposta imune humoral foi avaliada por titulação de anticorpos neutralizantes (AN) e se mostrou variável de acordo com o adjuvante utilizado na formulação vacinal. A associação de hidróxido de alumínio e saponina como adjuvantes se mostrou a melhor formulação vacinal, com indução de elevados títulos de anticorpos neutralizantes e conferindo 100% de proteção em modelo murino após o desafio. Esta formulação foi selecionada e testada em bovinos. Na espécie alvo, bovino, esta formulação vacinal foi capaz de induzir a produção de AN e resposta imune celular ativa após o desafio, com 100% de proteção contra os sinais clínicos na novilhas vacinadas e submetidas ao desafio por inoculação de VACV-GP2.

Palavras-chave: Vaccinia bovina, resposta imune, vacina, *Vaccinia virus*.

1. Introduction

Vaccinia virus (VACV) is the etiological agent of bovine vaccinia (BV), a zoonosis characterized by exanthematic lesions in cattle and humans, responsible for public health impacts and economic losses, especially in Brazil and India (Kroon et al., 2011; Singh et al., 2012).

Bovine vaccinia clinical course begins with the appearance of maculopapular rash that progress to papules which evolve to vesicles, pustules and subsequently scab lesions, which heals about 20 days after infection. (Lobato et al., 2005; Rehfeld et al., 2013). The lesions are mainly located in the teats and sometimes in the udder of infected cows. In humans, the lesions are frequently observed in the hands and arms of milkers, which come in direct contact with the cows' lesions. Fever, myalgia, headache and lymphadenopathy are some of described systemic clinical signs in humans. (Trindade et al., 2007a; Da Silva et al., 2008; Megid et al., 2008).

In Brazil, towards the end of the 1990s, there was an increase of reports describing an exanthematic disease affecting dairy cows and humans. In most of those reports, VACV was identified as the causative agent, characterizing the BV occurrence all over the country (Schatzmayr et al., 2000; Trindade et al., 2003, 2006; Nagasse-Sugahara et al., 2004; Leite et al., 2005; Lobato et al., 2005; Donatele et al., 2007; Megid et al., 2008; Silva-Fernandes et al., 2009; Quixabeira-Santos et al., 2011; De Assis et al., 2013; Oliveira et al., 2013, 2015).

According to the United States Department of Agriculture – USDA, in 2014, Brazil had the second largest number of milking cows in the world, behind only India. Among the BV affected regions in Brazil, the Southeast is noteworthy because of the large number of reported cases. The region is the second major milk producer in Brazil, producing about 12.16 billion liters per year, which corresponds to 34.6% of the total national production, and milking 34.4% of the dairy cows in Brazil (Instituto Brasileiro De Geografia E Estatística - IBGE, 2014). The emergence of BV outbreak configures a public health risk, and also generates economic losses to the milk production chain (Kroon et al., 2011).

Given the numerous cases of BV reported in the recent years, the development of a safe and effective vaccine has become an emerging demand. Besides being a zoonosis, another worrying factor is the rapid spread of the disease in the herd. In affected properties it is possible to observe high attack rate, which can reach up to 100% of dairy cows in a herd (Lobato et al., 2005). Few measures in relation to clinical recovery of animals could be taken, such as lesions disinfection to prevent secondary infections. Thus, immunization of susceptible animals would be the most important measure to prevent the occurrence of the disease in herds, mainly in the affected regions.

In the present study, a vaccine against bovine vaccinia was developed for the purpose of control and future eradication of the disease in cattle. BV is a zoonosis and the main route for human infection is through direct contact with infected cows. The control and eventual eradication of the disease in cattle could eliminate the main route of infection to humans, resulting indirectly in the control of the zoonotic disease.

Viral shedding in feces and milk has been identified in cattle naturally and experimentally infected (Abrahão et al., 2009b; D'anunciação et al., 2012; Guedes et al., 2012; Rivetti Jr. et al., 2013; De Oliveira et al., 2015). Control of the disease in cattle may also result in reduction of environmental contamination by VACV and the risks associated with ingestion of contaminated milk, as already demonstrated in murine model (Rehfeld et al., 2015).

The vaccine was produced using a VACV strain isolated from a lesion on the teats of an infected cow in Brazil. The strain was genetically characterized and named Guarani Property 2 (GP2) (Trindade et al., 2006). This VACV strain was cultivated in cell culture and subsequently subjected to viral inactivation process for use as the vaccine antigen. For vaccine formulations, the antigen was combined with adjuvants according to different formulations. Initially, potency tests using all the vaccine formulations were performed in Balb/c mice. Subsequently, the vaccine formulation that resulted in higher neutralizing antibody production and better disease protection in mice was selected and the efficacy assays were performed in the bovine target species.

2. Objectives

The main objective of this research was the development of vaccine formulations against BV using the inactivated VACV strain GP2 as antigen added by different adjuvants, followed by performing potency and efficacy tests in mice and cattle, respectively. For that, the specific objectives of the present study were to:

- Formulate inactivated vaccines against BV, using the VACV strain GP2 as antigen added by different adjuvants and evaluate the vaccines potency in Balb/c mice through neutralization assay and challenge;

- According to the results obtained in mice, choose the best vaccine formulation for cattle immunization and evaluate the efficacy of vaccine protection by serological neutralization assay and challenge with the homologous virus;

- Evaluate the humoral and cellular immune responses of the vaccinated cattle before and after challenge.

3. Literature review

3.1. The *Poxviridae* family

Members of the *Poxviridae* family are characterized by complex and large DNA viruses that replicate entirely in the cytoplasm of vertebrate and invertebrate cells. The poxviruses are divided into two subfamilies, *Chordopoxvirinae* and *Entomopoxvirinae*, based on vertebrate or insect host range, respectively (Moss, 2013). The subfamily *Chordopoxvirinae* consists of ten genera and the *Squirrelpox virus*, that was proven to be a poxvirus, although not classified within any of the currently recognized genera, signaling to the existence of a new genus (Darby et al., 2014; ICTV, 2014). The genera within the *Chordopoxvirinae* subfamily, the classified species and the type virus for each genus are demonstrated in Figure 1.

The majority of human pathogenic poxvirus infections are zoonosis; only *Variola virus*, the smallpox etiologic agent, and *Molluscum contagiosum virus* are strictly human pathogens (Moss, 2013). Considering the poxviruses, members from the same genus generally are genetically and antigenically related and have similar morphology and host range. The cross-protective relationship between members from the same genus was most explored within the *Orthopoxvirus* members, through the use of the *Vaccinia virus* (VACV) as vaccine antigen for the successful worldwide smallpox eradication (Fenner et al., 1988).

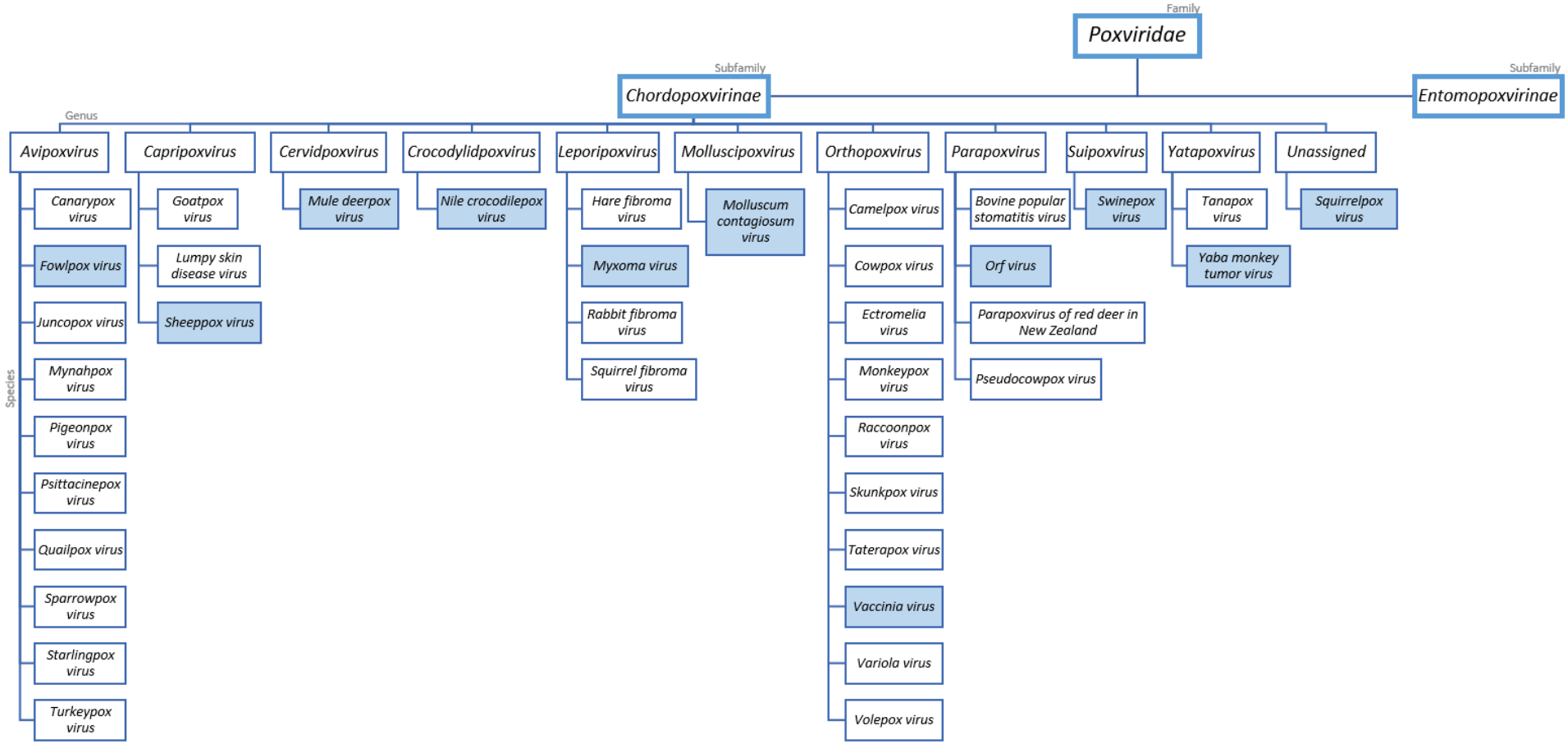

Figure 1 The *Chordopoxvirinae* (*Poxviridae*) classification. The blue filled boxes indicate the type virus for each genus. (Data from ICTV, 2014).

3.2. *Vaccinia virus* structure and morphology

The *Poxviridae* family comprises large and complex viruses. Electron microscopic investigations of VACV demonstrate that the virion is highly asymmetric, described variously as ellipsoidal, brick shaped, or barrel shaped with dimensions of approximately 360 x 270 x 250 nm (Moss, 2013).

The virus particle contains more than 100 proteins distributed between four distinct structures: the core, the lateral bodies, external membrane and envelope (Figure 2). The core presents a biconcave format and involves the viral DNA, enzymes and early gene transcription factors. The lateral bodies are arranged on both sides of the core, surrounded by a membrane (Moss, 2013). The outer membrane, of cellular origin, is formed by a lipoprotein bilayer and surrounds the core and lateral bodies. Surface tubular elements covering the outer membrane, are responsible for inducing the production of neutralizing antibodies during infection. A further outer membrane is the envelope, which has a lipoprotein nature and can be seen enveloping the virions (Buller; Palumbo, 1991).

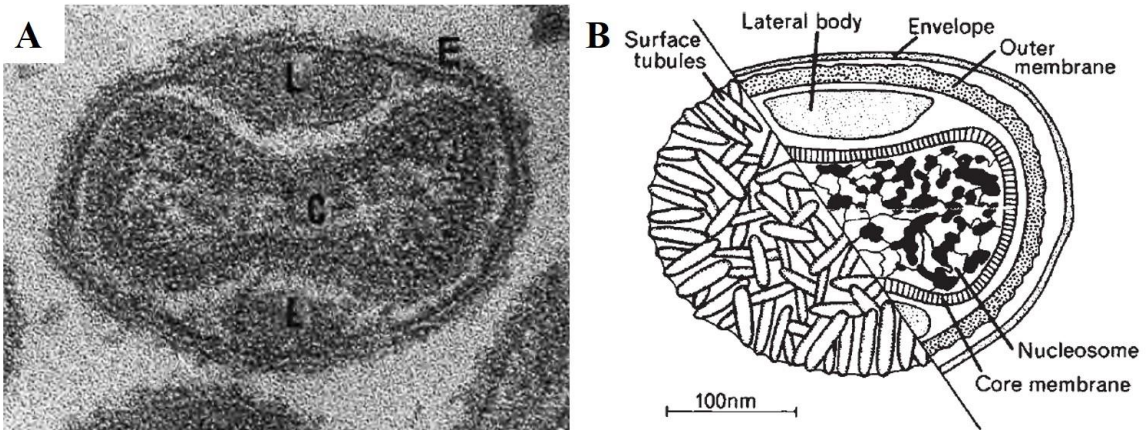

Figure 2 Morphological structure of *Orthopoxvirus*. A – Electron microscopic image of a thin sectioned intracellular mature virion (MV) of Vaccinia virus (VACV). It is showing the core (C), lateral bodies (L) and external membrane (E). B – Schematic representation of a poxvirus virion. Right-hand side: section of enveloped virion (EV). Left-hand side: surface structure of MV particle. (Buller; Palumbo, 1991; Moss, 2013).

3.3. *Vaccinia virus* replication cycle

Unlike most of DNA viruses, the members of *Poxviridae* replicate in the cytoplasm of infected cells. This characteristic is related to the presence of enzymes that synthesize and modify mRNA and a genome composed of a single linear double-stranded DNA molecule in the large complex enveloped virion. Early genes encode enzymes required for DNA replication, and as the early gene expression peaks, viral DNA replication ensues in cytoplasmic sites termed "virus factory" (Condit; Moussatche; Traktman, 2006).

Vaccinia virus is the prototype of orthopoxvirus and presents approximately 200 kb double-stranded DNA genome, with a hairpin loop at the ends, that encodes nearly 200 genes. Approximately half of the VACV encoded genes are early transcribed during infection. Proteins

translated from these early mRNAs serve to replicate the virus DNA, modify the host cell to the advantage of the virus and aid virus escape from the host innate immune response. After DNA replication has begun, the transcription of intermediate genes commences. Intermediate genes are fewer in number and encode mostly regulatory proteins that induce the transcription of late genes. The late genes encode most of the virus proteins that make up new virus particles and also enzymes that are packaged into virions to initiate transcription in the next infected cell (reviewed by (Roberts; Smith, 2008) .

The VACV has a complex morphogenic pathway that culminates in formation of two distinct infectious virus particles that are surrounded by different numbers of membranes, the mature virus (MV) and the enveloped virus (EV) (Moss, 2013).

Initially, during virus replication, the MV is produced, which is comprised of a single membrane surrounding the virus core, and remains within the cell until cell lysis. The MVs represent the majority of infectious viral particles. The remaining virions are formed when a subset of MVs are wrapped by modified trans-Golgi or endosomal cistern containing additional viral membrane proteins. Then, these particles are transported to the periphery of the cell where they would exit, surrounded by a second membrane and are exported from the cell before cell death, when they lose one of the membranes. These enveloped virions can be retained on the cell surface, which is called cell-associated EV or be released from the cell surface, through or not actin tails, forming the extracellular EV (Smith; Law, 2004; Roberts; Smith, 2008). Figure 3 illustrates an overview of the VACV replication cycle.

The differences between MVs and EVs affect virus attachment and entry into cells, egress from cells and virus dissemination. The EV forms are important for virus dissemination. Cell-associated EVs induce the formation of actin tails from the cell surface that drive virions towards uninfected cells, and extracellular EVs mediate longer-range dissemination *in vitro* and probably within the host also. In addition to their roles in virus dissemination, EVs help VACV to evade host antibody and complement, by being wrapped in a host-derived membrane. The MVs are thought to be important for long-term stability and transmission of the virus between hosts in the environment (Condit; Moussatche; Traktman, 2006; Roberts; Smith, 2008).

Different viral proteins are found within either membrane, and this makes MV and EV structurally, antigenically and functionally different. Several MV proteins, especially A27 and H3, are targets for neutralizing antibodies that prevent infection. The EV proteins that induces neutralizing antibodies are B5 and A33 (Pütz et al., 2006; Cohen et al., 2011; Matho et al., 2015).

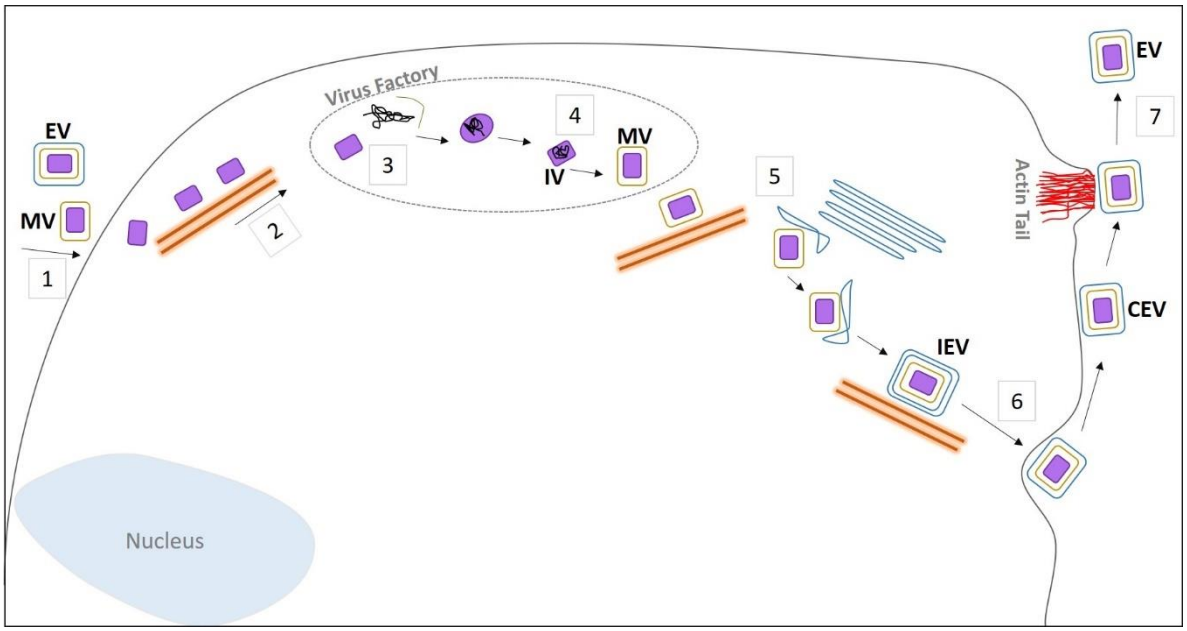

Figure 3 Overview of the VACV replication cycle in a single cell. (1) The virus binds and enters the cell, losing the membrane(s) and releases the naked viral core into the cytoplasm. (2) The core is transported on microtubules deeper into the cell. (3) Transcription of the early mRNAs leads to core uncoating and subsequent DNA replication. (4) within the viral factory, immature virions (IV) assemble and are processed to form mature virus (MV). The majority of MVs are released from the cell by cell lysis. (5) Some MVs are transported to sites of wrapping by early endosomes and the trans-Golgi network. (6) MV particles are wrapped by a double membrane to form intracellular enveloped virions (IEVs) that are then transported to the cell surface on microtubules. (7) The outer IEV membrane fuses with the plasma membrane to expose a cell-associated enveloped virus (CEV) at the cell surface. Polymerization of an actin tail beneath the CEV can occur to drive the virus into the neighboring cell, or the virus is released as an enveloped virus (EV). (Adapted from Roberts; Smith, 2008).

3.4. *Vaccinia virus* in the world

The genus *Orthopoxvirus* comprises ten species antigenically related (Figure 1) and presents wide geographical distribution with a variable spectrum of hosts. Among the orthopoxviruses, it is known that four of them can infect humans: *Cowpox virus*, *Monkeypox virus*, VACV and *Variola virus*. The only natural host of *Variola virus* is humans, and different from the other three viruses, is the only one not related to zoonosis (Moss, 2013; ICTV, 2014)

The smallpox, caused by *Variola virus*, was a devastating disease, responsible for approximately 50 million deaths only in the 19th century. During the smallpox era, overall mortality rates were approximately 30%, but other less common although more severe forms of the disease could present mortality rates above 96% (Rotz et al., 2001). Because of the very effective cross-protection between the orthopoxviruses, the *Cowpox virus*, and later, VACV were used in the 19th and 20th centuries to prevent smallpox infection and led to the term "vaccination". (Fenner et al., 1988).

The VACV has had an important role in human history due to its highly effective use as immunizing agent in the smallpox vaccination campaign that enabled the global eradication of this deadly disease in 1980 (Fenner et al., 1988).

Vaccinia vaccines, used against smallpox, are based on attenuated selected strains of VACV. Containing live VACV particles, the vaccinia vaccine strain of pustular lesions can be transmitted from person to person through physical contact. The recognition of adverse events associated with vaccinia vaccination and the decreased risk of smallpox led to the discontinuation of routine immunization for civilian population in 1983, and military personnel in 1990 (ROTZ et al., 2001). However, in December 2002, in response to the possible threat of intentional release of smallpox virus as a bioterrorism weapon, the United States government implemented a program to immunize select military and public health personnel against smallpox. Many soldiers, although immunized, remained for a long time releasing infectious viral particles presumably from scabs formed as a result of administration of the vaccine. For this reason, some cases of intra-familial VACV transmission have been described (Egan et al., 2004; Vora et al., 2008).

Vaccinia virus is the most studied poxvirus. After the smallpox eradication and vaccination suspension, VACV became widely used in research as a model for viral immune evasion strategies studies, and more recently in biotechnology, as a vector for the generation of recombinant vaccine candidates against many human and veterinary diseases (Xu et al., 2004; Jacobs et al., 2006; Brun et al., 2008; Bhanuprakash et al., 2012).

Around the world, after smallpox eradication, other zoonotic orthopoxviruses have emerged worldwide, such as *Cowpox virus*, in Europe, *Monkeypox virus*, which is endemic in many African countries and was accidentally introduced in the USA in 2003. In India and Brazil, VACV strains have emerged and are now endemic throughout these countries (Essbauer; Pfeffer; Meyer, 2010; Kroon et al., 2011).

Buffalopox was for the first time isolated in 1967 in Northern India and is associated with sporadic outbreaks in Asian buffalos (*Bubalus bubalis*) in India, Egypt, Pakistan and Nepal. The disease is characterized by severe local lesions affecting the udder and teats, leading to mastitis thereby undermining the productivity of milk animals (40–70% reduction) and thus having an impact on the dairy industry. Dairy workers may present lesions on hands, forearms and forehead accompanied by fever, axillary lymphadenopathy and general malaise (reviewed by Singh et al., 2012).

Phylogenetic analysis based on three genes confirmed that buffalopox virus is closely related to VACV, and taxonomically it was denominated to be a variant strain of VACV (reviewed by SINGH et al., 2012). Additionally, analysis of samples collected from cows presenting poxvirus lesions in the period of 2002–2006 throughout India revealed that based on sequence and phylogenetic analyses of HA gene the isolates were most closely related to VACV strains than *Cowpox virus* (Yadav et al., 2010).

In Brazil, the VACV outbreaks are associated with dairy cows and the dairy workers who have direct contact with those cows, and the zoonotic disease is named bovine vaccinia (BV). In South America, besides Brazil, VACV occurrence have only been reported in Argentina (Franco-Luiz et al., 2014) and Uruguay (FRANCO-LUIZ, 2016). However, so far no clinical signs have been reported neither in cattle nor in humans in these countries.

3.5. *Vaccinia virus* in Brazil and South America

The VACV studies in Brazil started with samples isolated in the 1960s during a Brazilian government effort to survey rural regions with reported zoonotic viruses' circulation. The history begins with *Cotia virus* (COTV), that was isolated initially from sentinel mice, in Cotia county, São Paulo, an area of forest in the southeastern region of the country. Phylogenetic analysis of COTV revealed the highest amino acid identity with different genera (*Cervidpoxvirus, Capripoxvirus, Suipoxvirus, Leporipoxvirus, Yatapoxvirus*), excluding its classification as an *Orthopoxvirus*. As COTV grouped as an independent branch within this clade, it was proposed that COTV could represent a new poxvirus genus (Afonso et al., 2012).

BeAn 58058 virus (BAV) was isolated in 1963 from blood of a *Oryzomis* rodent in the tropical rain forests surrounding Belém-do-Pará, Brazil, and was found to be antigenically related to the previously described COTV. Many years later, molecular studies of BAV characterized this sample as VACV (Fonseca et al., 1998; Marques et al., 2001). Later on, the sample SPAn232 (SAV), isolated from sentinel mice that had been exposed in the Cotia forest was studied. It was considered initially to be another COTV isolate, as the virus presented serological cross reaction with the viruses isolated previously. During the analyses, unexpectedly, the SAV isolate was also genetically characterized as a VACV (Fonseca et al., 2002).

Since the end of the 1990s, there was an increase of reports describing an exanthematic disease affecting dairy cows and humans, mainly in the Southeast region. The first poxvirus isolated and characterized as a VACV strain associated with episodes of exanthema affecting dairy cattle and milkers was sampled in the county of Cantagalo, state of Rio de Janeiro, during an outbreak affecting several farms, in 1999. The sample was named Cantagalo virus (CTGV) (Damaso et al., 2000). Then, in the same year, another outbreak of BV was reported in Araçatuba county, São Paulo state. The isolate was called Araçatuba virus (ARAV) and after phylogenetic analysis was identified as VACV strain (Trindade et al., 2003).

The Guarani Property 1 virus (GP1) and Guarani Property 2 virus (GP2) are isolates that were sampled from two different farms during an outbreak of BV in Guarani county, Minas Gerais state, in 2001 (TRINDADE et al., 2006). In 2003, another outbreak was declared in Minas Gerais, in another county (Passa Tempo), when was isolated the Passatempo virus (PSTV) (Leite et al., 2005). All these samples were isolated from lesions of injured cows. The VACV identification was based in clinical signs, virus isolation, serology and molecular studies (Leite et al., 2005; Trindade et al., 2006). Other VACV samples were isolated from humans' lesions during outbreaks in counties of Minas Gerais state, such as the Muriae virus, in 2000 (Trindade et al., 2007b). From 2001 to 2003, samples from exanthematic disease outbreaks in the states of Minas Gerais, Goias and Sao Paulo were studied. Specimens originating from Goias and Sao Paulo, after molecular characterization, clustered phylogenetically with Cantagalo virus and other VACV strains (Nagasse-Sugahara et al., 2004).

Since then, BV outbreaks affecting humans and cattle, with the isolation and identification of VACV strains as the etiologic agent, have been reported in at least 12 Brazilian states (Figure 4), such as Rio de Janeiro (Damaso et al., 2000; Schatzmayr et al., 2009); São Paulo (Trindade et al., 2003; Nagasse-Sugahara et al., 2004; Megid et al., 2008); Minas Gerais (Leite et al., 2005; Lobato et al., 2005; Trindade et al., 2006; Madureira, 2009); Espírito Santo (Donatele et al., 2007); Goiás (Nagasse-

Sugahara et al., 2004); Pernambuco; Tocantins (Medaglia et al., 2009); Rondônia and Mato Grosso (Quixabeira-Santos et al., 2011); Pará (De Assis et al., 2013); Bahia and Maranhão (Oliveira et al., 2013, 2015).

An outbreak of severe cutaneous disease associated with VACV infection in horses has occurred in February 2008, in the county of Pelotas, Rio Grande do Sul state, Brazil (Brum et al., 2010). Later on, biological and phylogenetic studies identified two different strains of VACV isolated not only from the same outbreak but also from the same animal biopsy. These VACV strains were named Pelotas virus 1 (PV1) and Pelotas virus 2 (PV2) (Campos et al., 2011; Cargnelutti et al., 2012). Other occurrences of VACV causing disease in horses were reported later, in Minas Gerais and Bahia states (Matos et al., 2013; Abrahão et al., 2016).

Genetic studies of Brazilian VACV isolates (BRA-VACV) show that some isolates share a similar deletion of 6 amino acids in the hemagglutinin (HA) protein, corresponding to 18 nucleotides of the A56R gene. Additionally, they cluster into two genetically distinct groups (Figure 5) (Trindade et al., 2007b; Drumond et al., 2008). From the previously mentioned BRA-VACV ARAV, CTGV, GP2, PSTV and PV2 are part of the same cluster, named Group 1. Group 2 is formed by BAV, SAV, GP1 and PV1 strains. This polymorphism is also reflected in the lysis plaque phenotype profiles in cellular culture and the virulence of these samples in mice (Balb/c). Infections caused by strains belonging to Group 1 generate small lysis plaques in cellular culture and do not cause clinical signs in infected mice, whereas the samples belonging to Group 2 present large lysis plaque size and cause clinical signs that may lead to death of infected mice (Ferreira et al., 2008).

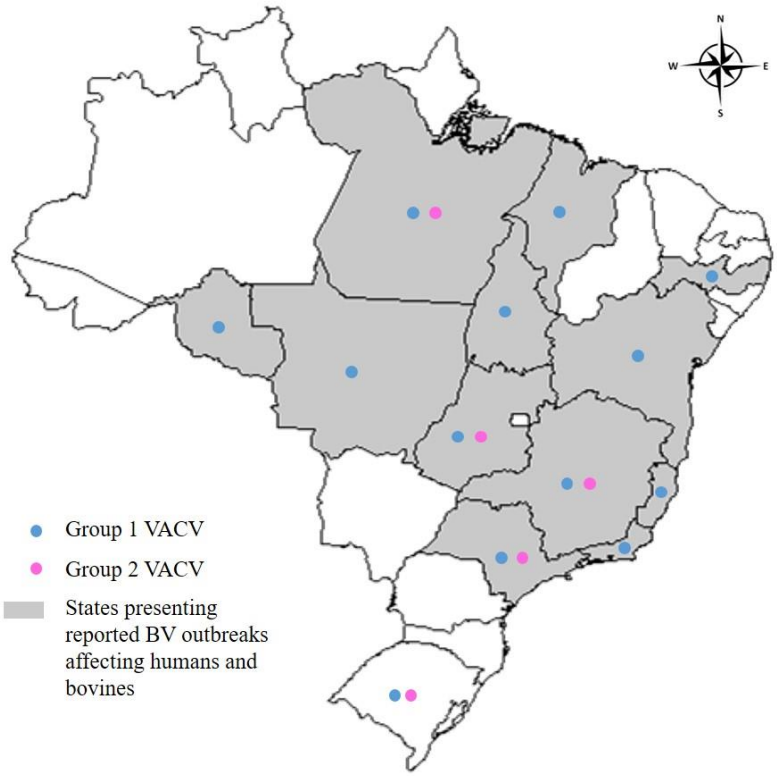

Figure 4 Geographical distribution of VACV identified in Brazil from 1999 to 2015. Grey colored states represent states where BV outbreaks were reported. Blue and pink dots indicate the group (Group 1 or Group 2) classification of Brazilian VACV isolates in each state.

A recent study of different isolates from BV outbreaks in Minas Gerais, Goiás, Espírito Santo and Bahia demonstrates the genetic and biologic variability among VACV and even within the same sample, which reinforced previous studies findings about the circulation of the two groups of BRA-VACV (Oliveira et al., 2015).

Despite the existence of two distinct VACV groups circulating in Brazil, Group 1 viruses have been more frequently isolated when compared to Group 2 viruses, in which 92% of the isolated clones were classified as Group 1, while only 8% were grouped in Group 2, based on sequencing of the A56R gene (Figure 4). However, there are some arguments about why this profile of virus circulation exist. It is suggested that in addition to the higher prevalence of Group 1 BRA-VACV samples there may be some aspects, such as the laboratory *in vitro* isolation systems, that favors the isolation and characterization of these viruses (Oliveira et al., 2015). In the other hand, the difficulty of Group 2 viruses detection may be related to their lower prevalence in the field, confirming the higher prevalence of Group 1 viruses' circulation in Brazil.

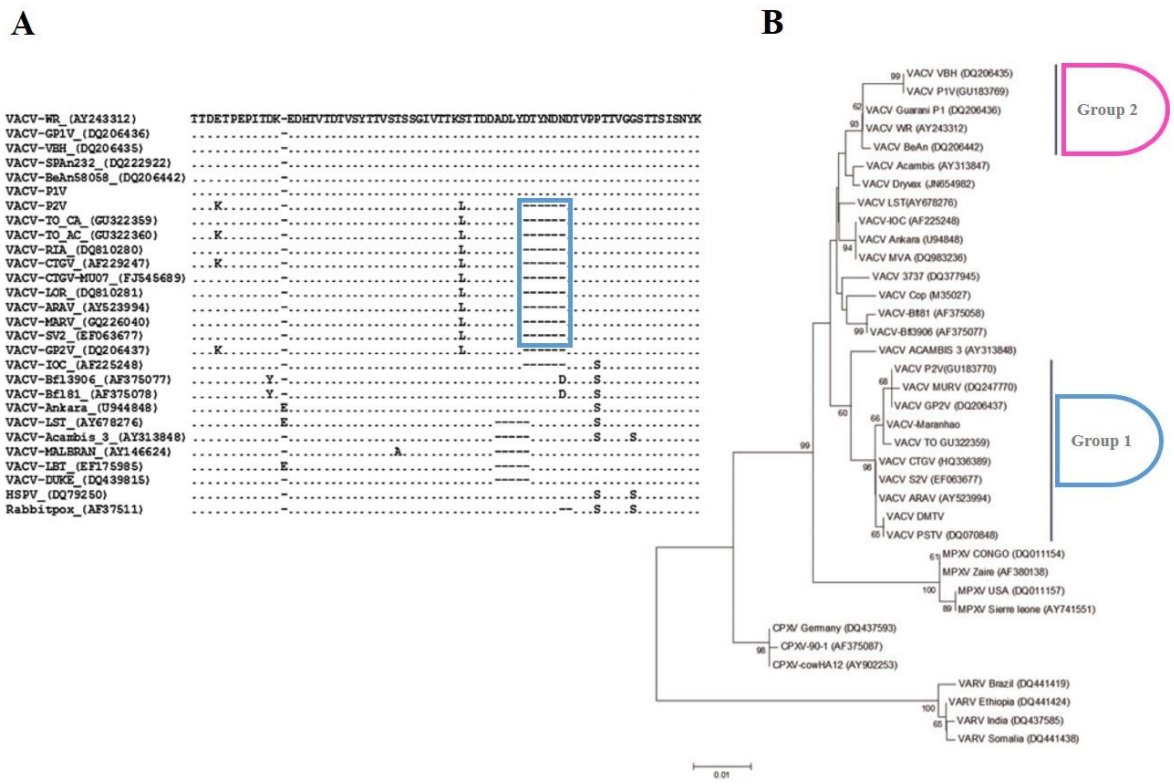

Figure 5 Molecular analysis of the ha gene of different strains of VACV. (A) Phylogenetic analysis of OPV strains based on ha gene sequences. The six-amino acid deletion signature are displayed in the ha genes of some Brazilian VACV strains. B- Phylogenetic tree generated from OPV A56R nucleotide sequences, including the Brazilian VACV isolates. A56R phylogenetic analyses showed that the Brazilian VACV isolates are split into two different branches (Groups 1 and 2). Alignment and maximum likehood tree was constructed using MEGA 4.0. Sources: (Campos et al., 2011; Oliveira et al., 2013).

The susceptibility of rodents to VACV infection, and isolation of poxviruses from sentinel, peridomestic and wild rodents have been reported (Fonseca et al., 2002; Abrahão et al., 2009a; Peres et al., 2013; Barbosa et al., 2014). The VACV was isolated from peridomestic rodents in a BV outbreak, and the phylogenetic analysis of the viruses isolated from rodent, bovine and human, showed a perfect homology between them, suggesting virus circulation between the three species (Abrahão et al., 2009a). Moreover, VACV DNA was also detected in blood samples from dogs and opossums (Peres et al., 2016). And in northern Brazil, VACV infection was detected in monkeys (*Cebus apella* and *Allouata caraya*) (Abrahão et al., 2010).

In Argentina, VACV DNA and neutralizing antibodies against orthopoxviruses were detected in bovine serum samples collected from farms located in Córdoba, Corrientes, Entre Ríos, and Santa Fe Provinces. Serum samples were collected from 100 animals (50 dairy and 50 beef cattle), and 12 samples (four from dairy cattle and eight from beef cattle) had neutralizing antibodies against orthopoxvirus. Of the 100 serum samples, five (three from beef and two from dairy cattle) were positive for *vgf* by real-time PCR. The HA sequences from the Argentinian isolates demonstrated 100% identity among themselves and exhibited higher identity with group 1 (98.2% identity) versus

group 2 (93.6% identity) isolates from Brazil. It is important to notice that, so far, no outbreaks of exanthematous VACV infection have been described in cattle or humans in Argentina (Franco-Luiz et al., 2014). The same research group has also identified VACV DNA and neutralizing antibodies against orthopoxvirus in bovine serum samples from Uruguay, which also has never reported BV outbreaks (Franco-Luiz, 2016).

3.6. Bovine Vaccinia

Bovine vaccinia (BV) is a zoonosis caused by VACV that occurs in Brazil. BV mainly affects milking cows and the dairy workers who have direct contact with those cows. Generally, dairy workers contract the infection through direct contact with animals during the milking process; therefore, the disease has been classified as an occupational zoonosis (Lobato et al., 2005; Silva-Fernandes et al., 2009).

The BV clinical course is characterized by the appearance of maculopapular rash that progress to papules which evolve to vesicles, pustules and subsequently scab lesions, which heals about 20 days after infection. (Lobato et al., 2005; Rehfeld et al., 2013). The lesions are mainly located on teats and sometimes in the udder of the affected cows. In humans, the lesions are frequently observed in the hands and arms of milkers, which come in direct contact with the cows' lesions (Figure 6). Systemic clinical signs, such as fever, myalgia, headache and lymphadenopathy are described in humans affected by VACV infections (Trindade et al., 2007a; Da Silva et al., 2008; Megid et al., 2008).

Lactating cows present most of the lesions located in the teats (Figure 6B). Mastitis and other secondary infections were identified in 43% of affected animals, and the reduction in milk production could reach up to 80% in some farms (Lobato et al., 2005). In farms with suckling calves in direct contact with the cows, it is common to observe sick calves, which can present lesions on the nuzzles, lips and oral mucosa (Figure 6C) (Leite et al., 2005; Lobato et al., 2005; Madureira, 2009). The painful lesions reduce the food intake, leading to weight loss (Lobato et al., 2005).

Figure 6 Lesions observed during BV outbreaks. A- Ulcerative lesions in hand of a dairy worker; B- Ulcerative lesions on teats of a lactating cow; C- Ulcerative lesions, progressing to scabs on the muzzle of a calf. Source: (Leite et al., 2005; Madureira, 2009).

The main route of viral transmission among cows occurs through the hands of milkers or the suction cups of milking equipment (Lobato et al., 2005). Between farms, the disease can be transmitted by the introduction of infected cattle in the herd or even by milkers who come in contact with sick animals in other properties (Lobato et al., 2005; Megid et al., 2008). A study conducted in

the northern region of Brazil showed the importance of animal trading and movement in the spread of BV in this region (Quixabeira-Santos et al., 2011).

The viral circulation in the wildlife and in peridomestic rodents has been studied and could be related to virus maintenance in the field or even transmission of the virus between farms (Abrahão et al., 2009a; Peres et al., 2013; Barbosa et al., 2014). VACV was also detected in blood samples from dogs and opossums (Peres et al., 2016). And in northern Brazil, VACV infection was detected in monkeys (Abrahão et al., 2010). Despite the evidence of VACV circulation in several wild and domestic animal species, VACV reservoirs and the role of wildlife in outbreaks remain unclear.

The elimination of VACV through feces has been demonstrated in experimentally infected cows with VACV GP2 strain (Guedes et al., 2012; Rivetti Jr. et al., 2013). An additional study assessed the horizontal transmission of VACV by exposing sentinel mice to wood shavings contaminated with bovine excrement. VACV DNA was detected in the sentinel mice feces, demonstrating the infectivity of virus particles eliminated in bovine feces and the potential circulation of the virus through bovine and rodents (D'anunciação et al., 2012).

The detection of VACV DNA and infectious particles in milk samples from BV outbreaks associated to the intermittently elimination of VACV in milk from experimentally infected cows call attention to the potential public health risk associated with the consumption of raw milk from BV outbreaks (Abrahão et al., 2009b; De Oliveira et al., 2015).

The actual prevalence of VACV contaminated milk, derived from infected cows and the real risk of infection by consuming contaminated milk and dairy products are still unknown. A study of experimentally infected lactating cows demonstrated that VACV DNA is present in milk up to the 25^{th} DPI, when the teats lesions have already healed (De Oliveira et al., 2015). The risks associated with ingestion of contaminated milk was also demonstrated in murine model (Rehfeld et al., 2015). However, to date, there are only two reports of exanthematous oral lesions in humans associated with the consumption of milk supposedly contaminated with buffalopox virus, an Indian VACV strain (Gurav et al., 2011; Singh et al., 2012).

In 2014, Brazil ranked the fifth position as milk producer in the world, behind the European Union, India, United States of America and China, according the United States Department of Agriculture – USDA. The Brazilian southeast region, the most affected region by BV, produce about 12.16 billion liters per year, which corresponds to 34.6% of the total national production, milking 34.4% of the dairy cows in Brazil (IBGE, 2014). The emergence of BV outbreaks in the country configures a public health risk, and also generates economic losses to the milk production chain (Kroon et al., 2011).

Given the numerous cases of BV reported in the recent years in Brazil and the wide occurrence in the country, as shown in Figure 4, the development of a safe and effective vaccine to be used in cattle has become an emerging demand. Besides being a zoonosis, another worrying factor is the rapid spread of the disease in the herd. In affected properties it is possible to observe high attack rate, which can reach up to 100% of dairy cows in a herd (Lobato et al., 2005). Few measures in relation to clinical recovery of animals can be taken, such as lesions disinfection to prevent secondary infections.

3.7. Immune response against *Vaccinia virus*

A viral infection can be understood as a competition between replication capacity and transmission of the virus and the mobilization of immune system to protect the host and to eradicate the pathogen. For this reason, the severity of disease and recovery from infection are related both to the infectious agent and the host immune response (Stanford et al., 2007).

The immune response in poxviruses infections led to protection through antibodies that help the control of infection by various mechanisms, such as virus neutralization, complement system activation, cytotoxicity and opsonization. The resolution of infection is associated with the activation of specific $CD8^+$ lymphocytes that attack the remaining infected cells (Panchanathan; Chaudhri; Karupiah, 2008).

The size and complexity of poxviruses make a significant target for the host immune response. Poxviruses have developed an array of molecules that are encoded by virulence genes and designed to directly subvert the defenses mounted by the host. These proteins can be classed into two groups based on whether they act: intracellularly or extracellularly (Seet et al., 2003).

Virotransducer proteins act intracellularly and function to interfere with the response to infection within the cell, including the induction of an antiviral state, the oxidative burst and apoptotic pathways. Virostealth proteins also act intracellularly and reduce the likelihood of detection by the immune system through downregulation of immune recognition molecules, such as major histocompatibility complex (MHC) class I and $CD4^+$. Viral proteins that act extracellularly, classed as viromimics, function to modulate the immune response and can be further classified into viroreceptors and virokines. Viroreceptors are secreted or cell surface glycoproteins that act to competitively bind host cytokines and chemokines thus interfering with their function. In contrast, virokines are viral mimics of host cytokines, chemokines and growth factors that act to subvert the host responses, which are detrimental to virus survival and to promote those responses that are favorable for viral replication and spread (reviewed by Stanford et al., 2007).

After VACV infection, a strong antibody response is generated. In mice, low levels of antibodies are identified up to the 7^{th} DPI. Then, high levels of IgM and multiple IgG isotypes are present from the 14^{th} DPI (Spriggs et al., 1992). Neutralizing antibodies are identified at the 20^{th} DPI and persist for more than three months (Xu et al., 2004; Coulibaly et al., 2005).

Our group has been studying the VACV infection in bovines. Serological studies of naturally infected dairy cows and their suckling calves identified that the peak of IgG and neutralizing antibodies occurs within the first month of infection, and, even at lower titers, they were detectable up to one year after the occurrence of outbreaks in the farms (Matos, 2012).

In lactating cows, after infection with VACV strain GP2, antibody response is generated up to the 10^{th} DPI, and by the 16^{th} DPI, neutralizing antibodies can be detected and peak at the 40^{th} DPI. IgG1 were detected primary than IgG2, and persisted in higher levels until the 20^{th} DPI (Matos, 2012). B cells and $CD4^+$ lymphocytes activation is significantly elevated post-infection, mainly in the 30^{th} DPI. The frequency of both T cells memory populations, $CD45R0^+CD3^+CD4^+$ and

$CD45R0^+CD3^+CD8^+$, was higher at the 30th DPI when compared to the day of infection. (Guedes et al., unpublished data).

Experimentally infected and post re-infected cows showed BV lesions. However, the lesions were milder and the BV clinical course was shorter, with complete healing up to the 10th DPI, when compared to that observed during the first inoculation (22nd – 32nd DPI). In addition, two cows infected 240 days before re-infection, which did not have antibody titers in day 0, had lesions that were more severe than the cows that were re-infected and had circulating antibodies (Rehfeld et al., 2013).

Considering humans vaccinated with attenuated VACV vaccines, memory B cells against VACV are generated after vaccination. Strong $CD8^+$ and $CD4^+$ lymphocytes responses can be detected from the 7th DPI. A further decline and stabilization in $CD8^+$ memory levels are identified after the 30th DPI, which maintains stable for more than 300 DPI. The $CD4^+$ memory cells levels against VACV kept stable up to seven months post infection, and most of $CD4^+$ effector cells produced IFN-γ. However, heterogeneity in this population has been identified with some cells producing TNF and/or IL-2, featuring a predominantly Th1 response (reviewed by Amanna; Slifka; Crotty, 2006).

There are few reports in the literature addressing the cellular immune response in human patients infected with BRA-VACV (Silva-Fernandes et al., 2009; Trindade et al., 2009; Gomes et al., 2012). Trindade and colleagues (2009) showed that a patient who presented typical lesions of bovine vaccinia, with the confirmed diagnosis of VACV, had a lower immune response to the virus. The PBMCs of this patient showed less proliferation rates and lower production of cytokines following stimulation with VACV *in vitro* when compared to PBMCs from control uninfected patients. Notably, the infected patients had IFN-γ production that was significantly lower than uninfected controls, which differs from that reported for other orthopoxviruses. Furthermore, T lymphocytes showed a reduced expression of cellular activation markers such as CD25, after stimulation *in vitro* with VACV, compared to T lymphocytes from uninfected patients.

After the first VACV infection, protection against diseases caused by orthopoxviruses is heavily dependent of antibody response, either in animal or human models (Galmiche et al., 1999; Hammarlund et al., 2003; Amanna; Slifka; Crotty, 2006). Edghill-Smith and colleagues (2005) demonstrated that the antibody response is necessary and sufficient for protection against *Monkeypox virus* in animals previously immunized with VACV vaccine.

Most of the immune response mechanisms against poxviruses is directed against the surface proteins of the two viral forms (MV and EV). MV virions are the most abundant forms in infected cells and it is believed to be the main form related to viral spread among hosts. The EV form relates to viral progeny spread within the infected host (Smith and Kotwal, 2002). In that way, antibodies against MV act to neutralize infectious viral particles just after infection of the host, and antibodies against EV prevent the spread of viral progeny within the host. Thus, the optimal protection against VACV is acquired when there is a presence of antibodies against proteins of both viral forms. The most important proteins related to the immune response against the MV form MV of VACV are A27, H3, L1, D8 and to the EV form are B5, A33 (Cohen et al., 2011; Matho et al., 2015).

3.8. *Vaccinia virus* vaccines

In 1796, Edward Jenner vaccinated an eight-year-old boy, James Phipps, with the material of a cowpox lesion from the milkmaid Sarah Nelmes. The vaccine was effective since the boy was challenged against smallpox two months later and did not develop disease (Riedel, 2005). Jenner decided to call this new procedure vaccination, derived from "vacca", the Latin word for cow, and "vaccinia" the Latin word for cowpox (Riedel, 2005). Early practitioners used a wide variety of poxviruses sampled from or grown on cows, sheep, horses, goats, pigs, and buffaloes. In 1881, Louis Pasteur proposed that vaccination should be the generic term used for preventive inoculations against any animal or human diseases. Vaccination quickly became widespread and by the 20th century almost all vaccines against smallpox virus contained VACV (Fenner et al., 1988). The origin of VACV has never been elucidated and remains obscure until today, but this virus eventually became the most studied poxvirus and has been used extensively as a research tool.

Throughout the history of smallpox vaccination, several strains of VACV with different degrees of virulence were used around the world. With the creation of the Global Program for Smallpox Eradication by the World Health Organization (WHO), less virulent strains became the official choice for mass vaccination (Rotz et al., 2001).

During the smallpox eradication program, the majority of the vaccine was produced on the skin of live animals including calves, sheep, buffaloes and rabbits. These vaccines are known as the first-generation of VACV vaccines. The New York City Board of Health (NYCBH) strain was used in the Americas and in West Africa. After the virus eradication, Dryvax® vaccine was the only commercially approved smallpox vaccine available for limited use in the United States until 2007. The Dryvax® vaccine was manufactured from the lymph fluid of calves' skin infected with the NYCBH strain (reviewed by Jacobs et al., 2009). Its supply has been replaced by ACAM2000® vaccine, a more modern product manufactured in laboratories by Acambis, now a division of Sanofi Pasteur (Greenberg; Kennedy, 2008).

The Lister or Lister/Elstree strain was developed at the Lister Institute in the United Kingdom. From 1968 to 1971, the Lister strain became the most widely used vaccine strain throughout the world (Rosenthal et al., 2001). Additional vaccine strains included the EM-63 (USSR), Paris strain (France), the Copenhagen (Cop) strain (Denmark), the Bern strain (Switzerland), the Ankara strain (Turkey), the Temple of Heaven and the Vaccinia Tian Tan (VTT) strains (China), and the Dairen strain (Japan) (Sánchez-Sampedro et al., 2015).

Brazil did not launch a national campaign against smallpox until 1962, and in 1966, the Global Program for Smallpox Eradication prompted and renewed a national program. At least four different vaccine strains: Paris, Lister, NYCBH and Malbran might have been distributed within Brazil during the WHO eradication campaign (reviewed by Trindade et al., 2007b). The major smallpox vaccine producer in Brazil was Instituto Oswaldo Cruz in Rio de Janeiro. VACV strain IOC was exclusively used by the Institute to manufacture the vaccine that was widely distributed in Brazil during the eradication campaign (Fenner et al., 1988). The vaccine was produced in calf skin or chicken eggs and usually had "take" rates higher than 98% and low rates of adverse effects. Unfortunately, poor documentation resulted in scant existing information about the origins of the VACV strain IOC (reviewed by Medaglia et al., 2015).

The use of live animals for the production of vaccine material has changed due to current unacceptability of this process and quality control issues regarding microbial contamination. This has led to the production of second generation smallpox vaccines using tissue culture systems or embryonated chicken eggs. ACAM2000™ is a second generation smallpox vaccine licensed for use in the United States as of August 2007. The vaccine strain was derived from plaque purification of a Dryvax® isolate that was subsequently multiplied in the Vero monkey cell line (reviewed by Jacobs et al., 2009).

Substantial work is being directed toward the development of safer, yet immunogenic vaccines. These third generation vaccines involve genetically altering the VACV genome in order to create non-replicating or highly attenuated VACV strains, which still retain their immunizing properties against smallpox. In the case of VACV, there are three current vaccine candidates that have been obtained by passage through alternative hosts, Lister clone 16m8 (LC16m8), Modified Vaccinia Ankara (MVA) and Dairen I Strain (DIs) (reviewed by Jacobs et al., 2009).

In the early 1980s, recombinant DNA technology revolutionized molecular biology, allowing the insertion of foreign DNA into poxvirus genomes. Poxviruses were no longer used only as successful smallpox vaccines, but also as vaccines against a wide range of heterologous diseases. It is of importance that, as all chordopoxviruses have a similar arrangement of genes, interchangeable promoters and conserved RNA polymerase and transcription factors, the principles developed for VACV expression vectors could be applicable to other poxviruses (Sánchez-Sampedro et al., 2015).

Vaccinia vaccines are based in attenuated processes, and as live viruses, the multiplication of the vaccine viruses may be exacerbated in children, elderly or other immunosuppressed patients and may even result in many secondary complications (Rosenthal et al., 2001; Rosenblatt; Stein, 2015). So, an inactivated vaccine becomes a viable alternative with no virus multiplication in the vaccinated patient. Additionally, due to viral inactivation, it does not enable recombination between the vaccine strain and other current poxvirus that might be infecting the same cell (Yao; Evans, 2003; Qin; Evans, 2014) nor the establishment and maintenance of these viruses in nature (Turner; Squires; Murray, 1970).

Several methods for viral inactivation have been developed, including inactivation by UV light, heat, pressure, and chemicals such as formalin, hydroxylamine, beta-propiolactone (BPL), paraformaldehyde and ethylenimine (Turner; Squires; Murray, 1970; Thornton, 1980; Frazatti-Gallina et al., 2004; Capodici et al., 2006). However, it is important to note that some of these viral inactivation methods can lead to protein denaturation, which may result in a less efficient immune response. Viral inactivation using UV light results primarily in destruction of the viral nucleic acid and nucleoproteins, with less destruction of envelope proteins. BPL is a lactone from the beta-hydroxypropionic acid, that reacts with DNA adenine causing mutations and inactivation of viruses, without compromising the structural viral proteins (Turner; Squires; Murray, 1970; Thornton, 1980).

Unlike attenuated vaccines, inactivated vaccines generally require the addition of adjuvants for effective action. The word “adjuvant” is derived from the latin word *adjuvare*, that means to help, assist or improve. Adjuvants act by modulating the immune system; stimulating cytotoxic T lymphocytes; targeting antigens to antigen presenting cells and/or the formation of antigenic deposits, and through sustaining release of antigen the immune system recognition (Mcvey; Shi, 2010).

Regarding the epidemic situation of BV in Brazil, two inactivated VACV vaccines have been already proposed. Ferreira (2008) described the development of inactivated VACV vaccines using as antigen purified VACV BAV strain particles associated to different adjuvants. The vaccine proved to be efficient to protect Balb/c mice immunized and subsequently challenged intranasally and by scarification. However, although there was protection, animals vaccinated and challenged intranasally with the VACV Western Reserve strain still showed some morbidity, marked by pilo-erection and weight loss during the first days post-challenge.

Abrahão (2010) proposed a prime-boost vaccination system using the purified VACV BAV strain inactivated by BPL in combination with a DNA vaccine. As a purified antigen was used in the development of this vaccine, only MV particles were selected during the purification process. However, as a DNA vaccine for protein B5, a major EV protein related to the production of neutralizing antibodies, was used in combination with the inactivated vaccine, neutralizing antibodies against both viral forms were detected and 100% of vaccinated mice were protect against death, although mild clinical signs have been observed.

These researches demonstrated that vaccine formulations using a Brazilian VACV strain inactivated with BPL, and preferably keeping EV and MV proteins, associated to adjuvants, are a promise to be used as a tool to prevent BV in Brazil.

4. Material and Methods

4.1. Experiment Workflow

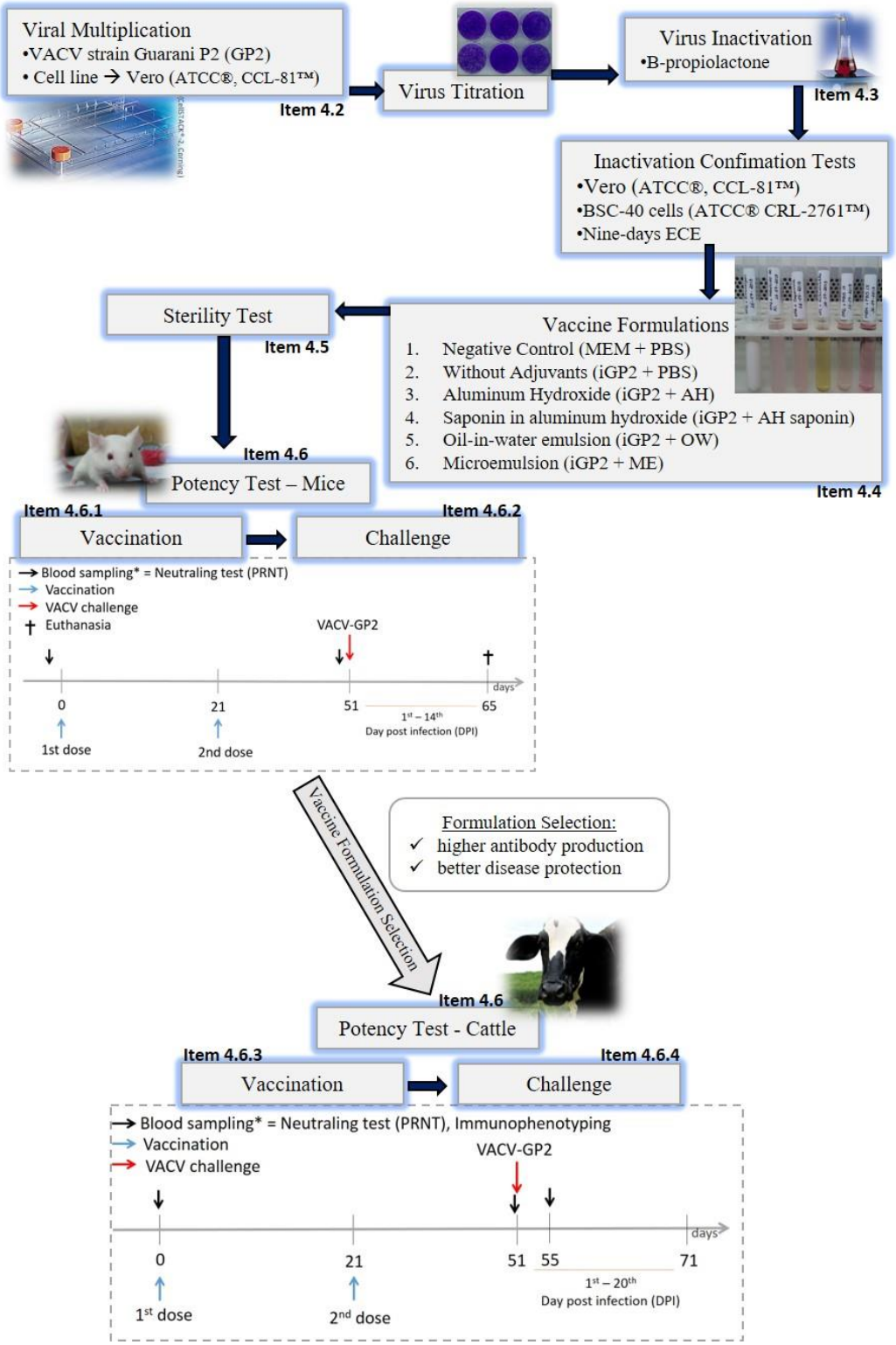

Figure 7 Schematic representation of the workflow of the methodology used in the thesis. The description of material and methods of each work phase is indicated by the numbered items in bold.

4.2. Ethics Approval

This study was approved by the Committee of Ethics in Animal Use from the Universidade Federal de Minas Gerais (CEUA/UFMG, protocol: 123/2012) (Supplement I).

4.3. Cell and Virus

The Brazilian VACV strain GP2, isolated from scabs of affected cow, biologically and genetically characterized as a Group 1 Brazilian VACV was used as viral antigen (Trindade et al., 2006). The VACV GP2 was cultivated in Vero cells (ATCC®, CCL-81™).

Vero cells were grown in 1272cm² flasks (CellSTACK®-2, Corning) in minimum essential Eagle medium (MEM) (Gibco™, Thermo Fisher Scientific, USA) containing 5% of heat inactivated fetal calf serum (FCS), potassium penicillin (100U/ ml) and streptomycin (100 μg/ ml). For infection, Vero cell monolayers with 90% cell confluence were washed twice with phosphate buffered saline (PBS) (NaCl 137mM; KCl_2 7mM; Na_2HPO_4 1.4mM; pH 7-7.2) and inoculated with the VACV GP2 at 0.01 multiplicity of infection (MOI) and incubated for 1h at 37ºC and 5%CO_2. After incubation, 250 ml of MEM supplemented with 1% FCS, penicillin (100 IU/ ml), streptomycin (100 μg/ml) and amphotericin B (2.5 μg/ ml) were added in each flask. Then, the flasks were kept under incubation at 37ºC and 5%CO_2 for 48h-72h, until a 90-95% of cytopathic effect was observed.

The viral suspension was frozen and thawed, pooled and stored at -80ºC in 50 ml aliquots. The aliquots were sonicated twice at pulse 3 and amplitude 50 for 30 seconds, between 60 seconds interval. The virus suspension was clarified by centrifugation at 3320xg for 15 minutes. An aliquot of the clarified virus suspension was collected for virus titration (Mota et al., 2011). The viral titer was expressed as plaque forming units per ml (PFU/ml).

4.4. Virus Inactivation

Pooled clarified VACV-GP2 supernatant was inactivated by adding 1% of beta-propiolactone (BPL) (Sigma-Aldrich®, Germany). The solution was stirred for two hours at 37ºC. The pH was monitored using a pH meter (Digimed, MD-20) and adjusted to pH 7.2-7.4 by addition of sterile K_2HPO_4 (1M), when necessary. Then, the solution was kept stirring at 25ºC for two hours. After the period of inactivation and hydrolysis of remaining BPL, the inactivated virus was stored at 4ºC (Thornton, 1980; Frazatti-Gallina et al., 2004).

To confirm the viral inactivation, the clarified VACV-GP2 inactivated suspension was inoculated in Vero and BSC-40 (ATCC® CRL-2761™) monolayers cells and embryonated chicken eggs (ECE) chorioallantoic membranes.

Monolayers of Vero and BSC-40 cells at a concentration of 50,000 cells/cm^2 were prepared on 6-wells plates and inoculated with 200 uL of the clarified VACV-GP2 inactivated suspension and a 1:2 PBS diluted solution in triplicate and kept under incubation at 37°C and 5% CO_2 for 1 hour. After the incubation period for possible remaining viral adsorption, 1.5 ml of MEM supplemented with 1% FCS, penicillin (100 IU/ ml), streptomycin (100 μg/ml) and amphotericin B (2.5 μg/ ml) were added. The plates were then incubated at 37°C, 5% CO_2 for 96 hours and the monolayer observed daily for cytopathic effect identification. Three consecutive passages of the supernatant were carried out.

Additionally, 100µl of the clarified VACV-GP2 inactivated solution were inoculated in the chorioallantoic membrane of nine days old ECE (Thornton, 1980). The inoculation was performed in triplicate. After incubation for 72 hours the ECE chorioallantoic membranes were collected and homogenized at a dilution of 1:9 in PBS. The solution was inoculated in BSC-40 cell monolayer for three consecutive passages, as previously described.

4.5. Vaccine Formulations

The vaccine formulations were prepared to present a final viral titer of $2x10^7$ PFU/ml, based on virus titration before inactivation. For that, the clarified BPL inactivated suspension of VACV strain GP2 was combined with different adjuvants. Negative vaccine control was prepared with the addition of MEM in substitution of the inactivated GP2 (iGP2) solution. The vaccine antigen control, in the absence of adjuvants, was prepared with the clarified inactivated virus suspension diluted in PBS. A total of six vaccines were formulated: MEM + PBS; iGP2 + PBS; iGP2 + aluminum hydroxide (AH); iGP2 + AH/saponin; iGP2 + oil-in-water (OW) emulsion; iGP2 + microemulsion (ME).

4.5.1. Aluminum hydroxide based vaccine

To formulate the aluminum hydroxide (AH) adjuvant based vaccine it was used the low-viscosity AH gel (Rehydragel LV® - Reheis, USA). Following the manufacturer instructions, the vaccine was composed of 10% of the AH gel. In the class II biosafety cabinet, the iGP2 solution was diluted in the PBS in a beaker containing a sterile magnetic bar. Then the AH gel was added, and the mixture was maintained stirring for 24 hours, at room temperature. At the end of the process, the vaccine was aliquoted in 100ml flasks, air-tight and stored at 4ºC until use.

4.5.2. Saponin in aluminum hydroxide based vaccine

The saponin in AH adjuvanted vaccine was prepared using a saponin adjuvant extracted from *Quillaja saponaria* (QSA® - Beraca, Brazil). For vaccine formulation, a final concentration of 10% of AH gel (Rehydragel LV® - Reheis, USA) and 40% of saponin adjuvant solution (2.5 mg/ml in PBS) were used. First, in the class II biosafety cabinet, the iGP2 solution was diluted in the PBS in a beaker containing a sterile magnetic bar. After homogenization, the AH gel was added, and the mixture was maintained stirring for 24 hours, at room temperature. Then, the saponin adjuvant solution, previously sterilized by filtration through a 0.22-micron filter, was added and kept stirred for 30 minutes. At the end of the process, the vaccine was aliquoted in 100ml flasks, air-tight and stored at 4ºC until use.

4.5.3. Oil-in-water emulsion based vaccine

The oil-in-water (OW) emulsion based vaccine was formulated using the Emulsigen® (MVP Technologies, USA) and the low-viscosity AH gel (Rehydragel LV® - Reheis, USA). The formula used was composed of 5% of the AH gel and 25% Emulsigen®. In the class II biosafety cabinet, the iGP2 solution was diluted in the PBS in a beaker containing a sterile magnetic bar. Then the AH gel was added and the mixture was kept at room temperature, under stirring, for one hour. After the homogenization, the Emulsigen® was added and stirring was continued for 1 hour at room

temperature. At the end of the process, the vaccine was aliquoted in 100ml flasks, air-tight and stored at 4ºC until use.

4.5.4.Microemulsion based vaccine

The microemulsion (ME) was prepared according to the titration with the co-surfactant (Leclercq et al., 2011). First, it was obtained a simple oil-in-water emulsion with a high concentration of surfactant. Then a co-surfactant was added until the microemulsion formation, characterized by its transparency. The microemulsion based vaccine was prepared with a final concentration of 30% Tween 80 (w/v), 5% isopropyl myristate (w/v) and 5% propylene glycol (w/v).

Under aseptic conditions, in the class II biosafety cabinet, the isopropyl myristate and Tween 80 were homogenized using a glass rod. Then, half of the volume of iGP2 solution diluted in PBS was dispersed in the oil phase using an Ultraturrax (IKA ® Ultra-Turrax T10 Basic, S10N - 10G), set at speed 6 for 15 minutes to form a single emulsion. Then, the co-surfactant (propylene glycol), with the rest of the diluted viral suspension was added to the single emulsion previously prepared and the homogenization was continued for another 25 minutes at the same speed until a transparent system was formed, indicating the formation of microemulsion. At the end of the process, the formulation was aliquoted in 100ml flasks, air-tight and stored at 4ºC until use.

4.6. Sterility Test

The sterility of the vaccines was evaluated by the aseptic transfer of 1 ml of each vaccine formulation in test tubes containing 5 ml of fluid Sabouraud and thioglycolate culture media, followed by incubation at 35-37°C for 14 days, with daily readings (OIE, 2012). The test was conducted in quadruplicate for each culture medium. Additionally, the tubes were kept under incubation temperature for up to 21 days.

4.7. Vaccine potency and efficacy tests *in vivo*

Potency tests were performed in Balb/c mice and the efficacy of the vaccine was performed in the target species, bovine. The formulated vaccines using different adjuvants were previously tested in Balb/c mice, using two-doses of vaccine application, serological tests and challenge with the homologous virus. According to the mice experiment results, the vaccine formulation that resulted in better protection was used for cattle immunization, followed by challenge.

All the animal experiments were carried out in accordance with regulations and guidelines of the CEUA/UFMG. The animal experiments were performed as described below.

4.7.1.Mice vaccination

Each vaccine formulation was tested in groups of ten four-weeks-old males Balb/c mice, from the Biotério Central, UFMG. The animals were vaccinated twice with intervals of 21 days, through the subcutaneous route. Vaccine doses of 100 µl, containing at least 2 x 10^6 PFU were applied.

The animals were kept for 30 days after the last dose of vaccine in cages and provided with commercial mouse food and water *ad libitum*. Serum samples were collected 25 days after the second vaccination.

The experiments were conducted at the Laboratório de Produção de Soros e Vacinas, Veterinary School, UFMG.

4.7.2.Mice VACV challenge

Thirty days after the second vaccination, Balb/c mice were challenged with the homologous vaccine virus strain, the VACV GP2. Before the procedure, mice were anesthetized by intraperitoneal injection of ketamine and xylazine (0.1mg/g e 0.001mg/g, respectively). Following anesthesia, both footpads were scarified using needles, forming ten horizontal scratches. Then, the left footpad was inoculated with 10 µl of viral suspension containing 10^5 PFU and the right footpad was inoculated with MEM, and used as negative control inoculation (Ferreira et al., 2009).

After challenge, the animals were weighed every other day and clinical signs were recorded daily. The clinical course was evaluated observing the main clinical signs that might occur, suggestive of VACV infection. A score was created, according to the evidence of VACV characteristic lesions. The presence of each type of lesion was scored as: edema =1; pustule =2; papule, vesicle or scabs =3. The absence of clinical signs was considered zero. The sum of score lesions observed by day, in all animals from each vaccination group was used as disease score. The group vaccinated with the control formulation (MEM + PBS) was considered the control group of infection. On the 15^{th} day post challenge (DPC), the mice were euthanized.

4.7.3.Cattle vaccination

The vaccine efficacy in cattle was carried out using the vaccine formulation that demonstrated the better performance for development of immune response and protection against clinical signs in mice. The induction of immune response was measured by neutralizing antibody titers in sera of the vaccinated mice, and disease protection was evaluated by the lowest recorded disease score after challenge.

The adjuvant control formulation was prepared similarly according to the vaccine, replacing the vaccine antigen by MEM. As negative control, a solution containing MEM replacing the viral antigen diluted in PBS was used, which replaced the adjuvant in this formulation.

Twenty-four cross-breed heifers, between 12 and 15 months old, seronegative for orthopoxviruses were previously identified by earring ID tags, and then randomly divided in three groups according to the vaccine formulation: negative control, adjuvant control and vaccinated. The animals were fed in pasture, supplemented with silage and mineral salt. This experimental phase was conducted at the Fazenda Modelo – UFMG, located in Pedro Leopoldo, Minas Gerais.

The heifers were vaccinated twice with interval doses of 21 days, through the subcutaneous route. Vaccine doses of 5ml, containing at least 10^8 PFU relative to the VACV strain GP2 titer before viral inactivation, were applied in the injection triagle, in the neck.

Blood samples were collected in vacuum tubes without anticoagulant and in anticoagulant tubes containing heparin (BD Vacutainer®) before the experiment and 30 days after the second dose of vaccination.

4.7.4.Cattle VACV challenge

To perform the VACV challenge, four heifers from the vaccinated group and four heifers from the negative control group were randomly selected.

Thirty days after the second vaccination dose, the randomly selected heifers were housed in microbiologically secure (NB-2) animal pens with dimensions 3,5m x 3,5m separated by wall, at the National Agricultural Laboratory (Laboratório Nacional Agropecuário – LANAGRO) from the Ministry of Agriculture, Livestock and Food Supply (Ministério da Agricultura, Pecuária e Abastecimento) in Pedro Leopoldo, Minas Gerais, Brazil. The animals were fed a complete balanced diet and water ad libitum. The experimental area is fenced, with restricted access. The waste (stools and bed) were disposed in 3m x 2.5m pits, while effluents from washing the pens were discarded in an exclusive septic tank for the stalls located within the experiment area.

For VACV challenge, the heifers were anesthetized by subcutaneous injection of xylazine (1mg/100kg) before the procedure. First, the teats and udder were cleaned with neutral detergent, followed by disinfection with ethanol 70% (v/v). The teats of each cow had their epidermis abraded with ten horizontal movements using a sandpaper (JX-91 (50) Sandpaper Doble A, Argentina). Then, the teats were inoculated with 50uL of a VACV GP2 viral suspension at 10^7 PFU/ml (Rehfeld et al., 2013).

After the VACV challenge, the animals were monitored daily up to 20 days. The BV clinical course was evaluated by the presence of the main clinical signs observed in VACV infection in bovines. A score was created according to the evidence of VACV infection characteristic lesions. The presence of each type of lesion was scored as: edema =1; pustule and healing =2; papule, vesicle, ulcer or scabs =3. The absence of clinical sign was considered zero. The sum of score lesions observed by day, in all animals from each vaccination group was used as disease score.

Blood samples were collected in vacuum tubes without anticoagulant or in anticoagulant tubes containing heparin (BD Vacutainer®) on the inoculation day and 4^{th} DPC.

4.8. Serological assay

4.8.1. Plaque reduction neutralizing test

Neutralizing antibodies (NA) assay was performed by plaque reduction neutralizing test (PRNT). Serum samples were preheated at 56°C for 30 min, diluted by 2-fold serial dilution (1:20 – 1:1280) and then incubated at 37°C for 60 min with equal volume of VACV-GP2 suspension containing 150 plaque form units (PFU). Serum/virus solutions were inoculated into confluent VERO cells monolayer on 6-well plates. The inoculum was adsorbed for 1 hour at 37°C, followed by the addition of 1.5 ml of maintenance medium. Virus inoculum, bovine positive and negative sera samples for antibodies against VACV obtained during BV outbreaks were used as controls (Lobato et al., 2005). After 72 hours at 37°C in a humidified atmosphere with 5% CO_2, the plates were stained with crystal violet, and the plaques were counted. The PRNT titer was defined as a reciprocal of the serum dilution resulting in 50% plaque reduction when compared with virus control. Titers less than 20 were considered negative. The NA titers were expressed in $\log_2$.

4.9. Cellular immune response

Bovine blood was collected in heparinized tubes before vaccination and 30 days after the second dose of vaccination. In the 4th DPC, blood samples were also collected for cellular immune response analysis. These samples were collected and stored between 18 to 24 hours at 4°C, until the peripheral blood mononuclear cells (PBMCs) separation process.

4.9.1. Peripheral blood mononuclear cells (PBMCs) separation

The PBMCs were separated using Histopaque®1077 (Sigma-Aldrich®, Germany) though density gradient (SOUZA et al., 2007). Briefly, heparinized blood was diluted to a proportion of 1:2 in PBS and applied onto the Histopaque® layer. The mixture was centrifuged at 1475xg for 40 minutes at room temperature. After centrifugation, the PBMCs were collected and washed three times with PBS by centrifugation at 830xg for 10 minutes at 4°C. After washing, the cells were resuspended in RPMI (GIBCO™, Life Technologies) supplemented with L-glutamine (1 mM), penicillin (100 IU/ ml), streptomycin (100 μg/ml) and amphotericin B (2.5 μg/ ml) to a final concentration of 10^7 cells/ml.

4.9.2. Peripheral blood mononuclear cells (PBMCs) *in vitro* stimulation

The PBMCs were stimulated by the viral antigen in 96-wells round bottom plates. First, $3x10^5$ cells were added to each well and incubated with 0.1 MOI of purified VACV GP2 at 37°C, 5% CO_2 for 1 hour. The non-stimulated cells were incubated with MEM, and used as control cells. After incubation, 150 μL of RPMI supplemented with 5% FBS, L-glutamine (1 mM), potassium penicillin (200 UI/ml), streptomycin (200 μg/ml) and amphotericin B (2.5 μg/ ml) were added and the plates were incubated at 37°C, 5% CO_2 for 12 hours.

4.9.3.Immunophenotyping markers

After incubation, plates were centrifuged at 830xg for 8 minutes under refrigeration (4°C). The supernatant was collected and the cells incubated with monoclonal antibodies conjugated with different fluorochromes (Table 1).

Table 1 Monoclonal antibodies conjugated with different fluorochromes used for phenotypic cell labeling.

MOLECULE MARKER	CELL PHENOTYPE FUNCTION	ANTIBODY CLONE	TARGET SPECIES	HOST	ISOTYPE	FLUOROCHROME	MANUFACTURER
CD3	T lymphocytes	MM1A	cattle	mouse	IgG1	-	VRMD
CD4	T helper lymphocytes	CC8	cattle	mouse	IgG2a	Alexa 647	AbD Serotec
CD8	Cytotoxic T lymphocytes	CC63	cattle	mouse	IgG2a	Alexa 647	AbD Serotec
CD21	B lymphocytes	CC21	cattle	mouse	IgG1	FITC	AbD Serotec
WC1	γ/δ T lymphocytes	CC15	cattle	mouse	IgG2a	FITC	AbD Serotec
CD25	Activation	IL-A111	cattle	mouse	IgG1	RPE	AbD Serotec
CD45R0	Memory	GC42A1	cattle	mouse	IgG1	-	VMRD
IgG1	Secondary antibody (marker)	A85-1	mouse	rat	IgG1	PE	BD

Cell populations were analyzed in a flow cytometer (FACSCanto II, Becton & Dickinson, USA) using the BD FACSDiva™ software (Becton Dickinson, USA). Data was analyzed using the FlowJo® version 10.1 software (TreeStar Inc., USA).

The lymphocyte population was determined based on cell size (FSC) and granularity (SSC) features observed in the flow cytometer acquisition. Then, according to the antibody and fluorochrome marker, the cell immunophenotyping was evaluated (Figure 8). To check the lymphocyte population gate, as a strategy, $CD3^+$ cells and $CD21^+$ were checked on the histogram by fluorescence, confirming the majority of $CD3^+$ and $CD21^+$ localized in the previous gate FSC x SSC as lymphocytes (Figure 9).

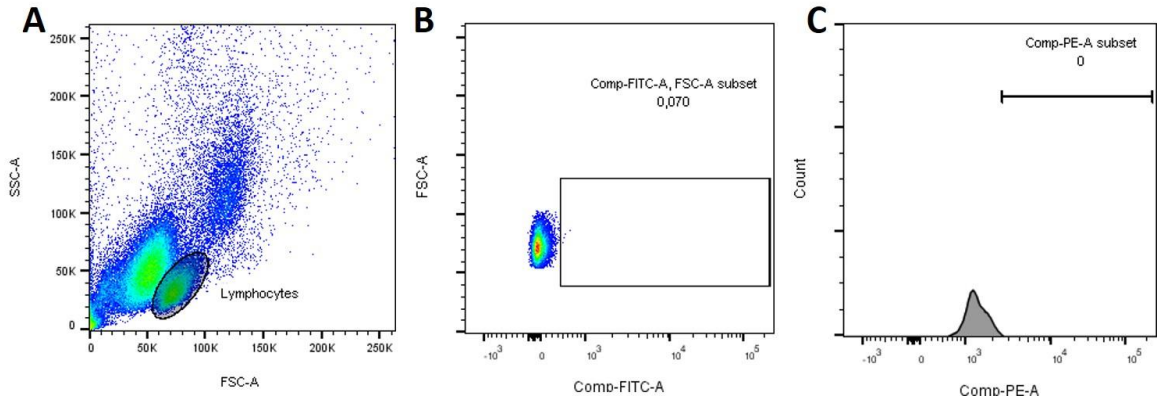

Figure 8 Gating strategies used to select specific lymphocytes subpopulations. A - The lymphocytes were selected based on their size (FSC) and granularity (SSC) flow cytometric features. B - Inside the lymphocyte gate, the lymphocytes subpopulations were analyzed according to the fluorochrome stained in the corresponding lymphocyte phenotype ($CD4^+$, $CD8^+$, $CD21^+$). C – Lymphocytes subpopulations expressing the activation marker (CD25) or memory marker (CD45R0) were then counted and quantified the mean intensity of fluorescence (MIF).

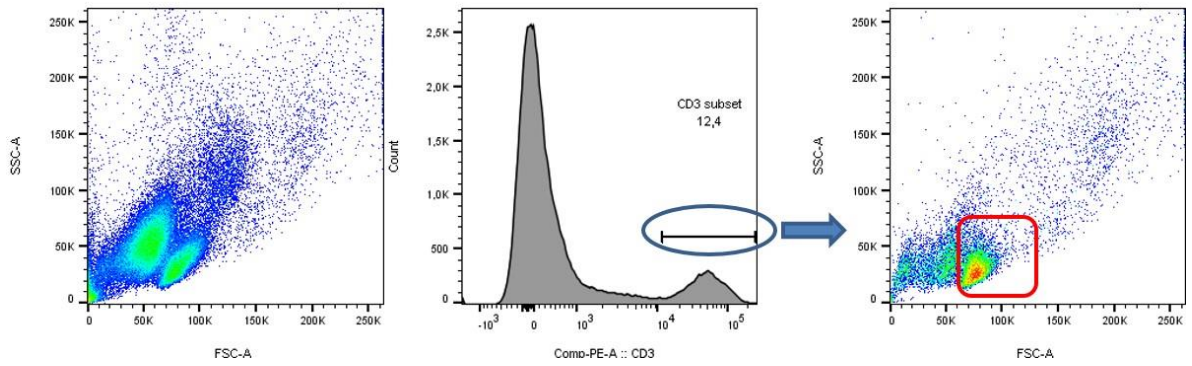

Figure 9 Gating strategies used to confirm the lymphocytes population gate. The lymphocytes were selected on histogram based on the phenotyping lymphocyte T (CD3+) or B (CD21+). Then, the size (FSC) and granularity (SSC) on flow cytometric features were checked.

4.10. Statistics

Data distributions were analyzed for normality by Shapiro-Wilk tests. Kruskal-Wallis test, with 95% confidence interval, complemented by Dunn´s multiple comparison test was used to compare the neutralizing antibody titers among vaccinated groups and the disease score observed among vaccinated groups of mice comparing to the infection control group.

Mice weights were compared among the vaccinated groups and the days after challenge, using two-way ANOVA, complemented by multiple comparisons using the Tukey test.

For bovine immunophenotyping variables, comparisons were performed through two-way ANOVA test, followed by the SIDAK´s multiple comparisons test. Bovine disease score data statistical analysis between control and vaccinated group after challenge was performed by Mann-Whitney test.

Results were considered significant when $p < 0.05$. Prism GraphPad program (version 6.01; GraphPad Software, USA) was used for statistical analysis and graphs drawing.

5. Results

5.1. Vaccine formulations

The produced VACV GP2 viral suspension was titrated and subsequently inactivated by BLP. Viral inactivation was confirmed after three passages in Vero cell culture and BSC-40. Additionally, the inactivated viral suspension was inoculated in chorioallantoic membrane of nine days-old ECE. No viral replication was observed in any of the isolation systems. After confirmation of the viral inactivation, the vaccines were formulated to present at least $2x10^7$ PFU/ml of vaccine.

The production processes of AH, saponin and OW emulsion based vaccines were simple and fast to execute. For the ME based vaccine production, it was required more equipment, but it was also simple. The visual aspects of the AH, saponin and ME based vaccines were fluid, homogeneous and clear, and offered no resistance in the syringe at the time of application. The OW emulsion vaccine presented cloudy and milky appearance, and during vaccination showed a slight resistance to the syringe (Figure 10).

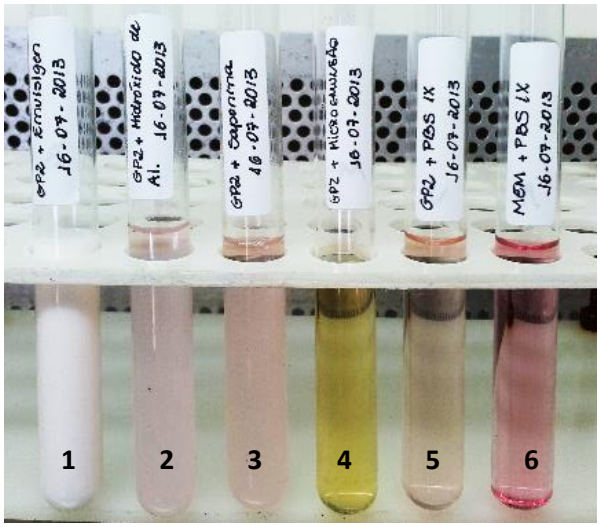

Figure 10 Visual appearance of the vaccine formulations produced. 1- iGP2 + OW emulsion; 2- iGP2 + AH; 3- iGP2 + AH saponin; 4- iGP2 + ME; 5- iGP2 + PBS; 6- MEM + PBS.

5.2. Vaccines potency test in Balb/c mice

After the first dose of the vaccines, a papule was observed through palpation at the vaccination site in all mice vaccinated with the saponin and OW emulsion vaccine formulations. The nodules could be felt through palpation up to the 15th day after application, then they regressed. The same was observed after administration of the second dose of these vaccines. Any other local or systemic adverse reaction was not observed, demonstrating the safety and innocuousness of the vaccines.

The first point analyzed to select an effective vaccine was the immune response elicited by each vaccine formulation. Neutralizing antibodies titers after vaccination were evaluated on the 25th day after the second vaccine dose. To test the ability of sera to neutralize virus, purified VACV GP2 were incubated with serially diluted sera of immunized mice, and titers of neutralizing antibodies were

determined by PRNT.

Figure 11 shows neutralizing antibody titers by animal. Neutralizing antibodies were not detected in the serum of animals from the negative control group (MEM + PBS) neither in the adjuvants control group (iGP2 + PBS). From the iGP2 + AH vaccine group, neutralizing antibodies were detected in the serum of only one mice, at low titer (20; log_2 = 4.32). Considering the different vaccine formulations, iGP2 + OW and iGP2 + AH saponin showed the highest number of seroconverted animals. In the iGP2 + AH saponin group, all animals seroconverted and 8 out of 10 had neutralizing antibody titers equal or above 80 (log_2 = 6.32).

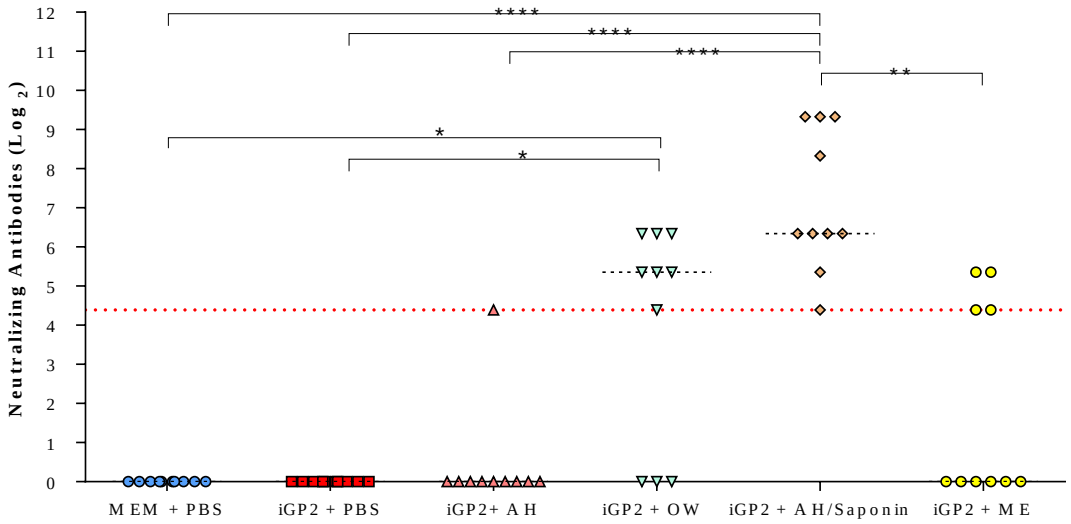

Figure 11 Neutralizing antibodies titers of Balb/c mice at the 25th day after the second vaccination. The antibody titers were transformed in log_2 and data are presented as scatter dot plot, representing each animal. Horizontal dotted lines indicate the neutralizing antibodies median for each vaccine formulation group. The red horizontal dotted line indicates the minimum neutralizing antibody titer (20; log_2 = 4,31) considered positive. Statistical difference by Kruskal-Wallis, followed by Dunn´s posttest analysis (*) p <0.05; (**) p <0.01; (****) p <0.0001.

Clinical disease protection elicited by the formulated vaccines was evaluated through the challenge phase, when the footpads were scarified and inoculated with VACV GP2 on the 30th day after the second vaccine dose. The animals were examined daily and weighed every other day, until the 15th DPC. No significant weight loss among challenged groups was detected throughout the study period.

Figure 12 summarizes the main lesions observed on footpads of the mice inoculated with VACV GP2. All mice from control group, vaccinated with the MEM + PBS formulation, became ill, presenting clinical signs of VACV infection. Edema was observed in inoculated footpads from the 1st to the 9th DPC. From the 3rd DPC, papules which evolved into vesicles from the 5th DPC were recorded. Four animals presented the evolution of the vesicles to pustules from the 7th DPC. Also from the 7th DPC scabs were forming and persisted in two out of the ten animals until the 14th DPC. The healing process was observed from the 11th DPC in the other mice from the same group.

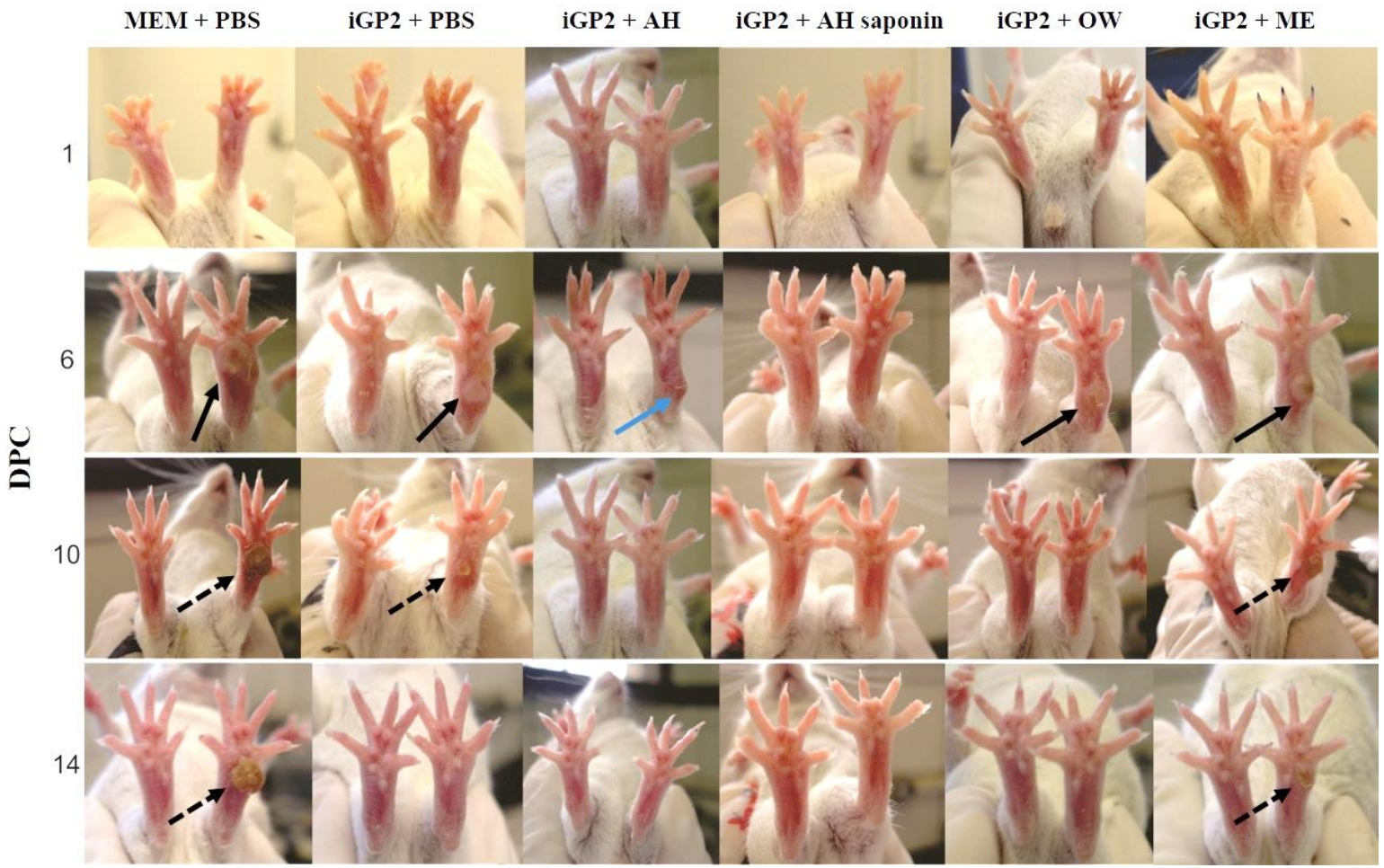

Figure 12 Panel of images of the footpads of Balb/c mice vaccinated and challenged with VACV strain GP2, at the 30th day post the second vaccination dose. Representative images from each vaccinated group at the 1st, 6th, 10th and 14th day post challenge (DPC) were selected. The mice´s right footpad was scarified and inoculated with MEM, and used as inoculation negative control. The left footpad was scarified and inoculated with 10^5 PFU of VACV strain GP2. Blue arrow indicates papule. Black arrows indicate vesicles. Black dash arrows indicate scabs formation.

Animals from iGP2 + PBS group presented similar clinical course as the control group, differing only in the absence of pustular lesions. No lesions were observed in four out of ten mice from the iGP2 + AH and iGP2 + ME groups, the other six mice from each group developed the disease similar to the recorded from the group iGP2 + PBS.

Characteristic VACV lesions were not evidenced in six out of the ten inoculated mice from the iGP2 + OW emulsion group. Two of them presented papules that regressed up to the 7th DPC. The other two animals developed the disease with the appearance of vesicles that evolved to pustules until scabs' formation. Then, these lesions healed quickly from the 8th DPC.

The group vaccinated with the vaccine formulation iGP2 + AH/saponin developed only edema in the first two days after viral inoculation, which was observed only in four from the ten inoculated mice. No vaccinated mice with this vaccine formulation developed characteristic lesions of VACV infection (Figure 12).

After challenge, the clinical signs were recorded daily. The clinical course was evaluated by presence of the main clinical signs observed in VACV infection. It was created a score, according to the evidence of VACV infection characteristic lesions. The sum of score lesions observed by day, in all animals from each vaccine group was used as disease score. The group vaccinated with the control formulation (MEM + PBS) was considered the control group of infection. Figure 13 represents the disease scores observed in each vaccinated group from the 1st to the 14th DPC, except the 12th DPC, when there was not clinical examination.

Statistical differences were observed when comparing the control group (MEM + PBS) with the iGP2 + OW ($p < 0.0.5$) and the iGP2 + AH/saponin ($p < 0.0001$) vaccinated groups. Although both vaccines conferred protection against the disease clinical course, it needs to be highlighted that iGP2 + AH/saponin vaccine protected 100% of the animals previously vaccinated and challenged with VACV-GP2 (Figure 13).

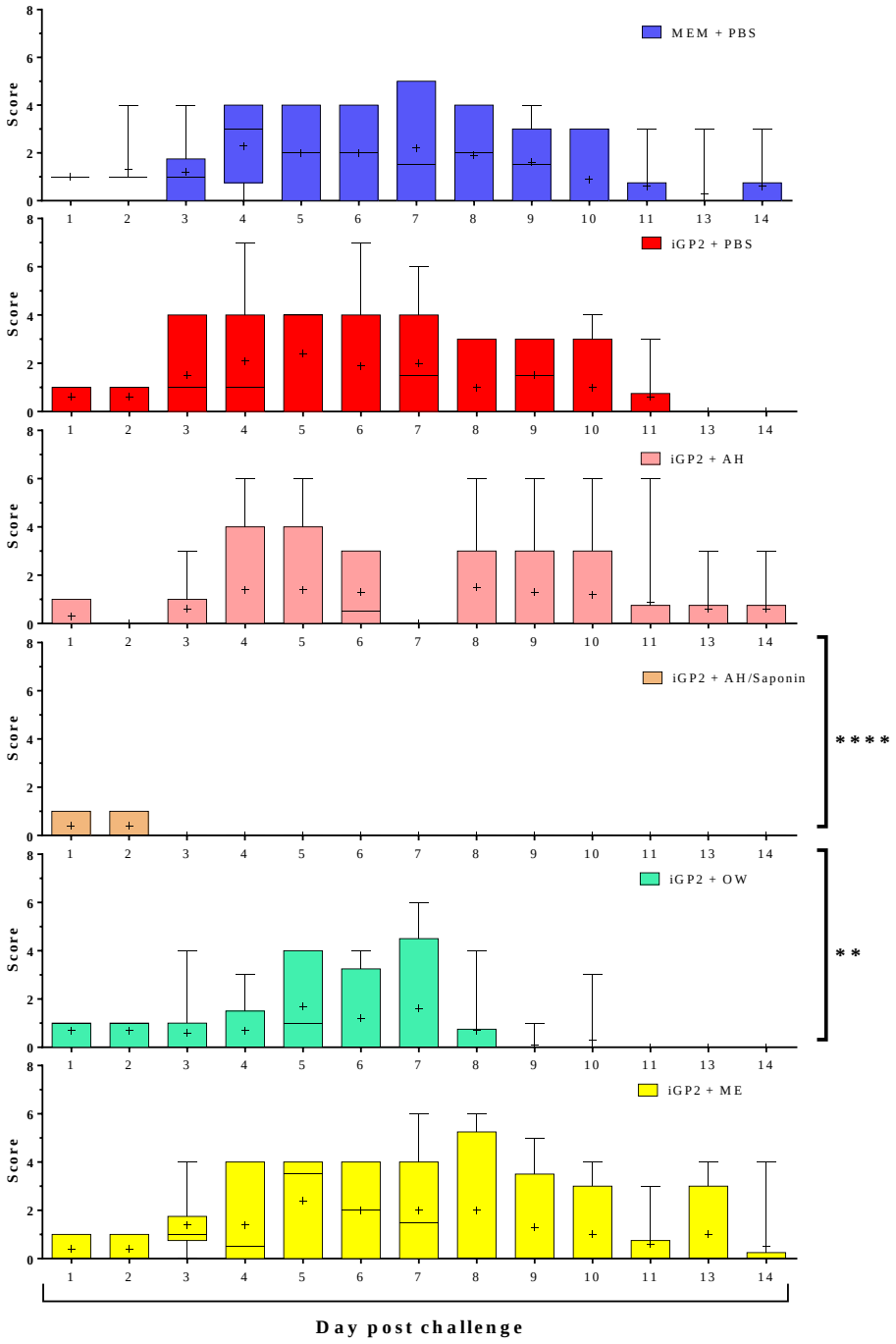

Figure 13 Box plot representation of VACV infection disease score recorded in each Balb/c mice vaccinated group, from the 1st to the 14th day post challenge (DPC) with VACV strain GP2. Horizontal bars indicate the median of disease score in each DPC. (+) Indicates the mean of disease score in each DPC. Boxes represent 5-95 percentile and vertical bars the maximum and minimum recorded disease score. The animals were not examined in the 12th DPC. (**) $p < 0.01$; (****) $p < 0.0001$ indicates statistical difference by Kruskal-Wallis test followed by Dunn´s posttest analysis when comparing with the challenge control group (MEM + PBS).

5.3. Vaccine efficacy in cattle

According to the mice experiment results, the vaccine formulation that resulted in higher antibody production and better disease protection was used for cattle immunization, followed by challenge with the vaccine antigen homologous virus. The iGP2 + AH saponin formulation was selected to be tested in bovine, as this vaccine demonstrated more intensive neutralizing antibody response in BAlb/c mice (Figure 11) and protected against clinical signs 100% of the mice challenged with VACV-GP2 as demonstrated by measurement of the disease score (Figure 12 and 13).

Before the experiment procedures, blood was collected for anti-orthopoxvirus neutralizing antibody investigation. All animals used in the experiment were negative through PRNT. Neutralizing antibody immune response was evaluated at the 30th day after the second dose of vaccination. Figure 14 shows the neutralizing antibody titers detected in each animal serum. As expected, the animals belonging to the control groups were negative by the PRNT. Neutralizing antibodies were detected in the serum of all vaccinated animals. The vaccinated heifers' antibody titers varied from 20 (log_2 = 4.32) to 320 (log_2 = 7.32).

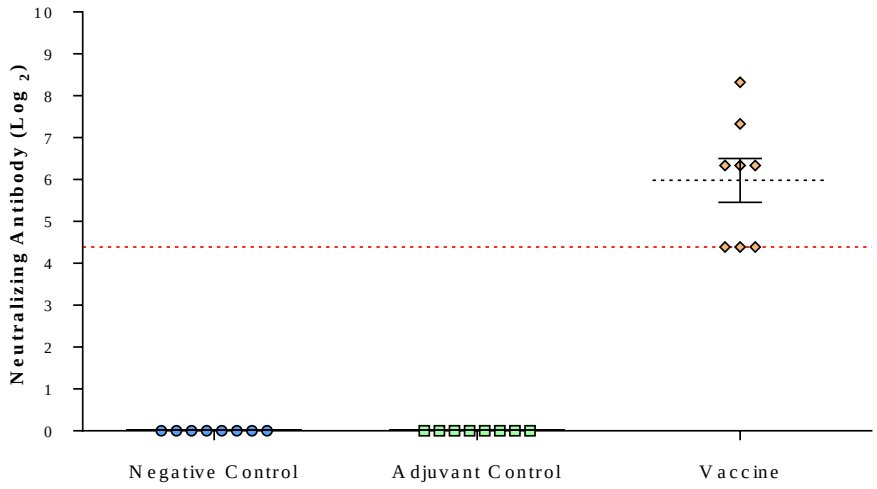

Figure 14 Neutralizing antibodies titers of heifers at the 30th day after the second vaccination. The antibody titers were transformed in log_2 and data are presented as scatter dot plot, representing each animal. Horizontal dotted lines indicate the neutralizing antibodies median for each group. The red horizontal dotted line indicates the minimum neutralizing antibody titer (20; log_2 = 4,31) considered positive.

Bovine cellular immune response after vaccination was evaluated by the PBMCs immunophenotyping from blood collected before the first vaccination and at the 30th day post second vaccination. To determine the specificity of the immune response generated by vaccination, the PBMCs were stimulated *in vitro* by incubation with purified VACV GP2.

The samples collected before vaccination were prepared and stained. However, the acquisition data was not uniform, rendering it impossible to be analyzed. Without the acquisition data before vaccination, the immunophenotyping analysis was proceeded comparing the vaccinated and control groups. Additionally, the WC1 antibody marker did not stain well. Because of this, WC1 marker was not analyzed in any time point of the experiment.

B cell frequency was evaluated by the CD21 surface molecule expression. No significant difference in the frequency of B cell population among groups and stimulated or non-stimulated cells were observed at the 30th day post second vaccination dose (Figure 15).

Following B cell analysis, the activation of this lymphocyte population was investigated by CD25 surface molecule expression on $CD21^+$ cells. The CD25 protein corresponds to the α-chain of the IL-2 receptor, expressed on activated T and B cells, as well as on macrophages. Binding of IL-2 to its receptor leads to proliferation and differentiation of lymphocytes into effector B or T cells. Through statistical analysis no differences in frequency of $CD21^+CD25^+$ cells, neither in the CD25 mean intensity of fluorescence (MIF) of the $CD21^+$ cells were detected (Figure 15).

A trend towards the increase of the CD25 MIF could be noticed mainly when analyzing the non-stimulated and the stimulated PBMCs in the vaccinated group (Figure 15C).

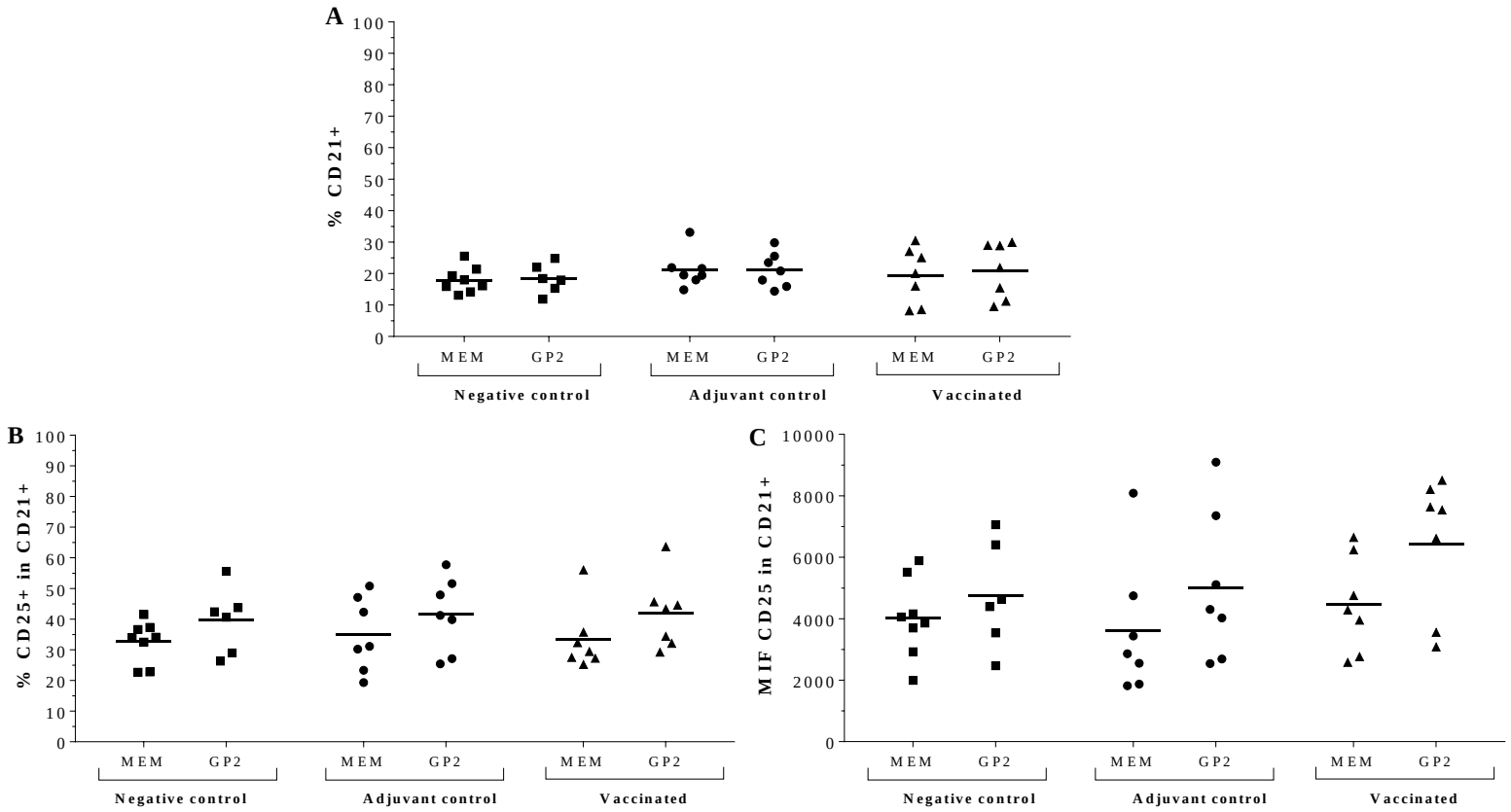

Figure 15 Peripheral blood B lymphocytes ($CD21^+$) from heifers at the 30th day after the second vaccination divided in three groups: negative control, adjuvant control and vaccine. The cells were non-stimulated (MEM) or stimulated with VACV GP2. Horizontal bars indicate mean values. A - Frequencies of peripheral blood B cells ($CD21^+$). B - Frequencies of $CD21^+$ cells expressing CD25. C - Mean Intensity of Fluorescence (MIF) of CD25 in $CD21^+$ cells.

No significant difference in the frequency of T cell populations, neither $CD4^+$ nor $CD8^+$, between groups and non-stimulated and stimulated cells were observed. The CD25 marker was measured in the $CD4^+$ and $CD8^+$ T cells populations. Frequencies of $CD4^+CD25^+$ cells and $CD8^+CD25^+$ was similar between groups. However, the MIF of CD25 producing cells was greater at the vaccinated group cells, and even higher in the stimulated cells from these group of animals (Figure 16).

In order to determine the main population of immune memory cells after vaccination, it was determined the expression of the surface protein CD45R0 on $CD4^+$ and $CD8^+$ lymphocytes (Figure 17). No significant difference in the frequency nor in the MIF of CD45R0 in each cell population was observed among groups and non-stimulated or stimulated cells.

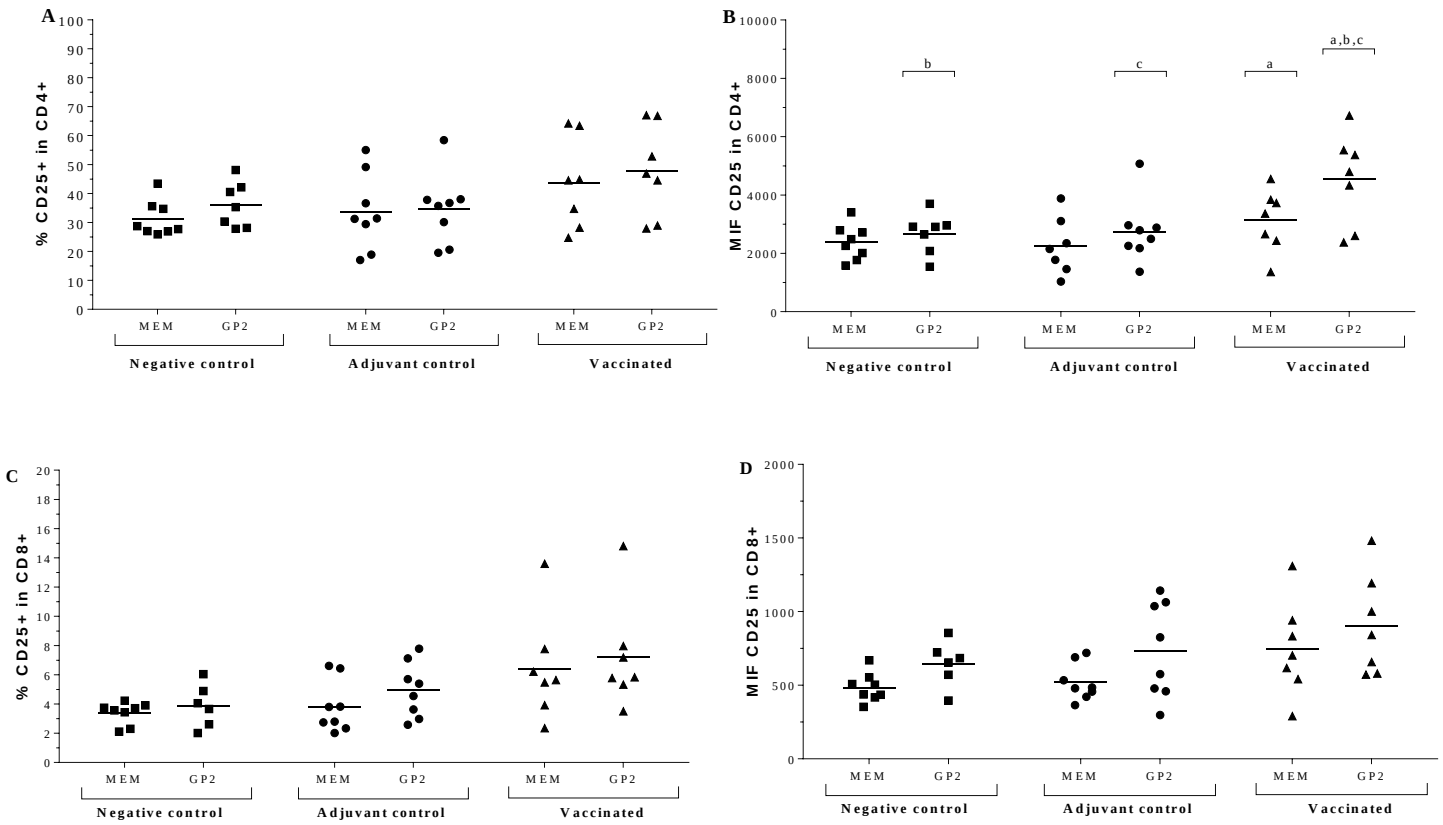

Figure 16 Peripheral blood T lymphocytes from heifers at the 30th day after the second vaccination divided in three groups: negative control, adjuvant control and vaccine. The cells were non-stimulated (MEM) or stimulated with VACV GP2. Horizontal bars indicate mean values. A - Frequencies of peripheral blood $CD4^+CD25^+$cells. B - Mean Intensity of Fluorescence (MIF) of CD25 in $CD4^+$ cells. C - Frequencies of $CD8^+CD25^+$ cells. D - Mean Intensity of Fluorescence (MIF) of CD25 in $CD4^+$ cells. Equal letters indicate statistical differences ($p < 0.05$). (two-way ANOVA, followed by SIDAK's multiple comparisons test).

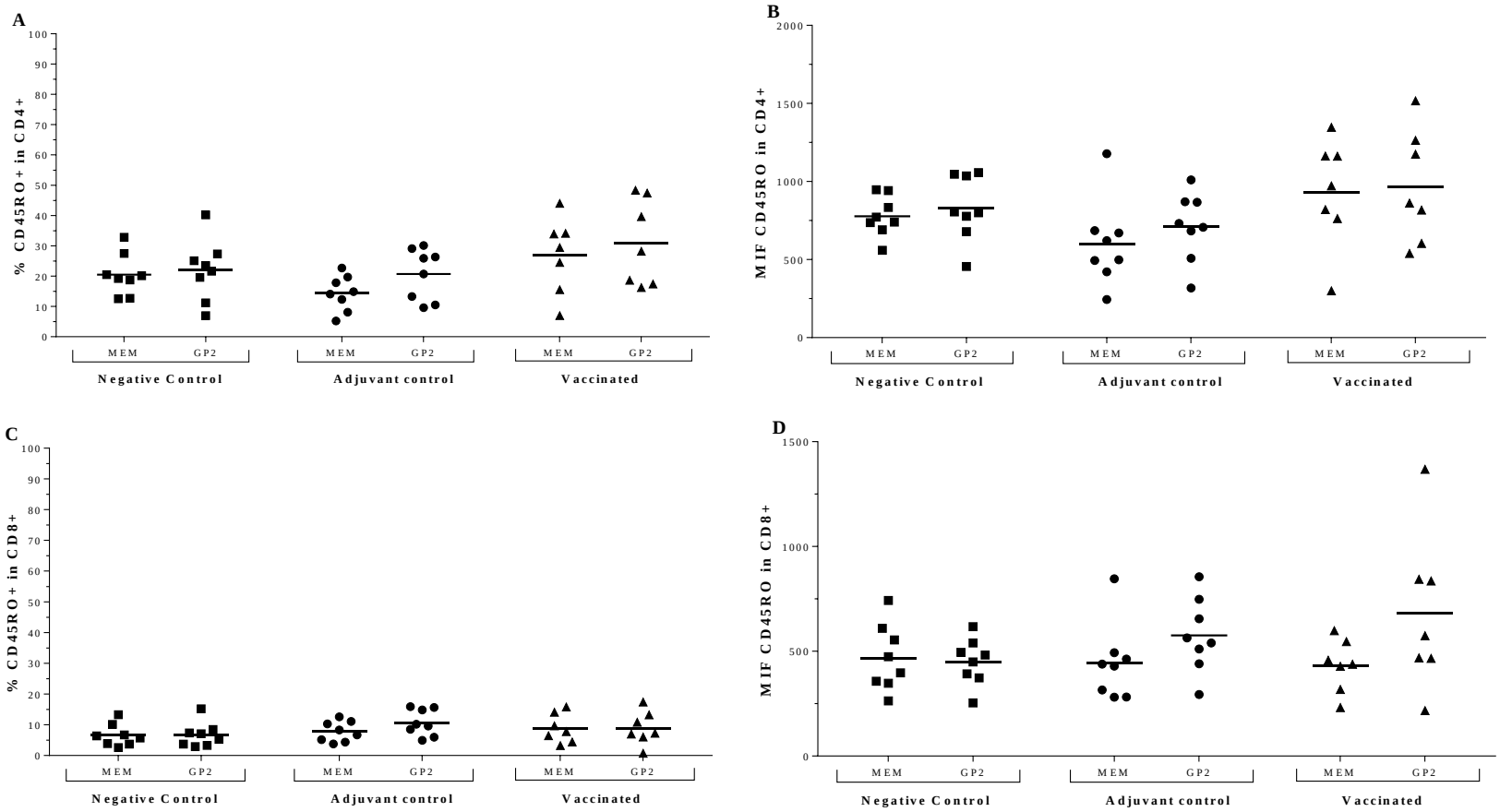

Figure 17 Peripheral blood T lymphocytes from heifers at the 30th day after the second vaccination divided in three groups: negative control, adjuvant control and vaccine. The cells were non-stimulated (MEM) or stimulated with VACV GP2. Horizontal bars indicate mean values. A - Frequencies of peripheral blood $CD4^{+}CD45R0^{+}$cells. B - Mean Intensity of Fluorescence (MIF) of CD45R0 in $CD4^{+}$ cells. C - Frequencies of $CD8^{+}CD45R0^{+}$ cells. D - Mean Intensity of Fluorescence (MIF) of CD45R0 in $CD4^{+}$ cells.

To verify disease protection after vaccination, VACV challenge was performed. Four heifers from negative control group and four heifers from vaccinated group were ramdoly selected and inoculated with VACV-GP2 at the 30th day post the second vaccination. The clinical course was evaluated by presence of the main clinical signs observed in VACV infection. It was created a score, based on the observation of lesions suggestive of VACV infection. The sum of lesions scores, observed by day, in all animals from each group was used as disease score. The disease scores recorded from the 2nd to the 19th DPC are demonstrated in Figure 18. Differences of disease score between control and vaccinated groups were identified by DPC (Figure 18).

The difference in BV evolution post challenge between the control and vaccinated groups was remarkable. Figure 19 shows the lesions observed in the heifers from the control and vaccinated groups at the 8th DPC. Characteristic BV lesions localized in the inoculated areas were observed in the heifers from control group. While ulcers were still observed at the 8th DPC in the teats from the heifers belonging to the control group, the animals from the vaccinated group presented signs of healing process. In animals from the vaccinated group, it seems that only the scarification lesions were observed, which evolved into complete healing up to the 10th DPC. No BV characteristic lesions, such as vesicles nor papules, were observed in the teats of heifers from vaccinated group.

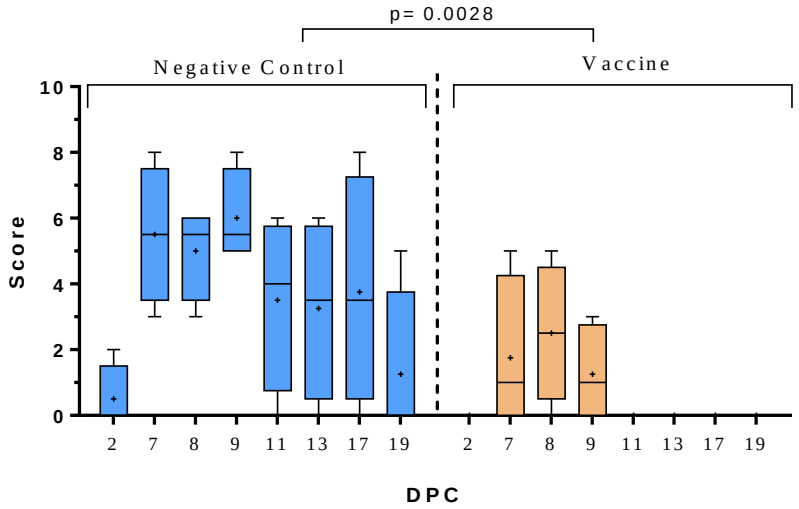

Figure 18 Box plot representation of VACV infection disease score recorded in bovine, from the 1st to the 19th day post challenge (DPC) with VACV strain GP2. Horizontal bars indicate the median of disease score in each DPC. (+) Indicates the mean of disease score in each DPC. Boxes represent 5-95 percentile and vertical bars the maximum and minimum recorded disease score. Statistical differences (p =0.0028) between groups identified by Mann-Whitney test.

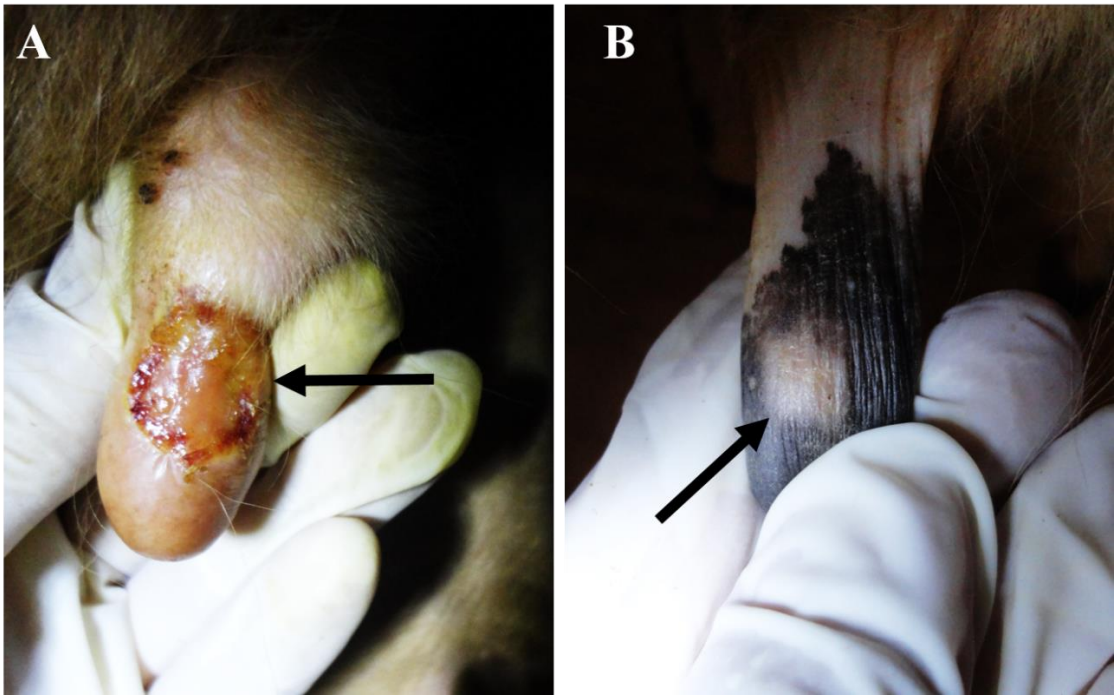

Figure 19 Lesions observed on the 8th day post challenge (DPC). A – Ulcer and early scabbing (arrow) on the teat of a heifer from the control group. B – Small scar (arrow) on teat of heifer from the vaccinated group.

The cellular immune response analysis was realized early after challenge, at the 4th DPC. Limitations regarding the size of challenged group animals need to be consider. However, descriptive analyzes of the acquired data indicate a tendency on the cellular profile of the groups after challenge.

Considering the B lymphocytes immunophenotyping, the percentage of $CD21^+$ cells and $CD25^+$ in $CD21^+$ cells between control and vaccinated groups were not statistical different. Moreover, when comparing the percentage mean of these cells populations before (30th day post second vaccination) and after challenge no statistical differences were noticed. Although any statistical difference between the MIF of CD25 in $CD21^+$ lymphocytes of control and vaccinated groups was not detected, a discrete variation between the groups was noted. Another interesting point was observed when comparing the data before and after challenge. A downward trend in the MIF of CD25 in $CD21^+$ cells was observed in the control group, indicating a negative modulation of immune response after challenge, which did not happen in the cellular immunophenotype profile of the animals from the vaccinated group (Figure 15 and Figure 20).

Analyzing the acquired cells data for T lymphocytes makers, although activated T helper $CD4^+$ and cytotoxic $CD8^+$ cells did not show differences in percentage, the CD25 marker MIF in both cells were notable greater after challenge in vaccinated group, and no difference was observed in control group. The higher MIF of CD25 between the two groups was observed at the 4th DPC (Figure 16, 21 and 22).

Although expected, the CD45R0 marker, which is considered a marker for memory T cells, did not present a tendency of increase in $CD4^+$ or $CD8^+$ cells comparing the data before and after challenge, and not even between the groups at the 4th DPC (Figure 17, 21 and 22).

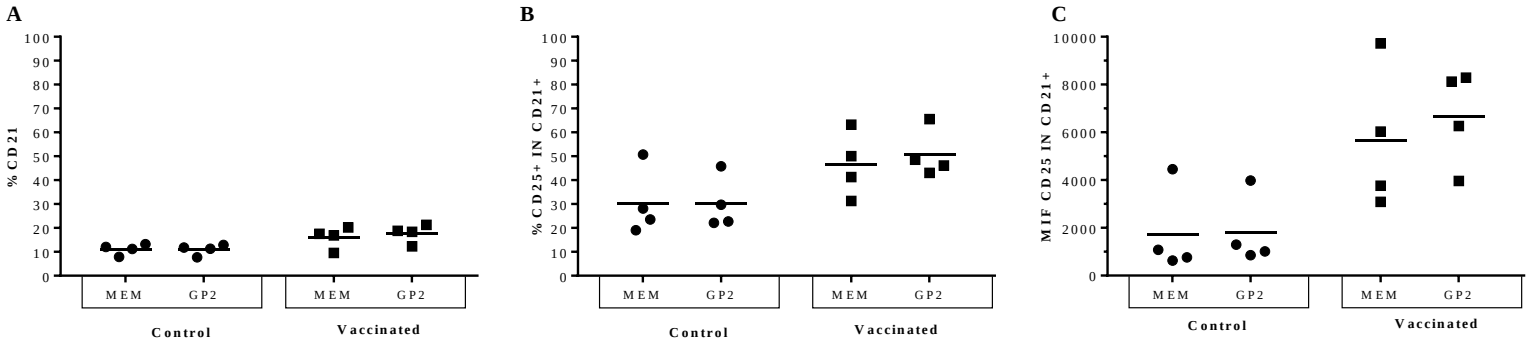

Figure 20 Peripheral blood B lymphocytes ($CD21^+$) from heifers at the 4^{th} DPC. The cells were non-stimulated (MEM) or stimulated with VACV (GP2). Horizontal bars indicate mean values. A - Frequencies of peripheral blood B cells ($CD21^+$). B - Frequencies of $CD21^+$ cells expressing CD25. C - Mean Intensity of Fluorescence (MIF) of CD25 in $CD21^+$ cells.

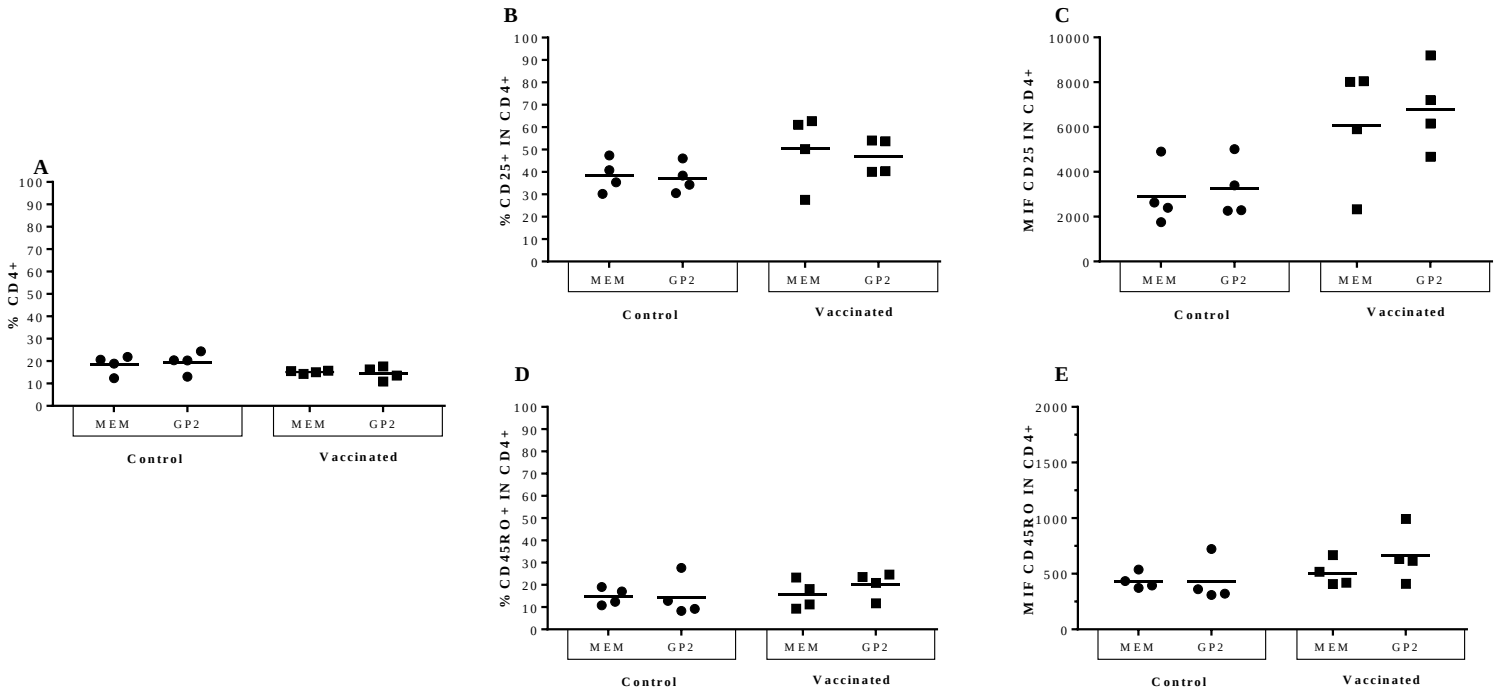

Figure 21 Peripheral blood T lymphocytes ($CD4^+$) from heifers at the 4th DPC. The cells were non-stimulated (MEM) or stimulated with VACV (GP2). Horizontal bars indicate mean values. A - Frequencies of peripheral blood T cells ($CD4^+$). B - Frequencies of $CD4^+$ cells expressing CD25. C - Mean Intensity of Fluorescence (MIF) of CD25 in $CD4^+$ cells. D - Frequencies of $CD4^+$ cells expressing CD45R0. C - Mean Intensity of Fluorescence (MIF) of CD45R0 in $CD4^+$ cells.

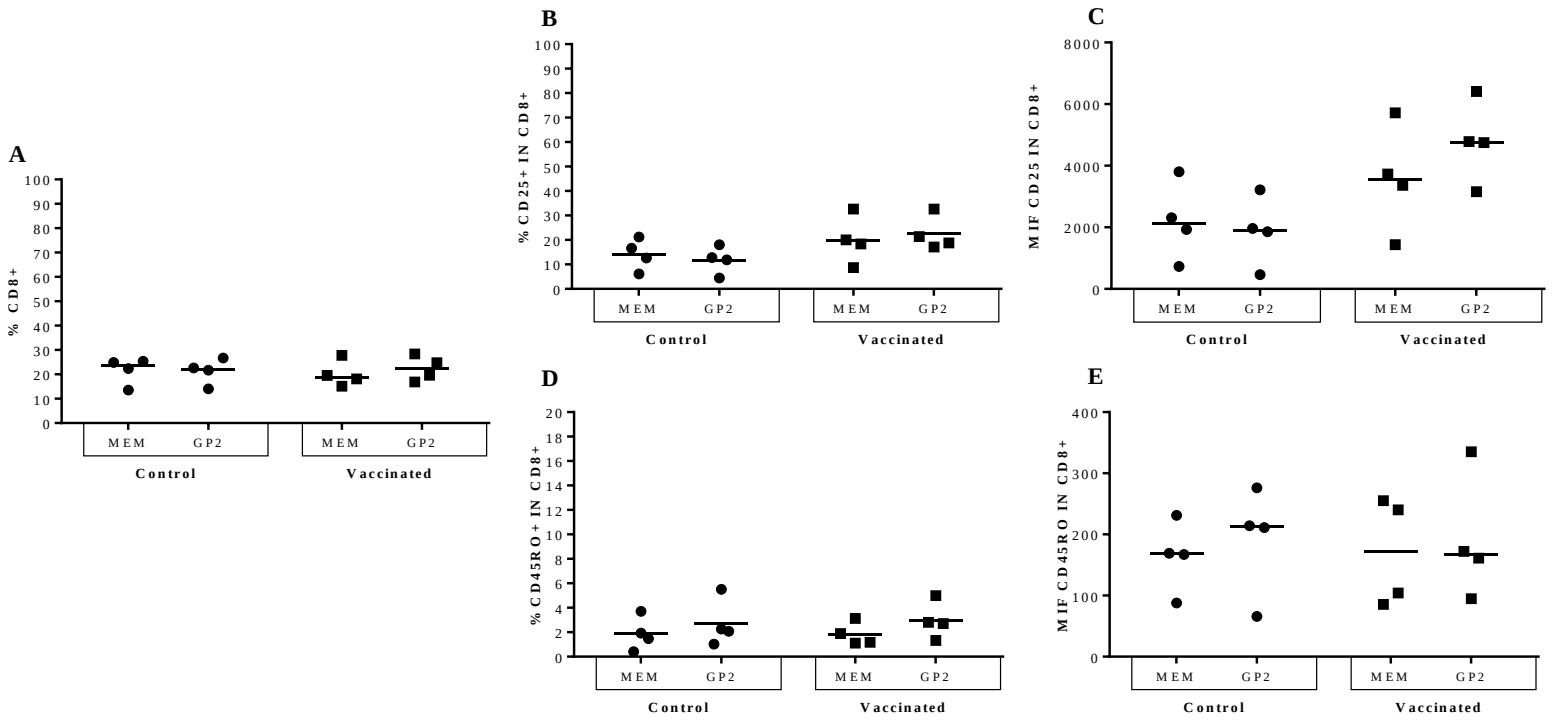

Figure 22 Peripheral blood T lymphocytes ($CD8^+$) from heifers at the 4th DPC. The cells were non-stimulated (MEM) or stimulated with VACV (GP2). Horizontal bars indicate mean values. A - Frequencies of peripheral blood T cells ($CD8^+$). B - Frequencies of $CD8^+$ cells expressing CD25. C - Mean Intensity of Fluorescence (MIF) of CD25 in $CD8^+$ cells.

6. Discussion

Given the numerous BV reported cases in Brazil since 1999, the development of a safe and effective vaccine has become an emerging demand. In the present study, a vaccine against bovine vaccinia was developed, for the purpose of control and future eradication of the disease in cattle. BV is a zoonosis and the main route for human infection is through direct contact with infected cows. The control and eventual eradication of the disease in cattle could eliminate the main route of infection to humans, resulting indirectly in the control of this zoonotic disease.

Critical attributes to be examined in the development of vaccines are efficacy, safety and tolerability. Regarding the vaccine formulations proposed in this work, no evidence of local or systemic toxicity was found even after two injections of any of the adjuvanted vaccines in the murine model. The iGP2 + AH/saponin, that was submitted to the efficacy tests in cattle did not cause any local toxicity, demonstrating that the developed vaccine presents good tolerability.

The VACV strain GP2 inactivated with BPL demonstrated to be a potential candidate for BV vaccine antigen. Moreover, the necessity of efficient adjuvants in activating a protective immune response need to be highlighted. Differences related to immune response and protection in murine model were observed between the vaccine formulations (Figure 11, Figure 12 and 13). The vaccine antigen alone (iGP2 + PBS) could not stimulate NA production in the murine model used, while the vaccine formulation iGP2 + AH/saponin induced high titers of NA and 100% of protection against clinical signs after challenge.

Saponin has been used in commercial veterinary vaccines as an adjuvant and there is a crescent number of experimental vaccines testing various extracts of saponins (Xiao; Rajput; Hu, 2007; De Costa et al., 2014; Cibulski et al., 2016; Yendo et al., 2016). Vaccines formulated using extracts of saponin as adjuvant have shown great advantage compared to the use of other adjuvants due to the stimulation of Th1 responses. Besides showing the stimulating effects of specific immunity components, saponins also stimulate non-specific immune activities, such as inflammatory response and monocyte proliferation (Rajput et al., 2007). Apart from the immune stimulating effects, adjuvants can also decrease the required amount of antigen to be incorporated into the vaccine, and thus reducing costs of production.

The existing attenuated VACV vaccines sound initially as a good choice to control BV, as they demonstrated high efficacy in promoting a protective immune response. However, the consequences related to vaccine viral escape and recombination between VACV vaccine viruses and wild VACV strains are unpredictable.

Although VACV reservoirs and the role of wildlife in BV outbreaks remain unclear, virus circulation in some species has been described, such as peridomestic and wild mice, capybaras, opossums and monkeys (Abrahão et al., 2009a, 2010; D'anunciação et al., 2012; Peres et al., 2013, 2016; Barbosa et al., 2014). Furthermore, there is a risk of human contact with the vaccine virus, such as by direct contact with the lesions or ingestion of contaminated milk, that can result in infection, associated with many side effects (Egan et al., 2004; Vora et al., 2008; Abrahão et al., 2009b; Silva-Fernandes et al., 2009; De Oliveira et al., 2015). Therefore, the use of live vaccine in cattle,

considering the epidemic situation of BV in Brazil, is definitely not recommended. The vaccine formulations developed in this work used the inactivated VACV, and demonstrated that in association with AH and saponin adjuvants, it could be a potential vaccine to control BV in Brazil.

In previous studies, Ferreira (2008) described the development of inactivated VACV vaccines using as antigen, purified VACV BAV strain particles associated to different adjuvants. The vaccine proved to be efficient to protect Balb/c mice immunized and subsequently challenged intranasally and by scarification. However, although there was protection, animals vaccinated and challenged intranasally with VACV Western Reserve strain still showed some morbidity, marked by pilo-erection and weight loss during the first days post-challenge. To minimize morbidity, Abrahão (2010) proposed a prime-boost vaccination system using the purified VACV BAV strain inactivated with BPL or heat inactivated in combination with a DNA vaccine. During the process of viral purification, only MV particles were selected, and then used as vaccine antigen. When in combination with a DNA vaccine for protein B5, a major EV protein related to NA production, NA against both viral forms were detected and 100% of vaccinated mice were protect against death, although mild clinical signs have been observed.

Most of the immune response mechanisms against poxviruses is directed against surface proteins of the two viral forms (MV and EV). In that way, antibodies against MV act to neutralize initial infection in the new host and antibodies against EV prevent the spread of the viral progeny within the host. Thus, the optimal protection against VACV is acquired when there are antibodies against proteins of both viral forms (Cohen et al., 2011). In this work, the vaccine antigen was produced using the clarified supernatant of VACV multiplication in cell culture. The presence of proteins from both MV and EV forms, associated to the adjuvant stimulus, might be related to the development of the potent disease protection observed in mice (Figure 12 and 13) and heifers (Figure 14Figure ***18*** Figure 19). However, further studies evaluating specific antibody response against each kind of virus particle are needed to confirm this hypothesis.

The VACV strain GP2 was chosen to be used as the vaccine antigen strain considering the wide distribution of Group 1 BRA-VACV in Brazil, most related to BV outbreaks. Additionally, information about pathogenesis and immunological response in cows experimentally infected with VACV strain GP2 was already available. The previous information about cattle infection by VACV strain GP2 demonstrates a systemic infection with activation of humoral and cellular immune response and helps to evaluate the vaccine potential immune response and protection in bovine species (Matos, 2012; Rehfeld et al., 2013; Rivetti Jr. et al., 2013; Guedes et al., unpublished data).

According to the mice experiment results, the iGP2 + AH saponin formulation was selected to be tested in bovine species, as this vaccine demonstrated more intensive neutralizing antibody response in BAlb/c mice and protected 100% of the mice previously vaccinated and challenged with VACV-GP2.

All vaccinated heifers, 30 days post the second vaccination, presented NA with titers varying from 20 ($\log_2 = 4.32$) to 320 ($\log_2 = 7.32$) (Figure 14). Considering VACV experimental infection in lactating cows, the antibody response was generated up to the 10^{th} DPI, and by the 16^{th} DPI neutralizing antibodies could be detected and peaked at the 40^{th} DPI, with titers varying from 20 to

80 (Matos, 2012). The developed vaccine, when applied twice with interval doses of 21 days, through the subcutaneous route, could induce the secretion of elevate titers of NA, even higher than observed in experimentally infected cows.

In experimentally infected cows, B and T $CD4^+$ lymphocytes activation was significantly elevated post-infection, mainly in the 30th DPI (Guedes et al., unpublished data). In this study, although frequencies of $CD21^+CD25^+$, $CD4^+CD25^+$ and $CD8^+CD25^+$ cells were similar between vaccinated and control groups, the expression of CD25 molecule in $CD4^+$ cells surface was greater in the PBMCs of the animals from the vaccinated group, and even higher when the PBMCs were stimulated, demonstrating VACV specific lymphocyte activation (Figure 16). Through statistical analysis no difference in the CD25 MIF of the $CD21^+$ cells were detected (Figure 15). However, a tendency towards a higher expression of CD25 in $CD21^+$ cells surface could be noticed mainly when analyzing the non-stimulated and the stimulated PBMCs in the vaccinated group (Figure 15C). The heifers' population was heterogenic, and the cell immunophenotyping of the groups demonstrated animals that were outliers within each group. These outliers may have interfered on the detection of differences between groups and stimulated or non-stimulated cells, mainly when analyzing the CD25 MIF of the $CD21^+$ cells.

The frequency of both T cells memory populations was expected to be greater in vaccinated animals, as recorded for experimentally infected cows (Guedes et al. unpublished data). However, neither frequency nor MIF of $CD45R0^+$ cells differences were observed between groups and non-stimulated or stimulated cells (Figure 17). In this analysis, the outliers within animals from each group may have also interfered in the detection of differences between groups.

Although T memory cells were expected, our findings about $CD45R0^+$ expression in T cells corroborate with previous studies that demonstrated differences in vaccinated and infected humans T cells phenotypes (Precopio et al., 2007; Medeiros-Silva et al., 2013). MVA is an attenuated VACV strain that exhibits very limited replication in mammalian cells and does not disseminate in the host. Studies of MVA vaccination in humans demonstrated that after vaccination VACV specific $CD8^+$ T cells revealed an unusual surface phenotype for memory cells, presenting predominance of $CD45R0^-$. Moreover, although VACV specific $CD8^+$ T cells co-expressed CD45RA and CD27, they were clearly not naïve (PRECOPIO et al., 2007). Medeiros-Silva and collaborators (2013) evaluating CD8+ and CD4+ T cells in VACV vaccinated and infected humans showed that the expression of CD45R0 by $CD4^+$ T cells was higher in the infected groups when compared to non-infected individuals, either previously vaccinated or unvaccinated. Furthermore, when the subpopulation of $CD8^+$ memory T cells were analyzed, there was a higher percentage of these cells only in the infected groups when compared with non-infected patients, either previously vaccinated or unvaccinated.

The developed iGP2 vaccine protection was identified by non-appearance of characteristic lesions of VACV infection after challenge in the vaccinated group. The scarification done previously of infection caused skin injuries, but the healing process of control and vaccinated groups were clearly different (Figure 18 and 19).

Experimentally infected and post re-infected cows showed BV lesions, however the lesions were mild and the BV clinical course was faster, with complete healing up to the 10th DPI, when

compared to that observed during the first inoculation (22^{nd} – 32^{nd} DPI). In addition, two cows infected 240 days before re-infection, which did not have antibody titers in day 0, had lesions that were more severe than the cows that were re-infected and had circulating antibodies (Rehfeld et al., 2013). In cows from farms affected by BV outbreaks, although antibody titers variation indicating viral circulation, no lesions were observed (Matos, 2012).

The cellular immune response analysis was realized early after challenge, at the 4^{th} DPC. Limitations regarding the size of challenged group animals need to be consider. However, descriptive analyzes of the acquired data indicate tendency on the cell profile of the groups after challenge. Difference between the MIF of CD25 in $CD21^+$ lymphocytes of control and vaccinated groups is notable. Another interesting point is observed when comparing the data before and after challenge. Downward trend in the MIF of CD25 in $CD21^+$ cells is observed in the control group, indicating a negative modulation of immune response after challenge, which did not happen in vaccinated group animals' cells (Figure 15 and 20). Interestingly, in humans, T lymphocytes of that patient showed reduced expression of cellular activation markers such as CD25, after stimulation *in vitro* with VACV, compared to T lymphocytes from uninfected patients (Trindade et al., 2009).

Analyzing the acquired cells data for T lymphocytes makers, although activated T helper $CD4^+$ and cytotoxic $CD8^+$ cells did not show differences in percentage, the CD25 marker MIF in both cells were notable greater after challenge in vaccinated group, and no difference was observed in control group. The higher MIF of CD25 between the two groups is observed at the 4^{th} DPC, indicating T cells activation in vaccinated group (Figure 16, 21 and 22)

The CD45R0 marker, which is an indicative memory T cells, did not present trends of increase in $CD4^+$ or $CD8^+$ cells comparing before and after challenge, and not even between the groups at the 4^{th} DPC (Figure 17, Figure 21 and Figure 22). Considering that the PBMCs immunophenotype were analyzed early after infection (4^{th} DPC), and that the increase of expression of CD45R0 in T cells is expected least 15 days post infection (Guedes et al., unpublished data), this hypothesis support the stability of CD45R0 molecule even in the VACV stimulated PBMCs.

7. Conclusion

The use of VACV strain GP2, inactivated by BPL in association with adjuvants was effective to induce humoral immune response against infection by the homologous VACV in Balb/c murine model. The induction of the humoral immune response, as measured by NA titration was variable according to the adjuvant used with the viral antigen. The vaccine formulation using AH associated with saponin as adjuvants induced production of high titers of antibody in all vaccinated mice, giving 100% protection in Balb/c murine model after challenge. In cattle, the use of the inactivated VACV strain GP2, formulated with the adjuvant based in AH and saponin, was able to induce the production of NA and demonstrated a tendency of early activation of cellular immune responses after challenge, conferring 100% protection against clinical signs in vaccinated heifers submitted to the challenge by inoculation of VACV-GP2.

8. References

ABRAHÃO, J. S.; GUEDES, M. I. M.; TRINDADE, G. S. et al. One more piece in the VACV ecological puzzle: could peridomestic rodents be the link between wildlife and bovine vaccinia outbreaks in Brazil? *PloS one*, v. 4, n. 10, p. e7428, jan. 2009a.

ABRAHÃO, J. S.; OLIVEIRA, T. M. L.; CAMPOS, R. K. et al. Bovine vaccinia outbreaks: detection and isolation of vaccinia virus in milk samples. *Foodborne pathogens and disease*, v. 6, n. 9, p. 1141–1146, 2009b.

ABRAHÃO, J. S. *Prevenção e controle do vaccinia virus no brasil : padronização de um sistema prime-boost para imunização e determinação de seus hospedeiros naturais*. 2010. 167p. Tese (Doutorado em Microbiologia) - Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte.

ABRAHÃO, J. S.; SILVA-FERNANDES, A. T.; LIMA, L. S. et al. Vaccinia virus infection in monkeys, Brazilian Amazon. *Emerging infectious diseases*, v. 16, n. 6, p. 976–9, jun. 2010.

ABRAHÃO, J.S; TRINDADE, G.S.; PEREIRA-OLIVEIRA, G. et al. Detection of Vaccinia Virus during an Outbreak of Exanthemous Oral Lesions in Brazilian Equids. *Equine Veterinary Journal*, p. n/a–n/a, 2016. <http://doi.wiley.com/10.1111/evj.12571>.

AFONSO, P. P.; SILVA, P. M.; SCHNELLRATH, L. C. et al. Characterization and next-generation genome sequencing of the unclassified Cotia virus SPAn232 (Poxviridae). *Journal of virology*, v. 86, n. 9, p. 5039–54, 2012.

AMANNA, I. J.; SLIFKA, M. K.; CROTTY, S. Immunity and immunological memory following smallpox vaccination. *Immunological reviews*, v. 211, p. 320–337, 2006.

BARBOSA, A. V.; MEDAGLIA, M. L. G.; SOARES, H. S. et al. Presence of neutralizing antibodies to Orthopoxvirus in Capybaras (Hydrochoerus hydrochaeris) in Brazil. *The Journal of Infection in Developing Countries*, v. 8, n. 12, p. 1646–1649, 2014.

BHANUPRAKASH, V.; HOSAMANI, M.; VENKATESAN, G. et al. Animal poxvirus vaccines: a comprehensive review. *Expert review of vaccines*, v. 11, n. 11, p. 1355–74, 2012.

BRUM, M. C. S.; ANJOS, B. L. Dos; NOGUEIRA, C. E. W. et al. An outbreak of orthopoxvirus-associated disease in horses in southern Brazil. *Journal of veterinary diagnostic investigation*, v. 22, n. 1, p. 143–147, 2010.

BRUN, A.; ALBINA, E.; BARRET, T. et al. Antigen delivery systems for veterinary vaccine development. Viral-vector based delivery systems. *Vaccine*, v. 26, n. 51, p. 6508–6528, 2008.

BULLER, R. M.; PALUMBO, G. J. Poxvirus Pathogenesis. *Microbiological reviews*, v. 55, n. 1, p. 80–122, 1991.

CAMPOS, R. K.; BRUM, M. C. S.; NOGUEIRA, C. E. W. et al. Assessing the variability of Brazilian Vaccinia virus isolates from a horse exanthematic lesion: coinfection with distinct viruses. *Archives of virology*, v. 156, n. 2, p. 275–83, 2011.

CAPODICI, J.; MAIGETTER, R.; REVAI, M.; LATZO, J. Large-scale beta-propiolactone inactivation of HIV for vaccines. *Bioprocess Int*, p. 36-41, 2006.

CARGNELUTTI, J. F.; SCHMIDT, C.; MASUDA, E. K. et al. Vaccinia viruses isolated from cutaneous disease in horses are highly virulent for rabbits. *Microbial Pathogenesis*, v. 52, n. 3, p. 192–199, 2012.

CIBULSKI, S. P.; SILVEIRA, F.; MOURGLIA-ETTLIN, G. et al. Quillaja brasiliensis saponins induce robust humoral and cellular responses in a bovine viral diarrhea virus vaccine in mice. *Comparative Immunology, Microbiology and Infectious Diseases*, v. 45, p. 1–8, 2016.

COHEN, M. E.; XIAO, Y.; EISENBERG, R. J. et al. Antibody against extracellular vaccinia virus (ev) protects mice through complement and fc receptors. *PLoS ONE*, v. 6, n. 6, p. 38–41, 2011.

CONDIT, R. C.; MOUSSATCHE, N.; TRAKTMAN, P. In a nutshell: structure and assembly of the vaccinia virion. *Advances in virus research*, v. 66, p. 31–124, 2006.

COULIBALY, S.; BRÜHL, P.; MAYRHOFER, J. et al. The nonreplicating smallpox candidate vaccines defective vaccinia Lister (dVV-L) and modified vaccinia Ankara (MVA) elicit robust long-term protection. *Virology*, v. 341, n. 1, p. 91–101, 2005.

D'ANUNCIAÇÃO, L.; GUEDES, M. I. M.; OLIVEIRA, T. L. et al. Filling One More Gap: Experimental Evidence of Horizontal Transmission of Vaccinia Virus Between Bovines and Rodents. *Vector-Borne and Zoonotic Diseases*, v. 12, n. 1, p. 61–64, 2012.

DA SILVA, A. C.; REIS, B. B.; RICCI, J. E. R. et al. Infecção em humanos por varíola bovina na microrregião de Itajubá, Estado de Minas Gerais: Relato de caso. *Revista da Sociedade Brasileira de Medicina Tropical*, v. 41, n. 5, p. 507–511, 2008.

DAMASO, C. R.; ESPOSITO, J. J.; CONDIT, R. C. et al. An emergent poxvirus from humans and cattle in Rio de Janeiro State: Cantagalo virus may derive from Brazilian smallpox vaccine. *Virology*, v. 277, n. 2, p. 439–449, 2000.

DARBY, A. C.; MCINNES, C. J.; KJÆR, K. H. et al. Novel host-related virulence factors are encoded by squirrelpox virus, the main causative agent of epidemic disease in red squirrels in the UK. *PloS one*, v. 9, n. 7, p. e96439, 2014.

DE ASSIS, F. L.; VINHOTE, W. M.; BARBOSA, J. D. et al. Reemergence of vaccinia virus during zoonotic outbreak, Pará State, Brazil. *Emerging Infectious Diseases*, v. 19, n. 12, p. 2017–2020, 2013.

DE COSTA, F.; YENDO, A. C. A.; CIBULSKI, S. P. et al. Alternative inactivated poliovirus vaccines adjuvanted with Quillaja brasiliensis or Quil-a saponins are equally effective in inducing specific immune responses. *PloS one*, v. 9, n. 8, p. e105374, 2014.

DE OLIVEIRA, T. M. L.; GUEDES, M. I. M. C.; REHFELD, I. S. et al. Detection of *Vaccinia Virus* in Milk: Evidence of a Systemic and Persistent Infection in Experimentally Infected Cows. *Foodborne Pathogens and Disease*, v. 12, n. 11, p. 898–903, 2015.

DONATELE, D. M.; TRAVASSOS, C. E. P. F.; LEITE, J. A. et al. Epidemiologia da poxvirose bovina no Estado do Espírito Santo , Brasil. *Brazilian Journal of Veterinary Research and Animal Science*, v. 44, n. 4, p. 275–282, 2007.

DRUMOND, B. P.; LEITE, J. A.; DA FONSECA, F. G. et al. Brazilian Vaccinia virus strains are genetically divergent and differ from the Lister vaccine strain. *Microbes and Infection*, v. 10, n. 2, p. 185–197, 2008.

EDGHILL-SMITH, Y.; GOLDING, H.; MANISCHEWITZ, J. et al. Smallpox vaccine-induced antibodies are necessary and sufficient for protection against monkeypox virus. *Nature Medicine*, v. 11, n. 7, p. 740–747, 2005.

EGAN, C.; KELLY, C. D.; RUSH-WILSON, K. et al. Laboratory-Confirmed Transmission of Vaccinia Virus Infection through Sexual Contact with a Military Vaccinee. *Journal of clinical microbiology*, v. 42, n. 11, p. 5409–5411, 2004.

ESSBAUER, S.; PFEFFER, M.; MEYER, H. Zoonotic poxviruses. *Veterinary Microbiology*, v. 140, n. 3-4, p. 229–236, 2010.

FENNER, F.; HENDERSON, D. A.; ARITA, I. et al. *Smallpox and its eradication.* 1988. 1421p.

FERREIRA, J. M. S.; DRUMOND, B. P.; ABRAHAO, J. S. et al. Brazilian vaccinia virus strains show a classical orthopoxvirus infection course and cross-protection. *Asian Pacific Journal of Tropical Medicine*, v. 2, n. 2, p. 21–26, 2009.

FERREIRA, J. M. S.; DRUMOND, B. P.; GUEDES, M. I. M. C. et al. Virulence in murine model shows the existence of two distinct populations of Brazilian Vaccinia virus strains. *PLoS ONE*, v. 3, n. 8, p. e3043, 2008.

FERREIRA, J. M. S. F. *Vaccinia virus: virulência de amostras brasileiras em camundongos balb/c e resposta imune humoral ao imunógeno inativado bean58058.* 2008. 152p. Tese (Doutorado em Microbiologia) - Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte.

FONSECA, F. G.; LANNA, M. C. S.; CAMPOS, M. A. S. et al. Morphological and molecular characterization of the poxvirus BeAn 58058. *Archives of Virology*, v. 143, n. 6, p. 1171–1186, 1998.

FONSECA, F. G.; TRINDADE, G. S.; SILVA, R. L. A. et al. Characterization of a vaccinia-like virus isolated in a Brazilian forest. *The Journal of general virology*, v. 83, p. 223–228, 2002.

FRANCO-LUIZ, A. P. M. *Vaccinia virus na américa do sul: caracterização de novos isolados e padronização de ensaio imunoenzimático*. 2016. Tese (Doutorado em Microbiologia) - Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte.

FRANCO-LUIZ, A. P. M.; FAGUNDES-PEREIRA, A.; COSTA, G. B. et al. Spread of Vaccinia Virus to Cattle Herds, Argentina, 2011. *Emerging Infectious Diseases*, v. 20, n. 9, p. 1576–1578, 2014.

FRAZATTI-GALLINA, N. M.; MOURÃO-FUCHES, R. M.; PAOLI, R. L. et al.. Vero-cell rabies vaccine produced using serum-free medium. *Vaccine*, v. 23, n. 4, p. 511–7, 9 dez. 2004.

GALMICHE, M. C.; GOENAGA, J.; WITTEK, R.; RINDISBACHER, L. Neutralizing and Protective Antibodies Directed against Vaccinia Virus Envelope Antigens. *Virology*, v. 254, n. 1, p. 71–80, 1999.

GOMES, J. A. S.; DE ARAJO, F. F.; TRINDADE, G. D. S. et al. Immune modulation in primary vaccinia virus zoonotic human infections. *Clinical and Developmental Immunology*, v. 2012, p. 974067, 2012.

GREENBERG, R. N.; KENNEDY, J. S. ACAM2000: A Newly Licensed Cell Culture-Based Live Vaccinia Smallpox Vaccine. *Expert opinion on investigational drugs*, v. 17, n. 4, p. 555–564, 2008.

GUEDES, M. I. M. C.; REHFELD, I. S.; DE OLIVEIRA, T. M. L. et al. Detection of Vaccinia Virus in Blood and Faeces of Experimentally Infected Cows. *Transboundary and Emerging Diseases*, v. 60, n. 6, p. 552–555, 2012.

GURAV, Y. K.; RAUT, C. G.; YADAV, P. D. et al. Buffalopox outbreak in humans and animals in Western Maharashtra, India. *Preventive Veterinary Medicine*, v. 100, n. 3-4, p. 242–247, 2011.

HAMMARLUND, E.; LEWIS, M. W.; HANSEN, S. G. et al. Duration of antiviral immunity after smallpox vaccination. *Nature medicine*, v. 9, n. 9, p. 1131–7, 2003.

ICTV. *International Committee on Taxonomy of Viruses (ICTV).* <http://www.ictvonline.org/virusTaxonomy.asp>. Accessed date: 20 jan. 2016.

INSTITUTO BRASILEIRO DE GEOGRAFIA E ESTATÍSTICA - IBGE. *Produção da Pecuária Municipal*, v. 42, p. 1 – 36, 2014.

JACOBS, B. L.; LANGLAND, J. O.; KIBLER, K. V. et al. Vaccinia virus vaccines: Past, present and future. *Antiviral Research*, v. 84, n. 1, p. 1–13, 2009.

JACOBS, N.; CHEN, R. J.; GUBSER, C. et al. Intradermal immune response after infection with Vaccinia virus. *The Journal of general virology*, v. 87, p. 1157–61, 2006.

KROON, E. G.; MOTA, B. E. F.; ABRAHÃO, J. S. et al. Zoonotic Brazilian Vaccinia virus: From field to therapy. *Antiviral Research*, v. 92, n. 2, p. 150–163, 2011.

LECLERCQ, S. Y.; DOS SANTOS, R. M. M.; MACEDO, L. B. et al. Evaluation of water-in-oil-in-water multiple emulsion and microemulsion as potential adjuvants for immunization with rabies antigen. *European Journal of Pharmaceutical Sciences*, v. 43, n. 5, p. 378–385, 2011.

LEITE, J. A.; DRUMOND, B. P.; TRINDADE, G. S. et al. Passatempo virus, a vaccinia virus strain, Brazil. *Emerging Infectious Diseases*, v. 11, n. 12, p. 1935–1938, 2005.

LOBATO, Z. I. P.; TRINDADE, G. S.; FROIS, M. C. M. et al. Outbreak of exantemal disease caused by Vaccinia virus in human and cattle in Zona da Mata region, Minas Gerais. *Arquivo Brasileiro de Medicina Veterinaria e Zootecnia*, v. 57, n. 4, p. 423–429, 2005.

MADUREIRA, M. C. *Vaccínia bovina no estado de Minas Gerais, 2005-2007*. 2009. 102p. Tese (Doutorado em Ciência Animal) - Escola de Veterinária, Universidade Federal de Minas Gerais, Belo Horizonte.

MARQUES, J. T.; DE SOUZA TRINDADE, G.; DA FONSECA, F. G. et al. Characterization of ATI, TK and IFN-alfa/betaR genes in the genome of the BeAn 58058 virus, a naturally attenuated wild Orthopoxvirus. *Virus Genes*, v. 23, n. 3, p. 291–301, 2001.

MATHO, M. H.; SCHLOSSMAN, A.; MENG, X. et al. Structural and Functional Characterization of Anti-A33 Antibodies Reveal a Potent Cross-Species Orthopoxviruses Neutralizer. *PLoS pathogens*, v. 11, n. 9, p. e1005148, 1 set. 2015.

MATOS, A. C. D. *Vaccinia virus: padronização de técnica para imunodiagnóstico e estudo da resposta imune humoral de bovinos.* 2012. 68p. Dissertação (Mestrado em Ciência Animal) - Escola de Veterinária, Universidade Federal de Minas Gerais, Belo Horizonte.

MATOS, A. C. D.; GUEDES, M. I. M. C.; REHFELD, I. S. et al. Outbreak of Vaccinia virus infection in horses in Minas Gerais state, Brazil. In: XXIV Congresso Brasileiro de Virologia e VIII Encontro de Virologia do Mercosul, 2013, Porto Seguro - *Virus Reviews & Research*, 2013.

MCVEY, S.; SHI, J. Vaccines in Veterinary Medicine: A Brief Review of History and Technology. *Veterinary Clinics of North America - Small Animal Practice*, v. 40, n. 3, p. 381–392, 2010.

MEDAGLIA, M. L. G.; MOUSSATCHÉ, N.; NITSCHE, A. et al. Genomic Analysis, Phenotype, and Virulence of the Historical Brazilian Smallpox Vaccine Strain IOC: Implications for the Origins and Evolutionary Relationships of Vaccinia Virus. *Journal of Virology*, v. 89, n. 23, p. 11909–11925, 2015.

MEDAGLIA, M. L. G.; PESSOA, L. C. G. D.; SALES, E. R. C. et al. Spread of cantagalo virus to northern Brazil. *Emerging infectious diseases***,** v.15, n.7, p. 1142-1143, 2009.

MEDEIROS-SILVA, D. C.; DOS SANTOS MOREIRA-SILVA, E. A.; ASSIS SILVA GOMES, J. De; DA FONSECA, F. G.; CORREA-OLIVEIRA, R. CD4 and CD8 T cells participate in the immune memory response against Vaccinia virus after a previous natural infection. *Results in immunology*, v. 3, p. 104–13, 2013.

MEGID, J.; APPOLINÁRIO, C. M.; LANGONI, H. et al. Short report: Vaccinia virus in humans and cattle in Southwest region of São Paulo State, Brazil. *American Journal of Tropical Medicine and Hygiene*, v. 79, n. 5, p. 647–651, 2008.

MOSS, B. In: KNIPE, D. M.; HOWLEY, P. M. (Ed.). *Fields Virology*. Sixth ed. Philadelphia: Lippincott Williams & Wilkins, 2013. p. 2129–2159.

MOTA, B. E. F.; GALLARDO-ROMERO, N.; TRINDADE, G. et al. Adverse events post smallpox-vaccination: Insights from tail scarification infection in mice with vaccinia virus. *PLoS ONE*, v. 6, n. 4, 2011.

NAGASSE-SUGAHARA, T. K.; KISIELIUS, J. J.; UEDA-ITO, M. et al. Human vaccinia-like virus outbreaks in São Paulo and Goiás States, Brazil: Virus detection, isolation and identification. *Revista do Instituto de Medicina Tropical de Sao Paulo*, v. 46, n. 6, p. 315–322, 2004.

OLIVEIRA, D. B.; ASSIS, F. L.; FERREIRA, P. C. P. et al. Group 1 Vaccinia virus Zoonotic Outbreak in Maranhao State, Brazil. *American Journal of Tropical Medicine and Hygiene*, v. 89, n. 6, p. 1142–1145, 2013.

OLIVEIRA, G.; ASSIS, F.; ALMEIDA, G. et al. From Lesions to Viral Clones: Biological and Molecular Diversity amongst Autochthonous Brazilian Vaccinia Virus. *Viruses*, v. 7, n. 3, p. 1218–1237, 2015.

PANCHANATHAN, V.; CHAUDHRI, G.; KARUPIAH, G. Correlates of protective immunity in poxvirus infection: where does antibody stand? *Immunology and cell biology*, v. 86, n. 1, p. 80–86, 2008.

PERES, M. G.; BACCHIEGA, T. S.; APPOLINARIO, C. M. et al. Serological study of vaccinia virus reservoirs in areas with and without official reports of outbreaks in cattle and humans in Sao Paulo, Brazil. *Archives of Virology*, v. 158, n. 12, p. 2433–2441, 2013.

PERES, M. G.; BARROS, C. B.; APPOLINARIO, C. M. et al. Dogs and Opossums Positive for Vaccinia Virus during Outbreak Affecting Cattle and Humans, Sao Paulo State, Brazil. *Emerging infectious diseases*, v. 22, n. 2, p. 271–273, 2016.

PRECOPIO, M. L.; BETTS, M. R.; PARRINO, J. et al. Immunization with vaccinia virus induces polyfunctional and phenotypically distinctive CD8(+) T cell responses. *Journal of Experimental Medicine*, v. 204, n. 6, p. 1405–1416, 2007.

PÜTZ, M. M.; MIDGLEY, C. M.; LAW, M.; SMITH, G. L. Quantification of antibody responses against multiple antigens of the two infectious forms of Vaccinia virus provides a benchmark for smallpox vaccination. *Nature medicine*, v. 12, n. 11, p. 1310–1315, 2006.

QIN, L.; EVANS, D. H. Genome Scale Patterns of Recombination between Coinfecting Vaccinia Viruses. *Journal of virology*, v. 88, n. 10, p. 5277–5286, 2014.

QUIXABEIRA-SANTOS, J. C.; MEDAGLIA, M. L. G.; PESCADOR, C. A; DAMASO, C. R. Animal movement and establishment of vaccinia virus Cantagalo strain in Amazon biome, Brazil. *Emerging infectious diseases*, v. 17, n. 4, p. 726–9, 2011.

RAJPUT, Z. I.; HU, S.; XIAO, C.; ARIJO, A. G. Adjuvant effects of saponins on animal immune responses. *Journal of Zhejiang University. Science. B*, v. 8, n. 3, p. 153–161, 2007.

REHFELD, I. S.; GUEDES, M. I. M. C.; FRAIHA, A. L. S. et al. Vaccinia virus transmission through experimentally contaminated milk using a murine model. *PLoS ONE*, v. 10, n. 5, p. 1–13, 2015.

REHFELD, I. S.; GUEDES, M. I. M. C.; MATOS, A. C. D. et al.. Clinical, hematological and biochemical parameters of dairy cows experimentally infected with Vaccinia virus. *Research in Veterinary Science*, v. 95, n. 2, p. 752–757, 2013.

RIEDEL, S. Edward Jenner and the history of smallpox and vaccination. *BUMC Proceedings*, v. 18, p. 21–25, 2005.

RIVETTI Jr., A. V; GUEDES, M. I. M. C.; REHFELD, I. S. et al. Bovine vaccinia, a systemic infection: evidence of fecal shedding, viremia and detection in lymphoid organs. *Veterinary microbiology*, v. 162, n. 1, p. 103–11, 2013.

ROBERTS, K. L.; SMITH, G. L. Vaccinia virus morphogenesis and dissemination. *Trends in microbiology*, v. 16, n. 10, p. 472–9, 2008.

ROSENBLATT, A. E.; STEIN, S. L. Cutaneous reactions to vaccinations. *Clinics in Dermatology*, v. 33, n. 3, p. 327–332, 2015.

ROSENTHAL, S. R.; MERCHLINSKY, M.; KLEPPINGER, C.; GOLDENTHAL, K. L. Developing new smallpox vaccines. *Emerging Infectious Diseases*, v. 7, n. 6, p. 920–926, 2001.

ROTZ, L. D.; DOTSON, D. A.; DAMON, I. K.; BECHER, J. A. Vaccinia (Smallpox) Vaccine: Recommendations of the Advisory Committee on Immunization Practices (ACIP), 2001. *MMWR. Recommendations and reports : Morbidity and mortality weekly report. Recommendations and reports / Centers for Disease Control*, v. 50, p. 1–7, 2001.

SÁNCHEZ-SAMPEDRO, L.; PERDIGUERO, B.; MEJÍ AS-PÉ REZ, E. The Evolution of Poxvirus Vaccines. *Viruses*, v. 7, n. 4, p. 1726–1803, 2015.

SCHATZMAYR, H. G.; COSTA, R. V. C.; GONÇALVES, M. C. R. et al. Infecções humanas causadas por poxvirus relacionados ao vírus vaccinia no Brasil. *Revista da Sociedade Brasileira de Medicina Tropical*, v. 42, n. 6, p. 672–676, 2009.

SCHATZMAYR, H. G.; SAMPAIO DE LEMOS, E. R.; MAZUR, C. et al. Detection of Poxvirus in Cattle Associated with Human Cases in the State of Rio de Janeiro: Preliminary Report. *Memorias do Instituto Oswaldo Cruz*, v. 95, n. 5, p. 625–627, 2000.

SEET, B. T.; JOHNSTON, J. B.; BRUNETTI, C. R. et al. Poxviruses and immune evasion. *Annual review of immunology*, v. 21, p. 377–423, 2003.

SILVA-FERNANDES, A. T.; TRAVASSOS, C. E. P. F.; FERREIRA, J. M. S. et al. Natural human infections with Vaccinia virus during bovine vaccinia outbreaks. *Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology*, v. 44, p. 308–13, 2009.

SINGH, R. K.; BALAMURUGAN, V.; BHANUPRAKASH, V. et al. Emergence and reemergence of vaccinia-like viruses: Global scenario and perspectives. *Indian Journal of Virology*, v. 23, n. 1, p. 1–11, 2012.

SMITH, G. L.; LAW, M. The exit of Vaccinia virus from infected cells. *Virus Research*, v. 106, n. 2, p. 189–197, 2004.

SOUZA, P. E. A; ROCHA, M. O. C.; MENEZES, C. A. S. et al. Trypanosoma cruzi infection induces differential modulation of costimulatory molecules and cytokines by monocytes and T cells from patients with indeterminate and cardiac Chagas' disease. *Infection and Immunity*, v. 75, n. 4, p. 1886–1894, 2007.

SPRIGGS, M. K.; KOLLERT, B. H.; SATOT, T. et al. b2-Microglobulin-, CD8+ T-cell-deficient mice survive inoculation with high doses of vaccinia virus and exhibit altered IgG responses. *Immunology*, v. 89, p. 6070–6074, 1992.

STANFORD, M. M.; MCFADDEN, G.; KARUPIAH, G.; CHAUDHRI, G. Immunopathogenesis of poxvirus infections: forecasting the impending storm. *Immunology and cell biology*, v. 85, n. 2, p. 93–102, 2007.

THORNTON, B. The antigenicity and immunogenicity of the virus. I: the production of high-titre vaccinia extracellular virus and its antigenicity after inactivation. *The British Journal of Experimental Pathology*, v. 61, p. 444–450, 1980.

TRINDADE, G. D. S.; DRUMOND, B. P.; GUEDES, M. I. M. C. et al. Zoonotic vaccinia virus infection in Brazil: Clinical description and implications for health professionals. *Journal of Clinical Microbiology*, v. 45, n. 4, p. 1370–1372, 2007a.

TRINDADE, G. S.; DA FONSECA, F. G.; MARQUES, J. T. et al. Aracatuba virus: a vaccinialike virus associated with infection in humans and cattle. *Emerging Infectious Diseases*, v. 9, n. 2, p. 155–160, 2003.

TRINDADE, G. S.; EMERSON, G. L.; CARROLL, D. S.; KROON, E. G.; DAMON, I. K. Brazilian vaccinia viruses and their origins. *Emerging Infectious Diseases*, v. 13, n. 7, p. 965–972, 2007b.

TRINDADE, G. S.; GUEDES, M. I. C.; DRUMOND, B. P. et al. Zoonotic vaccinia virus: clinical and immunological characteristics in a naturally infected patient. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*, v. 48, n. 3, p. e37–e40, 2009.

TRINDADE, G. S.; LOBATO, Z. I. P.; DRUMOND, B. P. et al. Short report: Isolation of two Vaccinia virus strains from a single bovine vaccinia outbreak in rural area from Brazil: Implications on the emergence of zoonotic orthopoxviruses. *American Journal of Tropical Medicine and Hygiene*, v. 75, n. 3, p. 486–490, 2006.

TURNER, G. S.; SQUIRES, E. J.; MURRAY, H. G. S. Inactivated smallpox vaccine . A comparison of inactivation methods Inactivation procedures. *Journal of Hygiene*, v. 68, p. 197–210, 1970.

VORA, S.; DAMON, I.; FULGINITI, V. et al. Severe Eczema Vaccinatum in a Household Contact of a Smallpox Vaccinee. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*, v. 46, n. 10, p. 1555–1561, 2008.

WORLD ASSEMBLY OF DELEGATES OF THE OIE. Tests for Sterility and Freedom From Contamination of Biological Materials. *OIE Terrestrial Manual*, n. May 2008, p. 105–114, 2012.

XIAO, C.; RAJPUT, Z. I.; HU, S. Improvement of a commercial foot-and-mouth disease vaccine by supplement of Quil A. *Vaccine*, v. 25, n. 25, p. 4795–4800, 2007.

XU, R.; JOHNSON, A.; LIGGITT, D.; BEVAN, M. J. Cellular and humoral immunity against vaccinia virus infection of mice. *The Journal of Immunology*, v. 172, p. 6265–6271, 2004.

YADAV, S.; HOSAMANI, M.; BALAMURUGAN, V. et al. Partial Genetic Characterization of Viruses Isolated from Pox-like Infection in Cattle and Buffaloes: Evidence of Buffalo Pox Virus Circulation in Indian Cows. *Archives of virology*, v. 155, n. 2, p. 255–261, 2010.

YAO, X.-D.; EVANS, D. H. High-Frequency Genetic Recombination and Reactivation of Orthopoxviruses from DNA Fragments Transfected into Leporipoxvirus-Infected Cells. *Journal of virology*, v. 77, n. 13, p. 7281–7290, 2003.

YENDO, A. C. A.; DE COSTA, F.; CIBULSKI, S. P. et al. A rabies vaccine adjuvanted with saponins from leaves of the soap tree (Quillaja brasiliensis) induces specific immune responses and protects against lethal challenge. *Vaccine*, v. 34, n. 20, p. 2305–2311, 2016.

Supplementary Information

UNIVERSIDADE FEDERAL DE MINAS GERAIS

CEUA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS[1]

CERTIFICADO
Certificamos que o Protocolo nº. 123 / 2012, relativo ao projeto intitulado “Desenvolvimento de vacina para prevenção da vaccínia bovina”, que tem como responsável Zélia Inês Portela Lobato, está de acordo com os Princípios Éticos da Experimentação Animal, adotados pela Comissão de Ética no Uso de Animais (CEUA/UFMG), tendo sido aprovado na reunião de 14/03/2013. Este certificado espira-se em 14/03/2018.

CERTIFICATE
We hereby certify that the Protocol nº. 123 / 2012, related to the Project entilted “Vaccine development to prevent bovine vaccinia”, under the supervision of Zélia Inês Portela Lobato, is in agreement with the Ethical Principles in Animal Experimentation, adopted by the Ethics Committee in Animal Experimentation (CEUA/UFMG), and was approved in 14/03/2013. This certificates expires in 14/03/2018.

FRANCISNETE GRACIANE ARAUJO MARTINS
Coordenador(a) da CEUA/UFMG

Belo Horizonte, 14/03/2013.

Atenciosamente.

Sistema CEUA-UFMG
https://www.ufmg.br/bioetica/cetea/ceua/

Universidade Federal de Minas Gerais
Avenida Antônio Carlos, 6627 – Campus Pampulha
Unidade Administrativa II – 2º Andar, Sala 2005
31270-901 – Belo Horizonte, MG – Brasil
Telefone: (31) 3499-4516 – Fax: (31) 3499-4592
www.ufmg.br/bioetica/cetea - cetea@prpq.ufmg.br

[1]Supplement I

[2]

INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL

06/05/2016 870160018315 16:14

00.000.2.2.16.0340791.4

Pedido nacional de Invenção, Modelo de Utilidade, Certificado de Adição de Invenção e entrada na fase nacional do PCT

Número do Processo: BR 10 2016 010341 0

Dados do Depositante (71)

Depositante 1 de 1

Nome ou Razão Social:	Universidade Federal de Minas Gerais
Tipo de Pessoa:	Pessoa Jurídica
CPF/CNPJ:	17217985000104
Nacionalidade:	Brasileira
Qualificação Jurídica:	Instituição de Ensino e Pesquisa
Endereço:	Av. Antônio Carlos, 6627 - Unidade Administrativa II - 2º andar- sala 2011
Cidade:	Belo Horizonte
Estado:	MG
CEP:	31270-901
País:	Brasil
Telefone:	(31) 34094774
Fax:	
Email:	patentes@ctit.ufmg.br

Dados do Pedido

Natureza Patente:	10 - Patente de Invenção (PI)
Título da Invenção ou Modelo de Utilidade (54):	"COMPOSIÇÃO VACINAL CONTRA INFECÇÃO POR VACCINIA BOVINA E USO"
Resumo:	A presente invenção se refere a uma composição vacinal compreendendo o antígeno Vaccinia virus amostra Guarani P2 (GP2) inativado preferencialmente por beta-propiolactona e adjuvantes à base de hidróxido de alumínio e saponina, sendo, preferencialmente, extrato oriundo de Quillaja saponaria. Refere-se também a seu uso para imunização de animais contra infecção por Vaccinia virus, a vaccínia bovina.
Figura a publicar:	1

[2] Supplement II