Priscilla Freitas Gerber

MOLECULAR AND SEROLOGICAL DIAGNOSIS OF HEPATITIS E VIRUS IN SWINE AND CHICKEN

A thesis submitted to the Escola de Veterinaria of Universidade Federal de Minas Gerais in partial fulfillment of the requirement of Doctor in Veterinary Science

Major: Preventive Veterinary Medicine
Major Professor: Zélia Inês Portela Lobato

Belo Horizonte
Escola de Veterinária - UFMG
2014

G362h Gerber, Priscilla Freitas, 1984-

Molecular and serological diagnosis of hepatitis E virus in swine and chicken / Priscilla Freitas Gerber. – 2014

53 p. :il.

Orientador: Zélia Inês Portela Lobato

Tese (Doutorado) – Universidade Federal de Minas Gerais, Escola de Veterinária

Inclui bibliografia

1. Suínos – Doenças – Teses. 2. Galinhas – Doenças – Teses. 3. Hepatite – Teses. I. Lobato, Zélia Inês Portela. II. Universidade Federal de Minas Gerais. Escola de Veterinária. III. Título.

CDD – 636.408 96

Tese defendida e aprovada em 10 de abril de 2014 pela Comissão Examinadora constituída por:

Profª. Zélia Inês Portela Lobato
Presidente - Orientadora

Prof. Amauri Alcindo Alfiéri
Universidade Estadual de Londrina

Drª. Maria Isabel Maldonado Coelho Guedes
Pós-Doutoranda - EV/UFMG

Dra. Sandra Yuliet Marin Gomez
Pós-Doutoranda - EV/UFMG

Dra. Alessandra Marnie Martins Gomes de Castro
Jovem Pesquisador - FMVZ/USP

DEDICATION

This thesis is dedicated to Sidney Gerber, vô Tote, vó Maria, and Lalinha (*in memorian*).

"Think of him still as the same. I say,
He is not dead—he is just away."

James Whitcomb Riley

ACKNOWLEDGEMENTS

It is extremely difficult to succeed without the support and guidance of others. Fortunately I had the chance to meet extremely generous mentors throughtout this journey. My most sincere gratitude and appreciation are dedicated to my supervisor, Prof. Zélia Lobato, for supporting and guiding me through the process of learning the scientific method and encouraging me to make my own discoveries. These past eight years of working under her guidance were a truly invaluable and rewarding experience. I also thank my committee members, Profs. Nelson Martins and Roberto Guedes, for the critical reading of this manuscript.

I am very thankful to Prof. Tanja Opriessnig for receiving me at Iowa State University and for providing me the opportunity to work with her, for her expert guidance and mentorship, for her encouragement and critical perspective that enriched my research. I am truly appreciative of the opportunity to have worked with her.

There are many others who have helped me accomplish this task. I thank Prof. Xiang-Jin Meng from Virginia Polytechnic Institute and State University, USA for providing samples and proteins used in this thesis. I thank Drs. Darrell Trampel, Chao-Ting Xiao and Luis Giménez-Lirola for their collaboration. I also acknowledge Kevin O'Neill, Cody Branstad, and Megan Ropella who contributed in numerous activities and helped me to manage the lab at ISU. It was an immense pleasure to learn and interact with all of them.

I am thankful to the *Vaccinia* research team and LPVA friends and colleagues for creating a wonderful and rewarding atmosphere. I am indebted to Dr. Maria Isabel Guedes for all her support and lab management at LPVA. I also thank Drs. Amanda Soriano, Alessandra Dias, Tércia Oliveira, Ana Carolina Matos, Izabelle Rehfeld and Aristóteles Gomes. Their scholarship and friendship has influenced me professionally and personally. I specially thank Grazielle Galinari for providing not only a great technical assistance but also for her supportive friendship. I also thank Prof. Marcos Bryan and Dr. Alessandra Castro (USP) for their feedback, advice and for giving opportunity to work together and for believing me and Profs. Leonaro Lara and Nelson Martins for facilitating the sample collection in chicken farms in Minas Gerais State.

In addition, I thank all my friends from Minas Gerais, Iowa, and beyond for their support. Special thanks to Vinícius and Orli Castro for their kind friendship and support over the years and Saleh Shahinfar for all caring and understanding throughout this process.

Finally, thank to my family, mom and Keilah, for always being supportive of my academic endeavors and for providing all love, understanding and comfort that I can always count on.

I received a scholarship from CAPES (grant # 5548110) during the first year of my internship abroad.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABREVIATIONS

BLSD – big liver and spleen disease
BLSV – big liver and spleen virus
CTV – cutthroat trout virus
DNA – deoxyribonucleic acid
ELISA – enzyme-linked immunosorbent assay
FMIA – fluorescent microbead immunoassay
HEV – hepatitis E virus
mHEV – mammalian hepatitis E virus
aHEV – avian hepatitis E virus
pHEV – piscine hepatitis E virus
HSS – hepatitis splenomegaly syndrome
ICTV – International Committee on the Taxonomy of Viruses
IgG – immunoglobulin G
IgY – immunoglobulin Y
kb – kilo bases
nm – nanometers
nt – nucleotide
ORF – open reading frame
RNA – ribonucleic acid
RT-PCR – reverse transcription polymerase chain reaction
WHO – World Health Organization

RESUMO

O vírus da hepatite E (HEV) tem sido identificado em várias espécies animais. Baseado no hospedeiro, os variantes genéticos podem ser divididos em HEV mamífero (mHEV), HEV aviário (aHEV) e HEV piscino. O objetivo do primeiro estudo foi comparar a performance de dois testes de PCR de transcriptase reversa (RT-PCR) para detecção dos quatro genótipos de mHEV em um único teste (testes A e B) e dois testes RT-PCR duplex para detecção e diferenciação do mHEV-3 e -4 (testes C e D). RNA extraído de 28 amostras de fezes de suínos experimentalmente inoculados com HEV-3 e 186 amostras de suínos a campo com exposição desconhecida ao HEV foram testados. Para os testes A, B, C e D, o RNA do HEV foi detectado respectivamente em 96,4%, 39,2%, 14,2% e 0% das amostras experimentais e em 67,2%, 36,4%, 1,1%, and 0,5% das amostras de campo. Os testes tiveram baixa concordância. Testes A e B tiveram maior taxa de detecção do que os testes C e D ($p < 0.05$). No segundo estudo, 40 amostras fecais foram coletadas de suínos de 7, 10, 13 ou 17 semanas em 10 granjas. Vinte e nove (72,5%) amostras foram positivas para o RNA do HEV através de RT-PCR. As seis sequências obtidas eram do genótipo 3. No terceiro estudo, 160 amostras de soro de galinhas entre 6 a 118 semanas foram coletadas em 3 granjas e testadas para anticorpos anti-aHEV através de um teste the micro-esferas fluorescentes. Anti-aHEV IgY form detectados em todas as granjas estudadas e em 17% das galinhas. Quarenta amostras de fezes de 8 granjas foram testadas para o RNA do aHEV e 3 (8%) amostras foram positivas para o gene da helicase. Este estudo mostra evidência da circulação do aHEV em galinhas no Brasil.

Palavras-chave: HEV, suíno, galinha, diagnóstico

ABSTRACT

Hepatitis E virus (HEV) has been identified in several animal species. Based on the host tropism the strains can be clustered into mammalian HEV (mHEV), avian HEV (aHEV), and in piscine HEV strains. The aim of the first study was to compare the performance of two single-plex reverse transcriptase (RT)-PCR assays for broad detection of all four mHEV genotypes (assays A and B) and two duplex RT-PCR assays for detection and differentiation of mHEV-3 and -4 (assay C and D). RNA extracted from 28 fecal samples from pigs experimentally inoculated with HEV-3 and 186 fecal samples from commercial pigs with unknown HEV exposure were tested. For assays A, B, C and D HEV RNA was detected respectively in 96.4%, 39.2%, 14.2%, and 0% of the experimental samples, and in 67.2%, 36.4%, 1.1%, and 0.5% of the field samples. Assays showed an overall poor agreement. Assays A and B had higher detection rates for HEV RNA than assays C and D ($p < 0.05$). In the second study, 40 fecal samples were collected from pigs at 7, 10, 13 or 17 weeks of age in 10 farms. Twenty nine (72.5%) samples tested positive for HEV RNA by RT-PCR. All 10 farms had at least one positive sample. All six yield sequences clustered in genotype 3. In the third study, 160 serum samples from chicken ranging from six to 118-weeks of age were collected on three farms and tested for aHEV antibodies by a fluorescent microbead-based assay. Anti-aHEV IgY were detected in 17% of the chickens. Forty pooled fecal samples from eight farms were tested for aHEV RNA by RT-PCR and three (8%) were positive for the helicase gene. This work provides evidence of circulation of aHEV in the Brazilian chicken population.

Key-words: HEV, swine, chicken, diagnostic

1. INTRODUCTION

> *"Since its discovery 30 years ago, hepatitis E has been a neglected disease in terms of research funding. In fact, it has been so neglected that it does not even make the WHO [World Health Organization] list of 'neglected tropical diseases'" (Scobie et al., 2013).*

Hepatitis E was first recognized in 1980s by retrospective studies of water-borne epidemics of hepatitis in India (Khuroo, 1980). This agent was initially known as the enterically transmitted non-A, non-B hepatitis virus and was subsequently named the hepatitis E virus (HEV) based on its enteric transmission and association with hepatitis epidemics (Reyes et al., 1991). Currently, HEV is recognized as an pathogen worldwide (Kamar et al., 2012). HEV infection in pregnant women may cause particularly severe illness with a mortality rate of 10-20% and there is evidence of HEV persistent infection in immunocompromised patients (Kamar et al., 2012). Thus far, at least four recognized and two putative genotypes of mammalian HEV (mHEV) have been identified worldwide. The genotypes 1 and 2 of HEV infect only humans, while genotypes 3 and 4 have an expanded host range and are zoonotic (Meng, 2013). Confirmed zoonotic human infections arise primarily from consumption of contaminated pork products and deer meats in industrialized countries (Meng, 2013). The infection by mHEV is widespread in pigs and it appears to be asymptomatic (Meng et al., 2012).

Epidemiology of mHEV has been investigated in Brazil, and swine HEV RNA has been identified in pigs from different regions (dos Santos et al., 2011; de Souza et al., 2012; Gardinali et al., 2012). Consumption of pork meat has been suggested as the most probable cause of an autochthonous case of acute hepatitis E in the country (Lopes dos Santos et al., 2010). Because of the great genomic heterogeneity between human and swine strains from different geographic origin (Lopes dos Santos et al., 2010; dos Santos et al., 2011; Gardinali et al., 2012; Passos et al., 2013), more studies to better understand HEV molecular epidemiology and its impacts on Brazilian pigs herds and the HEV zoonotic transmission are needed.

Avian HEV (aHEV) was identified in chickens displaying decreased egg production and increased mortality and it was considered the most economically significant pathogen affecting broiler breeder flocks in Australia in the 1990s (Payne et al., 1999). aHEV is genetically and antigenically related to human and swine HEVs (Payne et al., 1999; Haqshenas et al., 2001). Phylogenetic analysis revealed that aHEV forms a separate genus, consisting of at least three different genotypes and it is not considered a zoonosis (Meng et al., 2012). It has been shown that a considerable proportion of chicken flocks in North America, Europe, and Asia were seropositive to aHEV infection although seropositive flocks did not necessarily suffer from disease (Huang et al., 2002b; Peralta et al., 2009a; Kwon et al., 2012). Although there is serological evidence of aHEV circulation in Brazilian chickens based on testing of a limited number of samples (n = 25) (Vitral et al., 2005), the status of avian HEV infection in South American chickens is largely unknown. Therefore, it is important to investigate the HEV circulation in Brazilian chicken farms.

In this study, HEV infections were investigated in pigs and chickens from commercial farms in Minas Gerais state, southeast Brazil.

2. LITERATURE REVIEW

2.1 HEV biology and classification

2.1.1 Morphology, genome organization and replication

HEV is a spherical, non-enveloped virus of about 30 nm in size and with an icosahedral symmetry (Balayan et al., 1983; Xing et al., 1999). The viral genome is a single-stranded positive sense RNA strand of approximately 7.2 kb in length that consists of a short 5' non-coding region (NCR) and a 3' NCR, and three partially overlapping open reading frames (ORFs) (Tam et al., 1991). ORF1 encodes non-structural proteins. Putative functional motifs and domains such as methyltransferase, papain-like cysteine protease, helicase, and RNA-dependent RNA polymerase have been identified in ORF1 (Koonin et al., 1992) (Fig. 1). ORF2 encodes a capsid protein, and ORF3 encodes a small phosphorated protein that is associated with cytoskeleton (Zafrullah et al., 1997; Graff et al., 2005) (Fig. 1). aHEV is

genetically related to mHEV with conserved genomic organization and function despite a 600 nt deletion (Haqshenas et al., 2001; Huang et al., 2004). The capsid protein of aHEV contains both unique and conserved antigenic epitopes in comparison to the human and swine HEV capsid proteins (Dong et al., 2011).

The HEV replication cycle is currently not well understood due to the limited success in generating an efficient cell culture system (Berto et al., 2013; Okamoto, 2013; Rogee et al., 2013). HEV attaches to the host cell via a specific high-affinity receptor and enters the cytoplasm by clathrin-mediated endocytosis (Kapur et al., 2012). However, virtually nothing is known about the mechanism by which HEV enters susceptible cells, or about processing of proteins, and mechanisms of virus assembly and release (Okamoto, 2013). The new *in vitro* culture systems that support HEV replication and release of encapsidated RNA will facilitate the understanding of the biology of this virus.

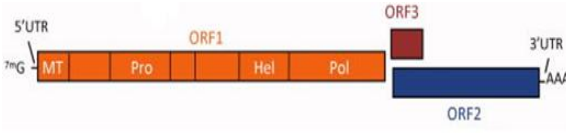

Fig. 1. HEV genome organization. The genomic RNA carries three open reading frames (orfs) that encode the nonstructural ORF1 (orange), capsid ORF2 (blue), and phosphorated ORF3 (brown) proteins. Reproduced from Aggarwal and Jameel, 2011.

2.1.2 HEV genetic diversity and nomenclature

The classification of HEV is currently in transition. According to the most recent report from the International Committee on the Taxonomy of Viruses (ICTV) (Meng et al., 2012), HEV belongs to the family *Hepeviridae*, which posses a single genus, *Hepevirus*, that includes four recognized genotypes and at least two putative new genotypes, along with a floating species of aHEV. All the viruses within the genus *Hepevirus* infect mammals and have been genetically identified from humans, pig, mongoose, deer, rat, rabbit, and ferret (Meng, 2011).

HEV genotypes 1 and 2 are restricted to humans and related to waterborne large outbreaks of hepatitis E in developing countries (Kamar et al., 2012). HEV genotype 1 occurs mainly in Asia (Lu et al., 2006); however, it has also been reported in autochthonous cases in Cuba (Villalba et al., 2008) and Venezuela (Garcia et al., 2012). HEV genotype 2 consists of a single Mexican strain and some African strains (Lu et al., 2006). HEV genotype 3 circulates worldwide and contains strains from sporadic and cluster cases, and from chronic cases of hepatitis E in humans (Kamar et al., 2012) and from several animal species including pig, deer, rat, mongoose, and rabbit (Meng, 2013). HEV genotype 4 circulates mainly in Asia; however, it has been reported recently in Europe (Hakze-van der Honing et al., 2011; Colson et al., 2012; Tesse et al., 2012) and includes strains from sporadic and cluster cases of hepatitis E in humans and animal HEV strains from pigs. The two putative new genotypes of HEV include strains from rat and ferret (Johne et al., 2010; Purcell et al., 2011; Raj et al., 2012), and a novel strain of HEV from wild boars in Japan (Sato et al., 2011; Takahashi et al., 2011). A tentative genus *Orthohepevirus* has been proposed to contemplate the above mentioned mammalian strains of HEV (Meng, 2013) (Table 1, Fig. 2). The geographic distribuition of the tentative genus *Orthohepevirus* genotypes 1 to 4 is shown on Fig. 3.

Avian HEV has only been reported in chickens and it is genetically and phylogenetically distinct from the mHEV, sharing only ~50% nucleotide sequence identity (Bilic et al., 2009). The tentative genus *Avihepevirus* has been proposed to include the three genotypes of avian HEV thus far recognized: genotype 1 in Australia and Korea, genotype 2 in the United States, and genotype 3 in Europe and China (Bilic et al., 2009; Marek et al., 2010; Kwon et al., 2012) (Table 1, Fig. 2).

The novel strain of HEV identified in fish, the cutthroat trout virus (CTV) (Batts et al., 2011) shares only 13% to 27% sequence identity with known mammalian and avian HEV strains and was grouped in the tentative genus *Piscihepevirus.* Bats strain (Drexler et al., 2012) was grouped in the tentative genus *Chiropteranhepevirus* (Meng, 2013) (Table 1, Fig. 2).

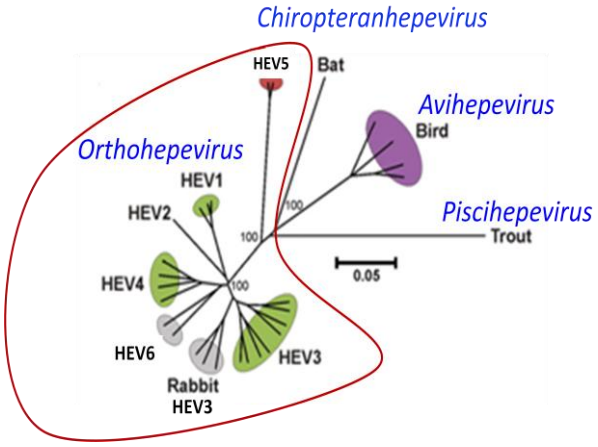

Fig. 2. Neighbor-joining phylogeny of the complete genomes of members of the *Hepeviridae* using the nucleotide percentage distance substitution matrix and complete deletion option in MEGA5. Adapted from Drexler et al. 2012.

Table 1. Nomenclature of the hepatitis E virus (HEV) as proposed by Meng et al. (2013).

Proposed genera	Natural hosts
Orthohepevirus	
Genotype 1	Human
Genotype 2	Human
Genotype 3	Human, domestic and wild pig, deer, mongoose, rabbit, rat
Genotype 4	Human, domestic and wild pig, cattle, sheep
Putative genotype 5	Rat, ferret
Putative genotype 6	Wild pig
Avihepevirus	
Genotype 1	Chicken (Australia, Korea)
Genotype 2	Chicken (USA, Canada)
Genotype 3	Chicken (Europe, China)
Piscihepevirus	
Cutthroat trout virus	Brown, Apache, and Gila trouts
Chiropteranhepevirus	
Bat HEV	Bat

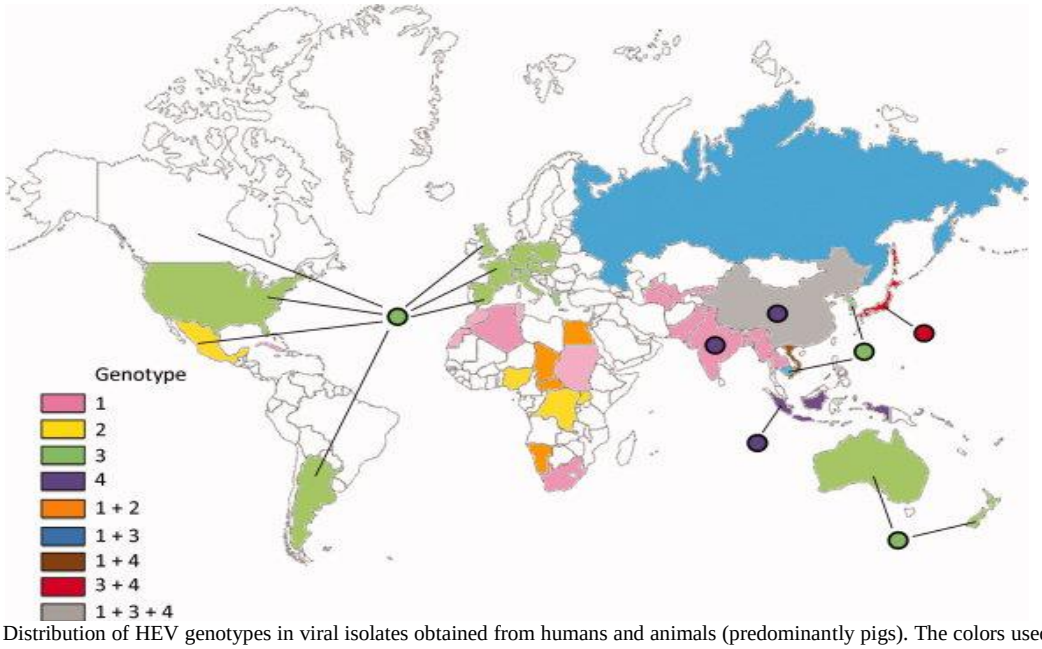

Fig.3. Distribution of HEV genotypes in viral isolates obtained from humans and animals (predominantly pigs). The colors used for a country and the circle associated with it represent the predominant HEV genotypes of human and animal isolates, respectively, from that country. The figure is based on data from Okamoto, 2007. Reproduced from Aggarwal and Jameel, 2011.

2.1.3 HEV classification: genotypes and subgenotypes

The ICTV does not include any consideration below the species level and this has been left to the initiative of specialty groups (Fauquet et al., 2005). The most current ICTV report recognizes four genotypes of HEV (Meng et al., 2012) and there is a new proposal to further divide the *Hepeviridae* family into four genera (Meng, 2013); however, it does not explain the methodology used for the classification and genotype demarcation. Different phylogenetic methods such as pairwise distances or bootstrap values of the resulting neighbor-joining or maximum likelihood trees have been used

(Arankalle et al., 1999; Worm et al., 2002; Lu et al., 2006; Zhai et al., 2006; Okamoto, 2007; Bouquet et al., 2012) contributing to differences in the classification among groups. Recently, there has been a controversy about whether isolates from rabbits and wild boar should be considered new genotypes or subtypes (Zhao et al., 2009; et al.; Cossaboom et al., 2011; Takahashi et al., 2011; Bouquet et al., 2012).

The rapid increase in sequencing of different genomic regions and different fragment sizes in addition to the lack of commonly agreed criteria to define different taxonomic categories have resulted in multiple genotype/subgenotype definitions applied to HEV (Arankalle et al., 1999; Worm et al., 2002; Lu et al., 2006; Zhai et al., 2006; Okamoto, 2007; Bouquet et al., 2012; Smith et al., 2013), complicating the comparison of molecular epidemiology data worldwide. There is no agreement in the assignment of isolates to particular virus subtypes that would result, for example, in recognition of 4 genotypes mHEV subdivided into 24 (Lu et al., 2006) or 12 (Zhai et al., 2006) subtypes. Lu et al. (2006) in a comprehensive analysis of the bootstrap support for phylogenetic groupings and the nucleotide distances between these groupings, suggested that genotypes 1, 2, 3 and 4 could be classified into five (1a-1e), two (2a-2b), ten (3a-3j) and seven (4a-4g) subtypes, respectively. To make this taxonomic assignment, Lu et al. (2006) examined not only HEV complete genomes but sequences from the 5' and 3' end of ORF1 regions and three separate ORF2 regions. The 5' end of the ORF2 was pointed as the region that better reflected the complete genomic sequence for HEV classification (Lu et al., 2006).

More recently, Smith et al. (2013) suggested a classification system based on the amino acid sequences of concatenated ORF1/ORF2 coding regions excluding the hypervariable region. Using this criterion, the *Hepevirus* genus would include four species, including HEV genotypes 1 to 4, and the putative genotype 6 as proposed by the ICTV (Meng et al., 2012), avian HEV, bat HEV and rat/ferret HEV. Cutthroat trout virus would represent a second genus.

aHEV nomenclature also lacks a consensus criteria and the designations given in different studies still give risen to confusion. Phylogenetic analysis of near-complete aHEV genomes from Australia, United States and Europe revealed three large clusters, corresponding to their geographical origin (Bilic et al., 2009). Subsequent analysis of a 130 nt fragment of the helicase gene using more isolates revealed greater diversity, and putative genotypes 4 and 5 were proposed (Marek et al., 2010). However, putative genotypes 4 and 5 strains clustered with aHEV genotype 3 when a 124 nt fragment of the capsid gene was used (Marek et al., 2010). More recently, a putative novel genotype was proposed based on a near-complete genome sequence from a Hungary isolate (Banyai et al., 2012); however, this sequence was not taken into consideration in the new *Avihepevirus* classification proposal (Meng, 2013). New genotypes have often been defined based on the presence of phylogenetic branches that have substantial bootstrap support. However, in many instances, there is a hierarchy of such branches so that it becomes difficult to decide which branches are taxonomically informative (Smith et al., 2013), leading to differences in the classification among groups.

The nomenclature and classification criteria of HEV need to be standardized to ensure that epidemiological, evolutionary and clinical observations among strains can be accurate and informative molecular epidemiological tools.

2.2 HEV pathogenesis

2.2.1 HEV infection in humans

Until recently, it was thought that HEV was only an acute self-limiting disease in the majority of patients (Aggarwal et al., 2011). However, awareness of HEV infection has expanded in the last few years and it is now known that the occurrence of HEV infection is underestimated, especially in developed countries (Kamar et al., 2013a). Clinical presentation of hepatitis E include acute icteric hepatitis, anicteric illness with nonspecific flu-like symptoms, and asymptomatic transaminase elevation (Aggarwal et al., 2011). The case fatality rate during epidemics was found to be between 0.2% and 4% (Kumar et al., 2013). However, fulminant hepatitis and high mortality are described, reaching 20% in cases of pregnant women infected with genotype 1 in developing countries and 70% in of patients infected with genotype 3 with underlying liver

disease (Kamar et al., 2012). HEV infection can become chronic in immunocompromised patients such as solid organ transplant recipients, patients receiving chemotherapy, and patients with HIV infection, and the course of disease progress to a chronic state and persistence of viral shedding in 65-80% of the cases and may rapdly progress to cirrhosis (Moal et al., 2013; Riveiro-Barciela et al., 2013; Zhou et al., 2013). Furthermore, HEV infection has been associated to extrahepatic manifestations, including neurologic symptoms, kidney injuries, pancreatitis, and hematological disorders (Kamar et al., 2013b).

To date, four major documented routes of HEV transmission have been reported: waterborne; foodborne; bloodborne, and vertical transmission (Kaba et al., 2013). Research among non-human primates has shown a direct association between infective dose and disease severity, but an inverse relationship to the incubation period (Tsarev et al., 1992; Tsarev et al., 1994). Incubation time ranges from 2 weeks to 2 months, viremia is transient and occurs mainly during the prodromic phase, and disappears at the onset of clinical symptoms, when viral shedding begins and regresses at the onset of jaundice within 2 to 3 weeks (Aggarwal et al., 2000). Vertical transmission from mother to fetus has been reported in 33 to 100% of the cases (Kaba et al., 2013). Human to human transmission of HEV is considered rare (Kaba et al., 2013). HEV infection is still an underdiagnosed disease and further studies are required to determine its prevalence, incidence, and control.

2.2.2 HEV infection in swine

Since its discovery in domestic swine in the United States in 1997, swine HEV strains have been identified worldwide in swine with widely variable prevalence (Meng, 2010). A retrospective study performed in Spain showed that HEV was endemic in the Spanish pig farms at least since 1985 (Casas et al., 2009). Studies of prevalence across Japan revealed that anti-HEV antibody is present in 93% of all domestic pig farms tested and that all swine HEV isolates belong to either genotype 3 or 4 (Takahashi et al., 2013). In Spain, the prevalence of anti-HEV antibodies on commercial swine farms reached 98%, while the anti-HEV prevalence in New Zealand and Brazil is 90% and 81%, respectively (Garkavenko et al, 2004; dos Santos et al., 2009). In the Netherlands, domestic pig farms carried a prevalence of 55% for HEV RNA in the feces, while 86.2% and 47.1% of 18 week-old pigs in Canada shed HEV virus in feces and were viremic, respectively, with a decrease as the pigs aged (Leblanc et al., 2007; Rutjes et al., 2007).

Natural and experimental infections with HEV genotypes 3 and 4 swine result in a subclinical course of infection with only mild microscopic lesions in the liver and associated lymph nodes (Meng et al., 1997; Halbur et al., 2001). Viremia lasts 1-2 weeks while fecal shedding lasts 3-7 weeks (Meng et al., 1997; Halbur et al., 2001). HEV infection in swine is age dependent with up to 86% pigs are viremic by 18 weeks of age (Leblanc et al., 2007). Studies from the United Kingdom, Spain, Japan and Brazil further demonstrated that the highest fecal virus shedding occurred by 10-12 weeks, 13-16 weeks, 1-3 months and 13 weeks of age, respectively (Nakai et al., 2006; de Deus et al., 2008a; McCreary et al., 2008; Gardinali et al., 2012). Seroconversion to HEV antibodies in swine occurs following the typical waning of maternal antibody levels around 8-10 weeks of age first with IgM anti-HEV antibodies peaking concomitant with fecal viral shedding followed by IgG anti-HEV antibodies peaking concomitant with clearance of virus from the feces (Nakai et al., 2006; de Deus et al., 2008a; McCreary et al., 2008; dos Santos et al., 2009). Transmission between swine is fecal-oral with large amounts of infectious HEV being shed in the feces, and direct contact between animals, with manure, and with potentially contaminated water sources in swine facilities contribute to transmission within a herd (Drobeniuc et al., 2001; Kasorndorkbua et al., 2004; Bouwknegt et al., 2008b; Feagins et al., 2008a). Although HEV infection in pigs does not pose as a major economical concern in swine production, the risk of zoonotic transmission to humans is an important public health concern (Pavio et al., 2010).

2.3 HEV environmental contamination and waterborne transmission

2.3.1 Waterborne and shellfish transmission

HEV is typically transmitted via fecal-oral route within an animal species, from animals to humans in infectious body fluids, and from contaminated water sources to human and other animals (Yugo et al., 2013). In developing countries, outbreaks usually occur during the rainy season, floods or the monsoon, conditions that facilitate the contamination of sources of drinking water by human excreta (Kaba et al., 2013). In both developing and developed countries, infectious HEV strains has been isolated from sewage, water treatment plants and rivers` water (Jothikumar et al., 1993; Vaidya et al., 2003; Borgen et al., 2008; Clemente-Casares et al., 2009; Rutjes et al., 2009). HEV contamination related to consumption of food items receiving sewage sludge, such as shellfish, that are most often eaten raw or slightly cooked have been reported in European and Asian countries (Cacopardo et al., 1997; ; Renou et al., 2008; Said et al., 2009; Song et al., 2010; Namsai et al., 2011; Diez-Valcarce et al., 2012). In Scotland, 92% of bivalve mussels collected were tested positive for HEV RNA with the viral sequences clustering with HEV genotype 3 identified in human and swine (Crossan et al., 2012).

In addition, HEV genotype 3 RNA has been detected on irrigated, field-grown strawberries (Brassard et al., 2012). Drinking water from non-public supplies was identified as a risk factor for acquiring hepatitis E in Spain (Galiana et al., 2008) and France (Renou et al., 2008). Therefore, as in developing countries, the environment should be considered as a potential source of HEV transmission in developed countries.

2.3.2 Environmental and animal handling transmission

Swine is a major reservoir for HEV and occupational contact with infected swine is a risk factor for zoonotic HEV transmission in humans. Professionals working in close proximity to swine, swine manure, or sewage may become infected with HEV during occupational activities (Galiana et al., 2008). Run-offs from animal facilities such as hog operations have been implicated in human HEV infections with the detection of infectious HEV genotype 3 in the animal manure and wastewater (Borgen et al., 2008; Rutjes et al., 2009). Swine veterinarians in the United States were shown to have 27% seropositivity to HEV in comparison to 16% of the normal blood donors (Meng et al., 2002). Swine workers in Valencia, Spain were found to be 5.4 times more likely to be positive for anti-HEV IgG than those not exposed to swine (Galiana et al., 2008). In the Netherlands, 11% of swine veterinarians were positive in comparison to 6% of non-swine veterinarians and 2% of the general population (Bouwknegt et al., 2008a). Similarly, swine farmers showed at least two-fold more chance to be seropositive than general population in Sweden and Maldova (Olsen et al., 2006; Bouwknegt et al., 2008a). In addition, an acute hepatitis E case was reported in a person who had been given a pet pig 2 months before the onset of symptoms. HEV sequences recovered from the patient were closed related to the sequence identified from the animal (Renou et al., 2007).

The multitude of novel strains of HEV in wildlife and other domestic animal species suggest additional mechanisms of transmission. For example, field workers at the Iowa Department of Natural Resources who work with a variety of wildlife species had a higher prevalence for HEV antibodies in comparison to normal blood donors (Karetnyi et al., 1999). In studies conducted in France and Japan, hunting has been found to be associated with the higher prevalence of anti-HEV antibodies in blood donors (Mansuy et al., 2008; Toyoda et al., 2008). Finally, contact with horses and pets have been associated with a higher prevalence of anti-HEV antibodies in multivariate analysis (Christensen et al., 2008; Kuniholm et al., 2009). While exposure to HEV, identified by the presence of anti-HEV antibodies in these populations, does not in itself indicate a disease, it does identify a potential route of transmission and exposure.

2.4 HEV foodborne transmission

Many sporadic and cluster cases of hepatitis E have been linked to the consumption of contaminated raw or undercooked animal meat and meat products (Masuda et al., 2005;

Matsubayashi et al., 2008). The strongest evidence of zoonotic transmission of HEV has come from studies conducted in Japan, and was associated with eating not thoroughly cooked deer or wild boar meat; HEV sequences recovered from leftover frozen meats and from patients infected with HEV were identical (Li et al., 2005; Pavio et al., 2010). Further evidence supporting the zoonotic transmission of HEV is through the consumption of contaminated raw or undercooked food products from pigs purchased in supermarkets or from wild boars (Borgen et al., 2008; Wichmann et al., 2008; Colson et al., 2010; Wilhelm et al., 2011; Miyashita et al., 2012). HEV sequences recovered from suspected commercial pig livers or pig liver sausages were closely related, or identical in some cases, with those recovered from patients infected with HEV (Matsubayashi et al., 2008; Colson et al., 2010; Wilhelm et al., 2011). In addition, based on case-control studies conducted in France and Germany, consumption of uncooked pig liver sausage, game meats or offal was identified as a risk factor for HEV infection (Wichmann et al., 2008; Colson et al., 2010; Legrand-Abravanel et al., 2010).

Approximately 2% of the pig livers sold in local grocery stores in Japan (Yazaki et al., 2003), 4% in Germany (Wenzel et al., 2011), 6.5% in the Netherlands (Bouwknegt et al., 2007), and 11% in the United States (Feagins et al., 2008a) tested positive for the HEV genotype 3 RNA. Investigations in pork product chains in Italy, the Czech Republic, Spain, and the United Kingdom revealed detectable, infectious HEV at both processing locations and point of sale (Di Bartolo et al., 2011; Di Bartolo et al., 2012). The potential widespread dissemination of HEV through pork production chains and the potential foodborne transmission are of significant concern (Purcell et al., 2010).

2.5 HEV diagnosis

2.5.1 Human HEV diagnosis

Diagnosis of hepatitis E relies on laboratory abnormalities in liver enzyme and liver function tests. Since cases of hepatitis E are difficult to distinguish clinically from other types of acute viral hepatitis, the diagnosis is established by a combination of serological and molecular techniques (Kumar et al., 2013).

Several assay systems have been developed for the detection of IgM and IgG anti-HEV antibodies, although there is considerable variability in their sensitivities and specificities (Aggarwal, 2013). The most frequently used format for anti-HEV assays has been indirect enzymatic immunoassays, because of its high sensitivity, relative ease of performance and adaptability to high-throughput testing (Aggarwal, 2013). Drobeniuc et al. (2010), in a pan-genotypic validation of six commonly available IgM assays have found that only two of these assays had sensitivity and specificity above 95%. Sensitivity of the different assays ranged from 72% to 98%, and specificity from 78% to 96%. κ-coefficients for agreement between results of various pairs of tests varied from 0.42 to 0.80 (mean = 0.53). Analytical sensitivities of various tests were compared using serial dilutions of a particular serum specimen and varied up to 15-fold. This lack of consistency makes comparison of the diagnosis of HEV infection using different tests difficult.

Analysis of HEV RNA by using nucleic acid amplification techniques is also used for diagnosis; this method can identify active infection and help confirm serologic results (Aggarwal, 2013). Several *in-house* conventional and real-time RT-PCR assays targeting either ORF2 or ORF3 genes have been developed. However, the sensitivity of these assays varies significantly, depending on the targeted region and viral genetic diversity (Abravanel et al., 2012). In a study published in 2011, a panel of 22 plasma specimens that were positive for HEV RNA and included serial 10-fold dilutions of four different strains of HEV genotypes 3 or 4 and two control specimens negative for HEV RNA were tested by 20 laboratories (using 12 assays) in 10 countries (Baylis et al., 2011). The majority (59%) of the 17 laboratories that provided method details were using the same assay (i.e., Jothikumar et al., 2006). The analysis revealed a 100–1,000-fold difference in sensitivity between different assays, and the assay developed by Jothikumar et al., (2006) presented the highest sensitivity (limit of detect detection of approximately 4 HEV genome equivalents per reaction). The high sensitivity of this assay associated to its ability to detect all four mHEV that infect humans contributed to its widespread usage in diagnostic laboratories. However, false negative

RT-PCR results have been reported using it and a modification in the probe has been suggested to restore the sensitivity of this assay (Garson et al., 2012). Accurate RT-PCR assays are needed to avoid misdiagnosed infections, because serological testing is less sensitive in immunocompromised patients.

2.5.2 Swine HEV diagnosis

Since HEV infection is asymptomatic in swine, HEV diagnosis is not performed routinely in pigs. HEV serology or detection of HEV RNA is performed usually in research laboratories that have developed assays adapted to swine (Engle et al., 2002; de Deus et al., 2008a; Casas et al., 2009; Zhang et al., 2011). However, pig is now a recognized reservoir for zoonotic HEV infection (Meng, 2013) and it is important to measure level of viral contamination in pig herds. HEV has been cited recently as a pathogen that should be eliminated from any herd used for xenotransplantation (Fishman et al., 2012), and therefore, appropriate diagnosis are required for detection and elimination of HEV in pigs used for this purpose.

Conventional or real-time RT-PCR assays have been developed in independent laboratories targeting ORF1, ORF2 or ORF3 of HEV (Wang et al., 1999; Huang et al., 2002a; Jothikumar et al., 2006; Gyarmati et al., 2007; Zwettler et al., 2012). Broadly reactive RT-PCRs developed to detect human HEV have also been used to monitor HEV in pig herds (Cooper et al., 2005; Andraud et al., 2013). Since only one serotype of HEV has been described, detection of anti-HEV antibodies in swine can be performed using commercial kits for humans adapted to swine or *in-house* ELISA tests based on genotypes 1 and/or 3 antigens (Zhang et al., 2011). Recently, a fluorescent micro-beaded immunoassay (FMIA) has been developed for the detection of anti-HEV IgG antibodies in pigs and compared to an in-house ELISA based on the same antigen, a recombinant HEV ORF2 protein (Owolodun et al., 2013). Results indicate an almost perfect agreement between the two assays; however the FMIA has the advantage of utilizing considerably less amount of antigen compared to an ELISA and also allows screening of antibodies to multiple pathogens or antigens simultaneously in one reaction well (Owolodun et al., 2013). Therefore, it reduces the time and labor required for assay performance and also requires a smaller amount of sample thereby further reducing cost.

Although various assays have been reported for the monitoring of HEV infection in pig herds, it is worth noting that these assays have not been validated due to the absence of appropriate gold standards (Pavio et al., 2010).

2.6 Avian HEV infection and disease

The first descriptions of an infectious disease in broiler breeders named big liver and spleen disease (BLSD) were reported in Australia in 1980s (Handlinger et al., 1988). Based on a 523-nt sequence, the causative agent, big liver and spleen virus (BLSV), was found to be genetically related to human HEV with about 62% nucleotide sequence identity (Payne et al., 1999). In this period, it was considered the most economically significant disease affecting commercial breeder flocks in Australia, causing reduced egg production and a slight increase in mortality (Payne et al., 1999). A similar disease, hepatitis-splenomegaly syndrome (HSS) was reported in Canada in 1991 (Ritchie et al., 1991) and a HEV-like virus was identified in the USA from chickens affected with HSS (Haqshenas et al., 2001). Based on the genetic relatedness of this novel chicken virus with HEV, it was designated avian HEV to distinguish from human HEV and swine HEV. aHEV shared about 80% nucleotide sequence identity with BLSV, and about 57-61% nucleotide sequence identity with mammalian HEVs (Haqshenas et al., 2001). These two previously identified syndromes (HSS and BLSD) are now recognized to be caused by variant strains of the aHEV (Bilic et al., 2009; Marek et al., 2010; Meng, 2013).

2.6.1 HEV infection in chickens

aHEV RNA can be detected in broiler breeders hens and laying hens with and without clinical signs (Huang et al., 2002b; Peralta et al., 2009a; Sun et al., 2004a). aHEV has been shown to cross species barriers and infect turkeys (Sun et al., 2004b), however, rhesus monkeys and mice are not susceptible to infection by aHEV under experimental conditions (Huang et al., 2004).

Based on serological evidence, aHEV is widespread in chicken flocks with seropositive rates of approximately 71% in the USA, 90% in

Spain, and 57% in Korea (Huang et al., 2002b; Peralta et al., 2009a; Kwon et al., 2012). aHEV infection in chickens is age-dependent with 17% of seropositive chickens under 18 weeks of age and 36% of seropositive adult chickens (Huang et al., 2002b; Zhao et al., 2013). Under natural conditions, chickens become infected at approximately 3-4 months of age (Sun et al., 2004a). The relatively low doses of virus that can be transmitted among chickens via the fecal-oral route has been speculated as the reason of the subclinical infection in the majority of chickens (Sun et al., 2004a). Differences in virus strain, virus dose, diet and age have been implicated as potential co-factors for the manifestation of the full spectrum of clinical disease (Agunos et al., 2006; Meng et al., 2008). Nevertheless, aHEV strains recovered from healthy chickens in normal flocks and previously considered to be avirulent were only slightly attenuated in an experimental infection model (Billam et al., 2009). There was no clear clustering of aHEV sequences based on whether viruses were sampled from chickens displaying clinical signs or not (Marek et al., 2010).

Clinical signs usually affects 30 to 72 weeks-old chickens and may include egg drop in some flocks up to 20%, enlargement of the liver and spleen, and acute death of affected chickens (Meng et al., 2008). In affected flocks, mortality rates can be up to 0.3-1.0% a week (Meng et al., 2008; Morrow et al., 2008). Post-mortem evaluations show enlarged livers, enlarged spleens, and serosanguineous fluid in their coelomic cavities accompanied by a drop in egg production and high mortality rates (Billam et al., 2005; Meng et al., 2008). Typical histopathological changes include necrotizing, hemorrhagic, and non-specific hepatitis (Meng et al., 2008). In specific-pathogen-free (SPF) chickens experimentally inoculated either by oronasal route or by intravenous route with aHEV strains recovered from a chicken with HSS developed microscopic liver lesions characterized by lymphocytic and heterophilic periphlebitis, phlebitis and fibrinoid necrosis (Billam et al., 2005; Billam et al., 2009). Also, approximately 25% of the infected chickens developed sub capsular hemorrhages or enlargement of the right intermediate lobe of the liver (Billam et al., 2005). Similar gross and microscopic findings have been described in chickens naturally infected with aHEV in field outbreaks of HSS (Agunos et al., 2006; Morrow et al., 2008).

2.6.2 aHEV diagnosis

aHEV diagnosis is primarily based on detection of avian HEV RNA by RT-PCR in feces, liver, or bile, and anti-aHEV IgY antibodies by ELISA (Meng, 2010). An indirect ELISA was developed by using a truncated ORF2 protein of aHEV genotype 2, and its cross-reactivity with human and swine HEVs was shown (Haqshenas et al., 2002). On the basis of this ELISA, several studies about the seroprevalence of avian HEV were carried out in North America, Europe and Asia (Huang et al., 2002b; Peralta et al., 2009a; Kwon et al., 2012). More recently, another in-house indirect ELISA using truncated ORF2 protein of aHEV genotype 3 has been developed and applied in a serological survey in Chinese chicken flocks (Zhao et al., 2013).The sensitivity and specificity of these assays are not known.

Most of the RT-PCR methods for detection of aHEV RNA are based on degenerate primers targeting ORF1 or ORF2 (Payne et al., 1999; Huang et al., 2002b; Sun et al., 2004a; Bilic et al., 2009; Troxler et al., 2011). Although the most used primers were developed for aHEV genotype 2 (Huang et al., 2002b; Sun et al., 2004a), it has been successfully used for detection aHEV genotypes 1 and 3 (Massi et al., 2005; Morrow et al., 2008; Peralta et al., 2009a; Marek et al., 2010; Kwon et al., 2012).However, the sensitivity of the RT-PCR assays for detection of different aHEV genotypes is not known since strains identified in different geographic regions are genetically heterogenic (Meng, 2010).

As subclinical infections with aHEV are common (Huang et al., 2002b; Peralta et al., 2009a), it has been suggested that a presumptive diagnosis of HSS can be made on the basis of clinical signs and gross and microscopic lesions (Meng et al., 2008) and detection of HEV RNA in bile or fecal samples (Peralta et al., 2009b). An important differential diagnosis of HSS is hemorrhagic fatty liver syndrome (HFLS) that occurs mostly in caged layers fed high-energy diets and results in accumulation of fat in the liver and liver hemorrhages (Agunos et al., 2006). These syndromes can be differentiated via histological examination, as hepatocytes in

HSS livers do not contain excessive fat and HFLS livers do not have massive necrosis (Agunos et al., 2006). Other causes of liver lesions and splenomegaly include bacterial septicemia, lymphoid tumours (lymphoid leukosis, Marek´s disease), and avian adenoviruses infections which have characteristic histological lesions. A fractured liver resulting from external trauma to the body wall should also be considered as a differential diagnosis and is macroscopically characterized by hemorrhage in the coelomic cavity with coagulated blood adhering to the fracture site on the liver surface (Meng et al., 2008). Until now, avian HEV appeared to be regarded as a minor causative agent in several diagnostic cases and ignored when other poultry diseases have been diagnosed. However, avian HEV should be tested as one of the potential causative agents since chickens showing HSS with decreased egg production have been identified worldwide when the reasons of decreased egg production and increased mortality in chickens are uncertain (Kwon et al., 2012).

2.7 HEV in other animals

Other known animal species infected by different HEV strains genetically identified thus far include rat, mongoose, rabbits, ferrets, cutthroat trout, bats, and deer (Meng, 2013). Anti-HEV antibodies have been detected in a number of other animal species including cattle, sheep, and goats with the potential to carry novel strains of HEV (Meng, 2013). The zoonotic potentials of these novel animal strains of HEV are not altogether understood.

HEV genotype 3 strains have been genetically identified in different species of deer and wild boar in Asia and Europe and foodborne transmission to humans have been reported (Tei et al., 2003; Sonoda et al., 2004; Takahashi et al., 2004; Li et al., 2005; Masuda et al., 2005; Nishizawa et al., 2005; de Deus et al., 2008b; Kaci et al., 2008; Reuter et al., 2009; Schielke et al., 2009; Kaba et al., 2010). Rabbits may also serve as reservoir hosts for HEV transmission to humans given the genetic identification of HEV strains related to the zoonotic genotype 3 from rabbits in China, the United States, and France (Lhomme et al., 2013). The ability of rabbit HEV to cause cross-species infection in a pig model has been demonstrated (Cossaboom et al., 2012).

2.8 HEV in Brazil and South America

2.8.1 HEV in humans

The first serological evidence of human HEV infection in South America was found in 1994 in Venezuela (reviewed by Echevarria et al., 2013). Most prevalence rates reported among either urban or rural populations ranged from 1 to 10% (Echevarria et al., 2013). In Brazil, the anti-HEV IgG positive detection rates in blood donors ranged from 2.0% to 3.0% and from 1.68% to 4.3% in the general population (Assis et al., 2002; Bortoliero et al., 2006; Carrilho et al., 2005; Kiesslich et al., 2002; Lyra et al., 2005; Parana et al., 2000; Silva et al., 2012; Trinta et al., 2001; Vitral et al., 2005). In a recent serological study conducted on individuals living in Mato Grosso state, contact with pig or pig carcasses was not associated with an increase in HEV exposure (Silva et al., 2012).

South American countries, including Argentina, Brazil, Chile, Peru, and Uruguay have diagnosed patients with acute hepatitis E by anti-HEV IgM and/or HEV RNA detection (Echevarria et al., 2013). Autochthonous hepatitis E cases due to HEV genotype 3 have been reported in Argentina, Brazil and Uruguay (Lopes Dos Santos et al., 2010; Mirazo et al., 2013; Munne et al., 2011; Passos et al., 2013). Anti-HEV IgM antibodies have been detected retrospectively from patients with acute non-A-C hepatitis in Brazil since the 1990s (Lyra et al., 2005). However, the first molecular identification and characterization of an autochthonous human acute HEV infection was only reported in 2010, and a foodborne zoonotic transmission has been pointed as the most probable cause of infection (Lopes Dos Santos et al., 2010). Recently, another retrospective study has identified HEV infection in renal transplant recipients with unexplained increase in alanine aminotransferase and aspartate aminotransferase levels in Brazil (Passos et al., 2013). Diagnosis for hepatitis E is still neglected in Brazil and public health strategies are needed to include HEV as a possible agent in cases of acute non-A–C hepatitis.

2.8.2 HEV in domestic animals

The first evidence of the circulation of HEV in commercial pig farms in South America were reported in Argentina in 2006, where HEV RNA detection rates ranged from 4% to 98% of pigs within farms (Munne et al., 2006b). The Argentinean strains were closely related to human HEV genotype 3 strains identified from sporadic cases in Argentina (Munne et al., 2006a), and from an acute case of hepatitis E in Brazil (Lopes Dos Santos et al., 2010). HEV genotype 3 has also been identified in Bolivia (Dell'Amico et al., 2011) and Chile (Ibarra et al., 2007).

In Brazil, HEV genome was detected in six of eight stool pools from piglets 40 to 60 days old in São Paulo state (Paiva et al., 2007). In Parana state, HEV RNA was detected in 62.5% pig farms and 15.3% fecal samples (Gardinali et al., 2012). More recently, two different HEV genotype 3 subtypes occurring in swine from Para state have been reported (de Souza et al., 2012). HEV strains isolated were always from genotype 3. HEV RNA has been detected in raw effluents from a slaughterhouse in Rio de Janeiro (dos Santos et al., 2011). Sequencing and phylogenetic analysis revealed that the sewage strains were closely related to human and swine HEV strains recorded in the state (dos Santos et al., 2009; Lopes Dos Santos et al., 2010).

Anti-HEV IgG has also been detected in cows (1.42%, 1/70), dogs (6.97%, 3/43), chickens (20%, 5/25), and rodents (50%, 2/4) in Brazil (Vitral et al., 2005). To the author's knowledge, this is the only report of serological detection of aHEV in South America and there is no report of HSS or aHEV occurrence in chickens in Brazil.

3. OBJECTIVES

The present study was divided in four chapters, with the following objectives:

1. To develop a broadly reactive RT-PCR assay capable of detecting mHEV genotypes 1-4 and to develop a duplex RT-PCR for detection and identification of mHEV genotypes 3-4;
2. Genetically identify and characterize HEV virus that may be found in pig fecal samples in Brazil;
3. To develop a fluorescent micro-beaded immunoassay for the detection of chicken HEV IgY antibodies;
4. To assess the serological profile of aHEV in chicken farms from Minas Gerais state and to determine the possible avian HEV circulation by RT-PCR.

CHAPTER 1
COMPARISON OF REAL-TIME REVERSE TRANSCRIPTASE (RT)-PCR ASSAYS FOR DETECTION OF SWINE HEPATITIS E VIRUS IN FECAL SAMPLES

ABSTRACT

Hepatitis E virus (HEV) is a major cause of acute viral hepatitis in people in many developing countries and is also endemic in many industrialized countries. Mammalian HEV (mHEV) isolates can be divided into at least four recognized major genotypes. Several nucleic acid amplification techniques have been developed for mHEV detection with great differences in sensitivity. The aim of this study was to compare the performance of two single-plex real-time reverse transcriptase (RT)-PCR assays for broad detection of all four mHEV genotypes (assays A and B) and two duplex real-time RT-PCR assays for detection and differentiation of mHEV genotypes 3 and 4 (assay C and D). RNAs extracted from 28 fecal samples from pigs experimentally inoculated with HEV genotype 3 and 186 fecal samples from commercial pigs with unknown HEV exposure were tested by all four assays. In experimental samples, HEV RNA was detected in 96.4% (assay A), 39.2% (assay B), 14.2% (assay C), and 0% (assay D) of the samples. In field samples with unknown HEV exposure, HEV RNA was detected in 67.2% (assay A), 36.4% (assay B), 1.1% (assay C), and 0.5% (assay D) of the samples. Assays showed an overall poor agreement ($\kappa = 0.19$ to 0.03) with differences in detection rates between assays ($p < 0.01$). Assays A and B that broadly detect HEV genotypes 1-4 had significantly higher detection rates for HEV RNA than the duplex assays C and D that were both designed to detect and differentiate between HEV genotypes 3 and 4.

Keywords: Diagnosis, multiplex, hepatitis E virus, HEV RNA, genotypes, real-time RT-PCR

1. INTRODUCTION

Hepatitis E virus (HEV) is the causative agent of hepatitis E in humans (Purcell et al., 2008). HEV infection in pregnant women may cause particularly severe illness with a mortality rate of 10-20%, and recently there are numerous reports of persistent and chronic HEV infection in immunocompromised patients such as organ transplant recipients (Kamar et al., 2012). Currently, HEV is classified in the genus *Hepevirus* in the *Hepeviridae* family (Meng et al., 2012). The virus is a non-enveloped, positive-sense, single-stranded RNA virus that encodes three open reading frames (ORFs). ORF1 encodes for non-structural proteins, ORF2 encodes the viral capsid, and ORF3, which overlaps with ORF2, encodes a multi-functional small protein (Meng et al., 2012).

HEV has been identified in several animal species including domestic pigs, chickens, deer, wild boars, mongooses, rabbits, rats, ferrets, bats, and fish (Meng, 2013) and, based on the host tropism, the strains genetically identified thus far can be clustered into mammalian HEV (mHEV), avian HEV (aHEV), and in piscine HEV (pHEV) strains. Within mHEV, there are at least four recognized genotypes capable of infecting humans. Genotypes 1 and 2 are associated with epidemics and restricted to humans in developing countries, whereas genotypes 3 and 4 can infect a wide variety of species including humans and pigs, and are associated with sporadic and cluster cases of human hepatitis E in both developing and industrialized countries (Kamar et al., 2012).While mHEV genotype 3 has worldwide distribution (Lu et al., 2006), genotype 4 were reported in Asia (Lu et al., 2006), and more recently in Europe (Colson et al., 2012; Hakze-van der Honing RW et al., 2011). In humans, infections with genotypes 1 and 2 are mainly transmitted via fecally-contaminated water while infections with genotypes 3 and 4 appear to occur primarily by food-borne zoonotic transmission through the consumption of raw or undercooked meat from pigs, wild boars or deer (Meng, 2013).

Due to its implication in public health and pork safety, several nucleic acid amplification techniques and immunoassays have been developed for mHEV detection; however, a reliable diagnostic procedure for mHEV is still needed (Purcell et al., 2008; Kumar et al., 2013). Serological studies comparing immunoassays widely used for mHEV diagnosis found 2.9- to 6.5-fold variation in anti-HEV antibodies detection rates (Drobeniuc et al., 2010; Rossi-Tamisier et al., 2013; Wenzel

et al., 2013), and only two of six commonly available IgM anti-HEV detection assays had sensitivities and specificities above 95% (Drobeniuc et al., 2010). Due to this overall low sensitivity, a combination of antibody detection and nucleic acid detection has been suggested for optimizing mHEV diagnosis (Huang et al., 2010; Baylis et al., 2011).

Considering the heterogeneity of mHEV strains circulating in humans and other animal species, several conventional reverse transcription (RT)-PCR and real-time RT-PCR assays have been developed for the detection of HEV RNA in various types of samples including sera, feces and environmental samples (Meng et al., 2001; Ahn et al., 2006; Enouf et al., 2006; Jothikumar et al., 2006; Gyarmati et al., 2007; Legrand-Abravanel et al., 2009). Comparisons of RT-PCR assays have shown a 10 to 1,000-fold variation in sensitivity when samples were tested in parallel in the same laboratory (Ward et al., 2009; Mokhtari et al., 2013). In a blinded study to investigate the performance of conventional and real-time RT-PCR assays in 20 laboratories that performed HEV RNA detection on a regular basis, variations in sensitivity in the order of 100- to 1,000-fold were found using a standard panel of HEV genotype 3 and 4 strains (Baylis et al., 2011). Currently, a real-time RT-PCR designed in 2006 (Jothikumar et al., 2006) is the most widely used assay for detection of HEV infection in humans (Baylis et al., 2011; Baylis et al., 2013) primarily based on the reported high sensitivity (limit of detection of 4 genome equivalents of HEV genome) and its ability to detect all four recognized mHEV genotypes that are capable of infecting humans (Garson et al., 2012).

Although real-time PCR assays targeting conserved regions can provide accurate detection of the HEV genomes and yield results more rapidly compared to conventional RT-PCR, commonly a second molecular method such as sequencing or subtyping is required to further characterize strains. Recently, a real-time duplex RT-PCR assay for detection and identification of HEV genotype 3 and 4 in amounts as low as 50 genomic equivalents copies per reaction has been reported (Zhang et al., 2013). This assay, targeting the ORF2/ORF3 overlapping region, was designed to allow for a sensitive and rapid detection of the zoonotic HEV genotypes to potentially facilitate epidemiological investigations and to better understand outbreak situations. The aim of this study was to compare the performance of two single-plex real-time RT-PCR assays for broadly detection of all 4 recognized mHEV genotypes (assays A and B) and two duplex real-time RT-PCR assays for detection and differentiation of mHEV genotypes 3 and 4 (assay C and D). Each single-plex and one duplex real-time RT PCR assays had been previously described while the other single-plex assay is an in-house assay.

2. MATERIAL AND METHODS

2.1 Experimental samples

The experimental protocol was approved by the Virginia Polytechnic and State University Institutional Animal Care and Use Committee and by Virginia Polytechnic and State University Institutional Biosafety Committee. Twenty-eight serial fecal samples were collected daily from two pigs experimentally inoculated with human HEV genotype 3 strain US-2 (GenBank accession number AF060669) or swine HEV genotype 3 strain Meng (GenBank accession number AF082843) from day post-inoculation (dpi) 2 to 14. The fecal samples were suspended in saline (10% w/v), and the fecal suspensions were stored −80 °C until use.

2.2 Field samples

A total of 186 fecal samples of pig origin were chosen arbitrarily from routine diagnostic cases submitted during May 2013 to the Iowa State University Veterinary Diagnostic Laboratory (ISU-VDL). These samples originated on 86 farms located in 12 US states, Iowa, Illinois, Indiana, Minnesota, Missouri, North Carolina, North Dakota, Nebraska, Ohio, South Dakota, Texas, and Wisconsin, with samples obtained from age group: suckling (1-2 weeks of age), nursery (3-7 weeks of age) and grow-finish pigs (8-25 weeks of age).

2.3 Sample processing and RNA extraction

Fecal samples of ~1 g were resuspended in phosphate buffered saline (PBS) to obtain a final 10% suspension (w/v), vigorously vortexed and centrifuged at 1500 × g for 10

min. Viral RNA extraction was carried out on 50 µl fecal supernatant using a MagMAX 96 Viral Isolation kit (Ambion, Foster City, CA, USA) according to the manufacturer's instructions on an automated extraction platform (KingFisher Flex; Thermo Fisher Scientific). Negative controls, using water as a sample, and positive controls, using fecal suspensions from a pigs infected either with mHEV genotype 3 or 4, were added to each extraction plate. The extracted RNA was stored at −80 °C until use.

2.4 Primers and probes

All primers and probes used in this study are listed in Table 1.

Primers and probes from assays B and D developed in this study were designed manually based on a multiple sequence alignment of mHEV genotypes 1-4 in GenBank. Sequences were aligned using CLUSTAL W within DNASTAR (Lasergene 8). A pair of primers (HEV5606F/HEV5427DR) and a probe (HEVGenP) located in the conserved ORF2/ORF3 overlapping region broadly reactive with mHEV genotypes 1-4 were designed (assay B). Additionally, probes specific for the detection of mHEV genotypes 3 or 4 (HEVg3 and HEVg4; assay D) were designed in this same region. Oligonucleotide primers/probes were analyzed for the absence of possible hairpins and dimers by Primer Express software (Version 3.0; Applied Biosystems).

Table 1. Primers and probes used in this study

Assay	Amplified region	Primer and probe	Sequence 5′–3′	Annealing temperature (°C)	Location[a]	Reference
A	ORF2/3	JVHEVF	GGTGGTTTCTGGGGTGAC	60	5311-5328	Jothikumar et al. (2006)
		JVHEVR	AGGGGTTGGTTGGATGAA		5363-5380	
		JVHEVP	FAM-TGATTCTCAGCCCTTCGC-BHQ		5334-5351	
B	ORF2/3	HEV5306F	GTTGATTCTCAGCCCTTCGC	60	5332-5351	This study
		HEV5427DR	TGGGMYTGRTCDCGCCAAG		5453-5471	
		HEVGenP	FAM-CCCCTATATTCATCCAACCAACCCCTT-BHQ		5329-5355	
C	ORF2/3	HEV-uni-F	TATTCATCCAACCAACCCCTT	60	5335-5355	Zhang et al. (2013)
		HEV-uni-R	GTCDCGCCAAGYGGAGC		5421-5437	
		HEV-3-CY5	QUASAR 670-GCCGATGTCGTTTCACAA-BHQ		5386-5403	
		HEV-4-FAM	FAM-CGCATCTGACATWCCARCCGC-BHQ		5371-5391	
D	ORF2/3	HEV5306F	GTTGATTCTCAGCCCTTCGC	60	5332-5351	This study
		HEV5427DR	TGGGMYTGRTCDCGCCAAG		5453-5471	
		HEVg3	QUASAR 670-TYGTWYCACAAYCCGGGGCTGG-BHQ		5393-5414	
		HEVg4	FAM-CATCYGACATWYCARCCGCMGCCG-BHQ		5373-5396	
Conventional nested RT-PCR	ORF2	3156N	AATTATGCYCAGTAYCGRGTTG	55	5737-5760	Huang et al.(2002)
		3157N	CCCTTRTCYTGCTGMGCATTCTC		6445-6467	
		3158N	GTWATGCTYTGCATWCATGGCT		6022-6043	
		3159N	AGCCGACGAAATCAATTCTGTC		6348-6369	

[a] Nucleotide positions are in accordance with GenBank accession number AF60669, except for the HEV genotype 4 probes that are in accordance with GenBank accession number HQ634346.

2.5 Construction of plasmid DNA standards for the real-time PCR reactions

Plasmid DNA standards were constructed by amplifying region at nucleotide positions 5311 to 5471 of a genotype 3 human HEV strain (US-2) (Schlauder et al., 1998) and region at nucleotide positions 5285 to 5445 of a genotype 4 human HEV strain (TW6196E) (Feagins et al., 2008b) using primers JVHEVF and HEV5427DR described in Table 1. Conventional RT-PCR reactions were carried out in a total volume of 20 µl using the QIAGEN OneStep RT-PCR kit (Qiagen, Valencia, CA, USA) according to the manufacturer's recommendations. Purified PCR products were cloned into the pGEM-T Vector (Promega, Madison, WI, USA) and transfected into TOP10 *Escherichia coli* bacteria (Invitrogen, Foster City, CA, USA) following the instructions of the cloning kit manual. Sequencing was performed on recombinant plasmids in both directions using the AB 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA) at the Iowa State University DNA Facility, Ames, IA, USA. The recombinant plasmid stocks were quantified using the NanoDrop spectrophotometer ND-1000 according to the manufacturer's instructions (NanoDrop Technologies Inc., Wilmington, DE, USA) and converted into copy numbers. The total numbers of copies in the plasmid stock was calculated as copy number = [(concentration of linearized plasmid)/(molar mass)] × $(6.0^{23} \times 10^{23})$. The plasmid DNA was used to generate standard curves using 10^1 to 10^8 genomic equivalent (GE) copies of plasmid. The GE titers of HEV were determined based on the standard curve.

2.6 Real-time RT-PCR assays

The real-time RT-PCRs were carried out in 96-well plates using the TaqMan One-Step RT-PCR Master Mix Reagent (Applied Biosystems, Foster City, CA, USA) in a 25 µl volume comprising 5 µl of extracted RNA and 20 µl of master mix according to the manufacturers' recommendation. All four assays (Table 1) were performed on the same day, and the same nucleic acid extracts were utilized. Single-plex assay B, capable of detecting mHEV genotypes 1-4, and duplex assay D, capable of detecting and differentiating of mHEV genotypes 3 and 4, utilized both the same forward and reverse primers. The concentrations of the primers and probe or probes (multiplex assays) were 400 and 200 nM for assay A; 800 and 200 nM for assay B; 400 and 200 nM for assay C; and 800 and 400 nM for assay D. One-step RT-PCR amplification was performed on an ABI 7500 real time PCR instrument (Applied Biosystems, Foster City, CA, USA) under the following conditions: 15 min at 50 °C for the RT reaction, 10 min at 95 °C followed by 45 cycles at 95 °C for 15 s for denaturation and 60 °C for 45 s for annealing and extension. A sample was considered negative if the cycle threshold (CT) was ≥41 amplification cycles. Quality control of the real-time RT-PCR process included negative (nuclease-free water) and positive (HEV RNA and HEV plasmid DNA) controls added to each PCR plate.

2.7 Efficiency, limit of detection, intra-assay and inter-assay precision of the PCR assays

Verification of assays sensitivity, specificity and precision were performed as proposed elsewhere (Rabenau et al., 2007). Standard curves of mHEV genotypes 3 and 4 ranging from 10^1 to 10^8 copies of HEV plasmid DNA were used to determine the efficiency, limit of detection, intra-assay and inter-assay precision of the real-time PCR assays. For assays A and B, which do not differentiate HEV genotypes, standard curves for genotypes 3 and 4 were tested separately. For assays C and B, which differentiate between HEV genotypes 3 and 4, the standard curves were tested as duplex assays or separately in single-plex assays. The amplification efficiency (E) for each assay was calculated according to the formula $E = [10\text{-}1/S] - 1$ to determine the performance of qPCR, where S indicated the slopes (S) of the regression lines. The limit of detection of each assay and the intra-assay variation were assessed with the standard curves tested in triplicate. Limit of detection was specified as the lowest amount of DNA standard that could be detected with 100% probability. The inter-assay variation was determined by three independent runs of the standard curves in triplicate.

2.8 Conventional nested RT-PCR

Twenty field samples tested by all four real-time PCR assays that presented discrepant

results, defined as a sample that exhibit a positive result in one assay and a negative result in another assay, were arbitrarily chosen for sequencing follow-up using a nested RT-PCR assay based on a partial HEV ORF2 fragment. Conventional nested RT-PCR were performed using primers previously described (Huang et al., 2002a) (Table 1). Briefly, for the first PCR reaction 6 μM each of primers 3156N and 3157N and QIAGEN OneStep RT-PCR kit (Qiagen, Valencia, CA, USA) were used. The thermal cycler conditions for the first reaction were as follows: 50 ˚C for 30 min; 95 ˚C for 15 min; 35 cycles of 94 ˚C for 30 sec, 55 ˚C for 30 sec, 72 ˚C for 1 min; final elongation step of 72 ˚C for 10 min. The second reaction was performed with 0.2 μM each of primers 3158N and 3159N and ReadyMix® Taq PCR Reaction Mix (Sigma-Aldrich, St. Louis, MO, USA). The thermal cycler conditions for the second PCR reaction were as follows: 95 ˚C for 5 min; 35 cycles at 94 ˚C for 30 sec, 55 ˚C for 30 sec, 72 ˚C for 30 sec; final elongation step at 72 ˚C for 7 min. The 348 bp second round PCR products were visualized after electrophoresis on a 1% agarose gel.

2.9 Sequencing and phylogenetic analysis

Sequencing of HEV RNA positive samples was performed directly on both strands at the Iowa State University DNA Facility, Ames, Iowa, USA. Sequences were aligned with published data using BLAST at the National Centre for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/). Sequences were compiled using Lasergene software and the Clustal W alignment algorithm (DNAStar, Madison, Wisconsin, USA). The nucleotide distance of the sequences was evaluated by neighbor-joining (NJ) using Lasergene MegAlign. Confidence in the NJ tree was estimated by bootstrap replicates. Sequences reported in this paper have been deposited in the GenBank database under the accession numbers KF719308 to KF719310.

2.10 Statistical analysis

Inter and intra-assay variances were computed using the C_T values, standard deviations and coefficient of variation of the standard curves. The variance was analyzed by a one-way repeated measures analysis of variance followed by Bonferroni's test for pairwise comparison. Cochran's Q test for matched data, followed by McNemar's test for pairwise comparisons were used to determine whether the proportions of RT-PCR positive samples were significantly different between assays. Differences between groups were considered significant if $p < 0.05$. A Kappa index was performed to determine the agreement of positive and negative results between assays. The strength of agreement was considered ≤ 0 = poor, 0.01-0.2 slight, 0.21-0.4 = fair, 0.41-0.60 = moderate, 0.61-0.80 = substantial, and 0.81-1 = almost perfect as previously described (Landis et al., 1977). Statistical analyses were performed using SAS version 9.2 (SAS Institute, Inc., Cary, NC, USA).

3. RESULTS

For assays C and D, which differentiate between HEV genotypes 3 and 4, there was no difference between the standard curves tested as duplex assays or single-plex regarding PCR efficiency, limit of detection and intra- inter-assay precision, therefore only results of the duplex assays were presented. A purine-pyrimidine mismatch was found in the probes used to detect HEV genotype 4 for both assays C (at 19^{th} base) and D (at 17^{th} base) regarding the strain used as control (C in virus at nucleotide position 5389/A in probes).

3.1 Evaluation of real-time RT-PCR assays

Standard curves were established for each real time PCR assay using the HEV genotypes 3 and 4 DNA controls serially diluted from 1×10^8 to 1×10^1 copies and amplified in triplicate. Efficiency, regression coefficient, slope and intercept for each assay are shown in Table 2. Assays A and B had a similar performance regardless of the HEV genotype used while assays C and D presented a decrease in efficiency of at least 11% when HEV genotype 4 was used (Table 2).

Table 2. Efficiency, regression coefficient, slope and intercept for real-time RT-PCR assays A, B, C and D obtained by quantification of serially diluted plasmid DNA containing HEV genotypes 3 or 4 ORF2 and ORF3 overlapping region from 1 x 10^8 to 1 x 10^1 copies

Variable	A		B		C		D	
	HEV-3	HEV-4	HEV-3	HEV-4	HEV-3	HEV-4	HEV-3	HEV-4
Efficiency (%)	95.9	98.7	88.2	90.7	95.6	82.1	93.3	82.3
Regression coefficient	0.992	0.99	0.991	0.992	0.990	0.993	0.990	0.991
Slope	-3.422	-3.352	-3.641	-3.565	-3.433	-3.843	-3.493	-3.833
Intercept	42.7	42.1	43.0	42.4	47.82	50.88	43.6	48.7

3.2 Limit of detection and inter- and intra-assay precision of the four real-time PCR assays

For each assay, inter-assay precision was assessed by calculating the standard deviation and coefficient of variation of C_T obtained for each standard dilution tested in three independent runs, the coefficient of variation was found to be < 7% for all assays (data not shown). Intra-assay precision was assessed by calculating the standard deviation and coefficient of variation of C_T obtained for each standard dilution tested in triplicate, the coefficient of variation was found to be < 4% (Table 3). Limit of detection of each assay was specified as the lowest recognized concentration of genotypes 3 or 4 mHEV DNA control serially diluted from 1×10^5 to 1×10^1 in triplicate (Table 3). However, the GE copy numbers do not reflect the number of RNA molecules, since the efficiency of the RT reaction was not directly determined.

Variation of detection limits was in order of 10 to 1,000-fold among assays. Assay A was able to detect 10^1 GE copies of the plasmid HEV DNA per reaction (4×10^3 copies ml^{-1}), assay B detected all 10^2 GE copy dilutions (4×10^4 copies ml^{-1}) and occasionally the 10^1 GE copies dilution, and assays C and D were able to detect all HEV DNA standards down to the 10^4 GE copy dilutions (4×10^6 copies ml^{-1}) (Table 3). Impact of HEV genotype was observed for assays C and D that could detect all 10^2 GE copy dilutions of genotype 3, but only 1/3 10^3 GE copy dilutions of genotype 4. Assays A and B detection was genotype independent.

Table 3. Limit of detection of four real-time RT-PCR assays and intra-assay precision results of 10-fold dilutions of HEV genotype 3 or HEV genotype 4 DNA plasmid controls tested in triplicate.

Assay	A				B				C				D			
	Detected samples	Mean Ct	SD	CV%	Detected samples	Mean Ct	SD	CV%	Detected samples	Mean Ct	SD	CV%	Detected samples	Mean Ct	SD	CV%
HEV-3[a]																
100,000	3/3	24.55	0.47	1.86	3/3	25.30	0.94	3.56	3/3	30.28	0.85	2.73	3/3	26.12	0.41	1.53
10,000	3/3	28.58	0.37	1.24	3/3	28.09	0.64	2.05	3/3	33.48	0.68	1.91	3/3	29.98	0.48	1.54
1,000	3/3	31.37	0.34	1.09	3/3	32.77	0.16	0.48	3/3	37.34	0.78	2.09	3/3	32.80	0.94	2.88
100	3/3	34.91	0.23	0.65	3/3	36.35	1.32	3.53	3/3	40.78	1.17	2.90	3/3	36.74	0.59	1.64
10	3/3	37.16	0.73	1.95	2/3	39.31	1.81	4.60	0/3				1/3	39.92		
HEV-4[a]																
100,000	3/3	23.78	0.66	2.77	3/3	25.86	0.18	0.69	3/3	31.85	0.17	0.53	3/3	30.77	0.90	2.82
10,000	3/3	28.44	0.39	1.36	3/3	29.25	0.72	2.31	3/3	35.86	0.12	0.32	3/3	34.03	0.33	0.93
1,000	3/3	31.59	0.68	2.16	3/3	32.81	0.09	0.24	1/3	39.54			1/3	38.21		
100	3/3	34.53	0.56	1.64	3/3	36.24	0.58	1.53	0/3				0/3			
10	3/3	37.36	0.13	0.35	2/3	40.22	0.51	1.26	0/3				0/3			

[a] Number of plasmid DNA copies per reaction

C_T: cycle threshold; SD: standard deviation; CV%: coefficient of variation

3.3 Detection of HEV RNA in experimental samples with known mHEV exposure by the four real-time RT-PCR assays

Detection of mHEV RNA in experimental samples evaluated in parallel using the same RNA extracts are shown in Fig. 1. The GE titers of mHEV were determined from the HEV genotype 3 standard curve included in each run and for each assay. Considering dpi 2 through 14, assay A presented the highest rate of cumulative positive detection (96.4%, 27/28) ($p < 0.05$), followed by assay B (39.2%, 11/28), which presented a positive detection rate higher than assays C (14.2%, 4/28) and D (0/28) ($p < 0.05$).

Considering samples from pigs infected with mHEV one of two genotype 3 strains, the detected viral loads ranged from 3.62 to 7.16 $\log_{10}$ HEV GE copies ml^{-1} 10% fecal samples for assay A; from 3.22 to 5.26 $\log_{10}$ HEV GE copies ml^{-1} in 10% fecal samples for assay B, and from 4.68 to 4.81 $\log_{10}$ HEV GE copies ml^{-1} for assay C (Fig. 1). In order to further investigate the reason of the low detection rates found with assay C and the lack of detection of any positive sample with assay D, the primer and probe sequences from each assay were compared to the genome sequence of each of the HEV strains used. Mismatches were not identified for any primer or probe (data not shown) indicating that the detection rates achieved were due to intrinsic differences in the limit of detection for each assay.

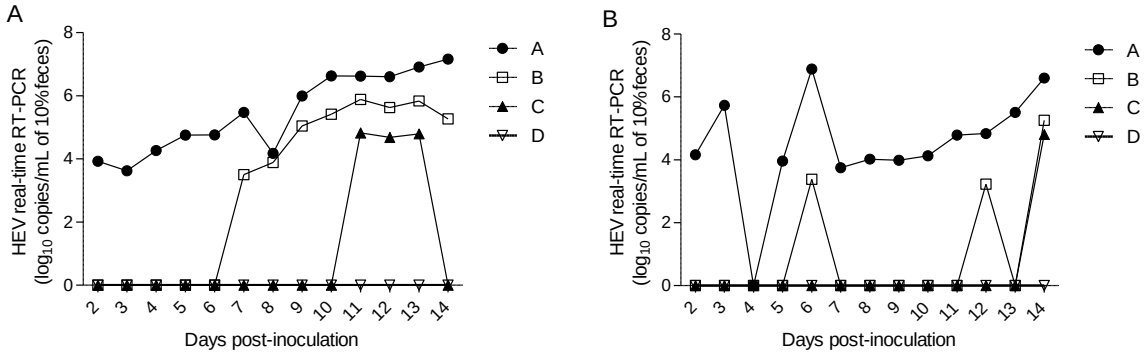

Figure 1.Comparison of four real-time RT-PCR assays (A, B, C and D) in detecting and quantifying HEV RNA on fecal samples after experimental inoculation of a pig with HEV genotype 3 strains US-2 (A) or Meng (B).

3.4 Detection of HEV RNA in field samples with unknown mHEV exposure by the four real-time RT-PCR assays

The rates of HEV RNA detection with the four assays on field samples are summarized in Table 4. Considering all age groups, the overall detection rate of HEV RNA positive samples was 67.2% (125/186) for assay A, 36.4% (68/186) for assay B, 1.1% (2/186) for assay C and 0.5% (1/186) for assay D. Assays showed an overall poor agreement ($\kappa = 0.19$ to 0.03) with difference in detection rates between assays ($p < 0.01$). Assay A presented the highest HEV RNA detection rate ($p < 0.01$). All positive samples with assays C and D were also positive with assays A and B. Regarding positive samples with assay B, 80.8% (55/68) were also positive by assay A, indicating that assay B identified additional 7.0% (13/186) positive samples that were not identified by assay A. However, assay A identified an additional 37.6% (70/186) positive samples that were not identified by assay B ($\kappa = 0.19$, $p < 0.01$).

Table 4. Detection rates for HEV RNA in fecal samples collected from pigs of unknown HEV status by real-time RT-PCR assays A, B, C and D.

Age-group	N. tested	A + (%)	B + (%)	C + (%)		D + (%)	
				HEV-3	HEV-4	HEV-3	HEV-4
Suckling	46	25 (54.3)[1,A]	17 (36.9)[A]	0[B]	0[B]	0[B]	0[B]
Nursery	86	62 (72.1)[A]	24 (27.9)[B]	0[C]	0[C]	0[C]	0[C]
Grow-finish	54	38 (70.3)[A]	27 (50.0)[B]	2 (3.7)[C]	0[C]	1 (1.8)[C]	0[C]
Total	186	125 (67.2)[A]	68 (36.6)[B]	2 (1.1)[C]	0[C]	1 (0.5)[C]	0[C]

[1]Different superscripts within the column indicate significant differences ($P < 0.05$) within assays.

3.5 Further evaluation of 20 field samples with discrepant results among real-time RT-PCR assays

Twenty samples with Ct values lower than 36 on the real-time RT-PCR assay were arbitrarily selected for amplification with a conventional nested RT-PCR assay (Table 5). Twelve of 20 (60%) samples positive in at least one real-time RT-PCR assay were also positive in the nested RT-PCR, and 3/12 samples that yielded a sequence were mHEV genotype 3 (Table 5).

Table 5. Detection of HEV RNA by conventional nested RT-PCR and real-time RT-PCR assays in 20 swine field samples.

	Real-time RT-PCR assay				Conventional nested
Sample	A	B	C	D	RT-PCR
19482	-	35.69	-	-	+ (w)
19702-C	-	36.18	-	-	+ (w)
19714	-	36.00	-	-	+ (w)
19762-B	33.02	34.46	-	-	+*
19775	36.97	-	-	-	-
19913	33.04	35.51	33.62	-	+*
19903	-	35.63	-	-	-
19912-B	36.06	-	-	-	+ (w)
19955	37.41	36.06	-	-	-
20354-A	35.99	38.99	-	-	-
20361-A	36.40	-	-	-	+
20383-B	36.39	33.32	-	-	-
20513	36.13	-	-	-	-
20517	36.88	-	-	-	+
20613	-	36.78	-	-	+ (w)
20467-E	36.59	38.05	-	-	+
20468-D	36.31	37.84	-	-	+*
20777-B	36.52	35.86	-	-	-
20792-B	37.23	36.46	-	-	+ (w)
20855	35.93	-	-	-	-

- , HEV RNA negative sample; +, HEV RNA positive sample; w, weak reaction; * HEV genotype 3 by sequencing

4. DISCUSSION

In this study, two single-plex real-time RT-PCR assays for detection of all four known HEV genotypes without differentiation and two duplex real-time RT-PCR assays for detection and differentiation of HEV genotypes 3 and 4 were evaluated. All assays were compared on the same real-time RT-PCR instrument at the same day, using the same RT-PCR enzymes, standard curves and nucleic acid extracts. Under these conditions single-plex assays A and B designed to broadly detect HEV genotypes 1-4 showed a significantly better performance ($p < 0.01$) than duplex assays C and D which both allow detection and differentiation of HEV genotypes 3 and 4 . Considering the detection rates in field samples, the single-plex real-time RT-PCR assays detected at least 34-fold more positive samples than the duplex real-time RT-PCR assays.

Design of a broadly reactive assay for detection of mHEV genotypes is a complex and challenging task due to the heterogeneity among the various HEV strains (Lu et al., 2006; Smith et al., 2013). Sensitivity of real-time assays can vary widely depending on target region and HEV genotype (Ward et al., 2009; Mokhtari et al., 2013). Previous comparison of conventional and real-time RT-PCR assays to detect HEV RNA have shown that targeting a more conserved region as ORF2/3 appears more reliable than the use of degenerate primers and probes targeting a less conserved region such as ORF2 (Ward et al., 2009; Mokhtari et al., 2013; Vasickova et al., 2012). In this study, all the real-time RT-PCR assays used target the overlapping of ORF2 and ORF3; however, with the exception of assay A, assays B, C, and D used degenerate primers and probes. Degeneracies may reduce the sensitivity and specificity of an assay due to factors such as a

lower effective concentration of each primer or difficulties in estimating the suitable annealing temperatures and primer lengths (Rose et al., 2010), which could partially explain the poor results achieved with assays B, C and D when compared with assay A in the present study. Analysis of full-length genomes of various human and animal mHEV strains revealed that the HEV genome could vary even in conserved regions (Lu et al., 2006; Smith et al., 2013) and this genetic variability complicates reliable detection of mHEV subtypes. In fact, recent studies found polymorphisms in the probe-binding site region of the most widely used real-time RT-PCR assay for HEV detection (assay A) (Jothikumar et al., 2006), and a modification of the probe and subsequent increase of the melt temperature, restored detection of the polymorphic strains (Garson et al., 2012; Baylis et al., 2013). Employment of more than one set of primers targeting different loci of the HEV genome could perhaps increase the likelihood of HEV detection (Vasickova et al., 2012). The same strategy has been used for detection of other highly variable RNA viruses such as influenza A virus (Hoffmann et al., 2010) and porcine reproductive and respiratory syndrome virus (PRRSV) (Kleiboeker et al., 2005; Wernike et al., 2012).

The best PCR performance on experimental samples was observed for assay A (27/28), followed by assay B (11/28) and C (4/28). Assay D could not detect a single sample. In similar PCR comparisons as presented here, assay A was determined to be the most suitable, reproducible and reliable assay for the detection of HEV RNA (Ward et al., 2009; Baylis et al., 2011; Mokhtari et al., 2013). It is well recognized that fecal samples as used in this study could contain metabolic compounds possible interfering with the RT-PCR reactions. The addition of an internal control to monitor the presence of such inhibitors would ensure reliability of negative HEV RNA results. Although such a control was not included in this study, all assays used the same acid nucleic extracts and issues with the viral RNA extraction recovery can therefore be excluded. No mismatch could be found for any primer or probe when compared to the mHEV strains used to infect the pigs, indicating that the detection rates achieved were due to intrinsic differences in the limit of detection for each assay. Moreover, it is worth noting that the single-plex assay B and the duplex assay D developed in the present study used the same primer pair and the difference in the positive detection rates between them (11/28 vs. 0/28, $p < 0.01$) are likely due to the differences in the nucleotide composition of the targeted region for the probes.

HEV genome variability may also influence the quantification of its RNA. Comparison of analytical sensitivities of the assays based on the detection of the plasmid DNA standards showed that the sensitivities of assay A and B were independent of HEV genotypes (3 or 4), and assay A was 10-fold more sensitive compared to assays B, C and D based on the genotype 3 standard curve detection. However, the sensitivities of assays C and D for HEV genotype 4 were 100-fold lower than the sensitivities yielded by using HEV genotype 3 within the same assay which could be partially explained by a single mismatch in the probe region. Although a study has reported that probes with up to two mismatches showed little variation in the PCR efficiency and nucleic acid quantification compared to probes that were fully complementary (Yao et al., 2006), another study has shown that a single mismatch in the probe binding region can result in a quantification error up to 33% (Süß et al., 2009). Due to the usage of plasmid DNA for quantification, reverse transcription reaction, as one of the crucial steps of RT-PCR was dismissed and the limit of detection presented here cannot be fully compared to assays in which there is usage of RNA standards.

In summary, real-time RT-PCR assays A and B that broadly detect HEV genotypes 1-4 showed better results for RNA detection than the duplex assays C and D that there both designed to detect and differentiate between HEV genotypes 3 and 4. Assay A presented the overall best performance among the tested assays.

CHAPTER 2
DETECTION AND CHARACTERIZATION OF HEPATITIS E VIRUS IN DOMESTIC PIGS IN SOUTHEAST BRAZIL

ABSTRACT

Hepatitis E virus (HEV) infections in domestic pigs were investigated in ten farms in southeast Brazil, Minas Gerais state. A total of forty pooled fecal samples were collected, one pool corresponding to pigs at 7, 10, 13 or 17 weeks of age in each farm. Twenty nine of 40 (72.5%) samples tested positive for HEV RNA using a real-time RT-PCR. All 10 farms had at least one positive sample and the detection of HEV RNA ranged from 60% at 7 weeks of age to 90% at 13 weeks of age. Phylogenetic analysis of a 304 nt fragment of the ORF2 gene revealed that all six yield sequences clustered in genotype 3, described previously in human and swine infections in Brazil.

Keywords: hepatitis E virus (HEV), swine, zoonosis, phylogeny

1. INTRODUCTION

Hepatitis E virus (HEV) is the causative agent of hepatitis E that can leads to acute viral hepatitis in humans (Purcell et al., 2008). Mammalian HEV is classified in four recognized genotypes and at least two putative new genotypes (Meng et al., 2012). Genotypes 1 and 2 are associated with waterborne epidemics in humans in Asia, Africa and Mexico (Emerson et al., 2003). Genotypes 3 and 4 are associated to foodborne sporadic and cluster cases of hepatitis E related to consumption of raw or undercooked pork, deer, and wild boar meat products (Meng, 2013). Genotype 3 is distributed worldwide while genotype 4 is found mainly in Asia (Lu et al., 2006). More recently, genotype 4 has been described in Europe through swine surveillance (Hakze-van der Honing et al., 2011) and has been associated to autochthonous hepatitis E cases in France (Colson et al., 2012; Tesse et al., 2012).

HEV genotype 3 has been reported in pig farms and slaughterhouses in different regions of Brazil with great genomic heterogeneity between strains from different states (de Souza et al., 2012; dos Santos et al., 2009; dos Santos et al., 2011; Gardinali et al., 2012). Recently, autochthonous hepatitis E cases have been reported involving an acute case of non-A-C hepatitis (Lopes dos Santos et al., 2010) and three cases of transplant recipients in Brazil (Passos et al., 2013). The described acute human case of HEV isolate presented high degree of nucleotide sequence similarity to swine HEV sequences described previously in Brazil and a probable zoonotic transmission has been suggested (Lopes dos Santos et al., 2010).

Further studies addressing the genetic variability of HEV strains circulating in swine herds would broaden the knowledge in molecular epidemiology of HEV infection in Brazil and alert for the introduction of new genotypes and variants. The aim of this study was to detect and characterize HEV in pigs from different farms in Minas Gerais state.

2. MATERIAL AND METHODS

2.1 Samples

A total of 40 pooled fecal samples were collected between May and August 2012 on 10 farrow-to-finish farms located in the west, south, southeast, metro area and zona da mata regions of the Minas Gerais state, Brazil. Fresh fecal samples were collected directly from the floor (2 g of fresh fecal material from five sites in each pen) from four pens on each farm, corresponding to pigs at seven, 10, 13 and 17 weeks of age. The samples size calculation was based on a pool size of five, an assumed HEV prevalence of 40% on a herd, a pool level test sensitivity of 70%, and a herd sensitivity of 95% according to an adaptation by using the Australian Biosecurity EpiTools (http://epitools.ausvet.com.au/). The minimum of three pools required for detecting disease if present was rounded up to four samples per farm. Fecal samples were homogenized and an aliquot of 0.4 g was resuspended in 4 ml phosphate-buffered saline (PBS), vigorously vortexed and centrifuged at 1500 × *g* for 10 min. Samples were stored at −80°C until use.

2.2 RNA extraction

RNA was extracted as described on Chapter 1, Section 2.2.

2.3 RT-PCR

A real-time RT-PCR were performed using the primers and probe JVHEV-F (5'-GGTGGTTTCTGGGGTGAC -3'); JVHEV-R (5'- AGGGGTTGGTTGGATGAA -3'); JVHEV-P (5'- FAM-TGATTCTCAGCCCTTCGC-BHQ-3') as described on Chapter 1, Section 2.2. One HEV-positive sample by real-time RT-PCR from each farm was selected for amplification by a conventional nested RT-PCR using the primers targeting an ORF2 fragment as previously described (Huang et al., 2002a).

2.4 Sequencing and phylogenetic analysis

Sequencing of HEV RNA positive samples was performed directly on both strands at the Iowa State University DNA Facility, Ames, Iowa, USA as previously described on Chapter 1, Section 2.3. Sequences reported in this paper have been deposited in the GenBank database under the accession numbers KF719311 to KF719316.

3. RESULTS

3.1 HEV detection

Overall, 29/40 (72.5%) fecal samples tested positive for HEV RNA using the real-time RT-PCR. All 10 farms had at least one positive sample, with an average of three positive samples per farm (Fig. 1). The detection of HEV RNA was similar among the tested ages, ranging from 6/10 (60%) HEV RNA positive at 7 weeks to 9/10 (90%) at 13 weeks. Pigs at 10 and 17 weeks old presented 7/10 (70%) of positive samples, respectively.

3.2 Phylogenetic analysis

From each farm, one HEV-positive sample was selected and amplified using a conventional nested RT-PCR and 6/10 PCR products of correct size were obtained and sequenced. Sequencing results showed that strains within this study had 87.9 to 96.1% nucleotide identity for the 304 nt fragment of the 5' end of ORF2 gene (Fig. 1). The sequences found in this study had the highest nucleotide identity (88.1-93.4%) to BRAsw-107, a swine Brazilian HEV strain found on a high-density pig producing region on south of Brazil (Paraná state) (Gardinali et al., 2012). Nucleotide identity of sequences described in this study ranged from 84.5 to 91.9% when compared to Brazilian human strains Brazilh2 and Brazilh3 (Passos et al., 2013), and from 80.5 to 85.4% when compared to swine HEV strains SE, SW2, SW3, BRsw-B, and 029 described in other Brazilian regions (Sao Paulo, Rio de Janeiro, Mato Grosso, and Para states) (de Souza et al., 2012; dos Santos et al., 2009; Paiva et al., 2007). Between 73.4 and 88.5% identity was found with other mammalian HEV strains (Fig. 2).

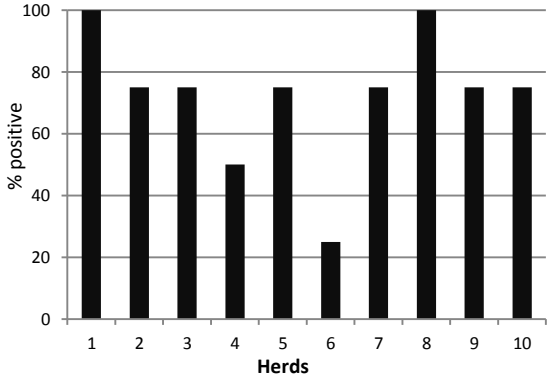

Figure 1. Percentage of HEV RNA positive fecal pools in each of 10 farms located in Brazil by real-time RT-PCR

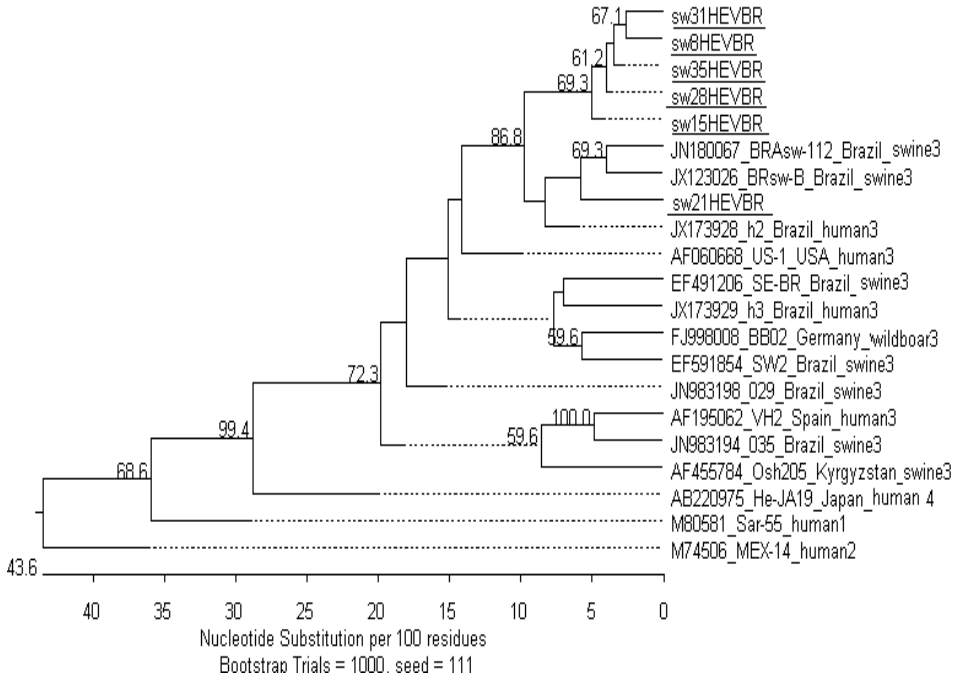

Figure 2. Phylogenetic tree based on 304 nt region of the 5' end of HEV isolates. Sequences that were obtained in this study are underlined. The phylogenetic tree was constructed by the NJ method. Significant bootstrap values are indicated as a percentage for 1000 replicates.

4. DISCUSSION

In this study, HEV RNA was detected and characterized in different pig herds in Minas Gerais State, Brazil. Screening of the pooled porcine fecal samples from different age groups resulted in positive detection of HEV RNA in all investigated farms and 72.5% of the samples tested. The HEV RNA detection rate in this study was higher than in previous surveys conducted in Brazil, which found 8% (12/151) positive fecal samples collected from pigs in a slaughterhouse in the Para state (de Souza et al., 2012), and 15% (26/170) positive fecal samples in a survey in suckling to finish pigs in the Parana state (Gardinali et al., 2012). However, when considering only pigs from 9 to 17 weeks of age from the latter study, 62% (16/26) fecal samples were HEV RNA positive (Gardinali et al., 2012), which is similar to the present study in which samples from 7 to 17 week old pigs were tested. Although HEV RNA has been detected in pigs of all age groups, HEV infection and fecal shedding occurs mostly frequently in growing pigs between 2 and 4 months of age (Fernandez-Barredo et al., 2006; McCreary et al., 2008; Berto et al., 2012; Gardinali et al., 2012). In addition, differences in the extraction and RT-PCR assays could partially explain differences between studies. Alternatively, the collection method used in the present study, pooled samples collected directly from the floor, could have increased the likelihood of HEV RNA detection when compared to sample protocols on individual pigs.

HEV positive samples were further characterized by sequencing of a portion of the ORF2 region and phylogenetic comparison with available sequences from humans and other pigs. All HEV strains detected in the present study clustered with genotype 3 and were genetically most closely related to HEV strains previously described in humans and pigs in Brazil (dos Santos et al., 2009; Gardinali et al., 2012; Passos et al., 2013). Interestingly, a HEV strain reported in this study shared 91.9% nucleotide identity with a HEV strain identified in a kidney transplant patient who was hospitalized in 2006 in the São Paulo state (southeast Brazil) (Passos et al., 2013), and 93.4% identity with a pig strain identified in

2009 in the Paraná state which is located in south Brazil (Gardinali et al., 2009). As the zoonotic transmission of HEV from pigs to humans is of concern, the present study provides insight on the sequences of HEV isolates currently circulating in the pig population in Brazil.

HEV RNA was found in raw effluents from a pig slaughterhouse in the Rio de Janeiro state in southeast Brazil (dos Santos et al., 2011). Taking the high HEV RNA detection rates (72.5%) in fecal samples in the present study in account, pig manure could represent a direct exposure source for pig handlers as well as an important source for environmental contamination. Studies conducted in Spain and the Netherlands have reported that HEV sequences that cluster with sequences from human hepatitis E cases could be recovered from urban sewage and river water (Clemente-Casares et al., 2009; Rutjes et al., 2009).

The present study reinforces previous evidence of high circulation of HEV on pig farms and the genomic heterogeneity among swine genotype 3 HEV strains in Brazil. Understanding the genomic heterogeneity of HEV in pigs provides important information about the circulation and geographical patterns of HEV strains.

CHAPTER 3
DEVELOPMENT OF A FLUORESCENT MICROBEAD-BASED IMMUNOASSAY FOR THE DETECTION OF ANTIBODIES AGAINST AVIAN HEPATITIS E VIRUS

ABSTRACT

The objective of this study was to develop a fluorescent microbead immunoassay (FMIA) for the detection of IgY antibodies against avian hepatitis E virus (HEV). The diagnostic performance of the FMIA was evaluated on samples of chickens with known (n = 96) and unknown aHEV (n = 310) exposure. The results on the experimental samples with known avian HEV exposure indicate that the FMIA have a specificity and sensitivity of 100%. The earliest antibody response was detected 14 days post inoculation. The overall prevalence of avian HEV IgY antibodies in field samples from chickens with unknown avian HEV exposure was 46.8% (145/310). A higher seroprevalence was found in chickens older than 50 weeks ($p < 0.05$). The ORF2 FMIA has great potential as an ELISA alternative for detection of antibodies against avian HEV.

Keywords: Fluorescent microbead immunoassay, aHEV, serology, chickens

1. INTRODUCTION

Hepatitis E virus (HEV) is a member of the genus *Hepevirus* within the family *Hepeviridae*, which are non-enveloped, single-stranded RNA viruses with icosahedral symmetry consisting of at least four recognized genotypes of mammalian HEV (mHEV) and a separate floating species consisting of at least three genotypes of avian HEV (Bilic et al., 2009; Marek et al., 2010). The viral genome consists of two non-coding regions and three open reading frames (ORFs): ORF1 encodes the non-structural viral proteins, ORF2 encodes for the capsid protein, and ORF3 which partially overlaps ORF2 and encodes a cytoskeleton-associated phosphoprotein (Huang et al., 2004).

Avian HEV was first detected in chickens with big liver and spleen disease in Australia (Payne et al., 1999). A similar disease, hepatitis-splenomegaly syndrome (HSS) was described in North America (Ritchie et al., 1991) and subsequently associated to avian HEV (Haqshenas et al., 2001). Avian HEV has now been detected worldwide including United Kingdom, Hungary, Czech Republic, Ukraine, Germany, Spain, Poland, Israel, China, Korea and Tawain (Huang et al. 2002b; Morrow et al., 2008; Peralta et al., 2009a; Bilic et al., 2009; Marek et al., 2010; Zhao et al., 2010; Kwon et al., 2012; Hsu et al., 2014). In both broiler breeder hens and egg laying hens aged from 30 to 72 weeks, the HSS is characterized by an increase in mortality, a decrease in egg production, bloody fluid in the abdomen and an enlarged liver and spleen (Meng et al., 2008). It has been shown that a considerable proportion of the chicken flocks (71%) in the United States were seropositive to avian HEV infection (Huang et al., 2002b), although seropositive flocks did not necessarily suffer from HSS (Sun et al., 2004a).

Currently, the methods for detecting avian HEV infection include conventional reverse transcriptase (RT)-PCR (Bilic et al., 2009; Huang et al., 2002b; Sun et al., 2004a), real-time RT-PCR (Troxler et al., 2011), commercial and *in-house* enzyme-linked immunosorbent assay (ELISA) (Hsu et al., 2014; Huang et al., 2002b; Zhao et al., 2013), and agar gel immunodiffusion (AGID) (Handlinger et al., 1988; Morrow et al., 2008). RT-PCR is a common method used for molecular epidemiological studies of avian HEV in chickens from many different countries (Bilic et al., 2009; Huang et al., 2002b; Kwon et al., 2012; Marek et al., 2010; Peralta et al., 2009a; Sun et al., 2004a). Compared to detection of viral RNA by RT-PCR, serology is rather inexpensive and less technical and therefore is ideal for screening large number of samples and can provide important and useful tool for understanding avian HEV epidemiological features.

The fluorescent microbead immunoassay (FMIA) is an evolving novel diagnostic tool with an increasing application in veterinary serology (Clavijo et al., 2006; Wagner et al., 2011a; Wagner et al., 2011b; Kunita et al., 2011a; Lin et al., 2011; Gimenez-Lirola et al., 2012; Langenhorst et al., 2012; Chen et al., 2013a; Owolodun et al., 2013; Roerig et al.,

2013; Gimenez-Lirola et al., 2014). The FMIA uses multiple fluorescent microspheres, and each bead set can be conjugated to different antigens as the solid phase for the detection of antibodies or antigens in biological samples. An advantage of this technology is that FMIA screening of samples for serum antibodies to multiple pathogens or antigens simultaneously in one reaction well using a small amount of sample. Therefore, the savings in cost of time, labor and reagents could be substantial. In addition, there are indications that FMIA tests can improve sensitivity compared to conventional assays such as ELISAs (van Gageldonk et al., 2008; Giménez-Lirola et al., 2012; Gimenez-Lirola et al., 2014). The development of a sensitive and specific FMIA for the detection of avian HEV IgY antibodies is described using samples from chickens infected experimentally with avian HEV and field samples from chickens with unknown avian HEV status.

2. MATERIALS AND METHODS

2.1 Experimental samples

Ninety-six serum samples from 36 specific-pathogen-free (SPF) chickens from a previous aHEV study (Billam et al., 2009) were used in the present study. Briefly, 24 chickens were intravenously inoculated with aHEV genotype 2 strain prototype US (Haqshenas et al., 2001) or strain VA (Sun et al., 2004). Blood samples were collected before inoculation and weekly thereafter for three (n = 12) or four (n = 12) weeks. Twelve chickens where sham-inoculated and served as negative controls. To verify successful challenge in the chickens infected experimentally, seroconversion was determined by using an ELISA described previously (Huang et al., 2002). Specifically, seroconversion started at day post-inoculation (dpi) 14 and at dpi 28 all inoculated chickens had seroconverted to IgY anti-aHEV. Serum from an additional 12 sham-inoculated chickens served as negative controls. A total of 36 negative control samples (all samples collected on dpi 0) and 60 samples (dpi 7–28) from chickens infected experimentally were tested.

2.2 Field serum samples

A total of 310 serum samples were collected from 16 to 112-weeks old chickens from seven commercial layer chicken flocks located in Iowa, Illinois, South Dakota, and Michigan, USA. All houses were samples in each flock (n = 5 to 11, average n = 8). Five serum samples were collected from chickens in different points of each house (n = 25 to 55/flock; average n = 42). Samples were collected at the same day within flocks. At the time of sample collection, none of the flocks reported HSS clinical signs.

2.3 Fluorescent microbead immunoassay (FMIA)

2.3.1 Antigen and coupling to carboxylated paramagnetic microbeads

A truncated recombinant protein spanning 268 aa of the C-terminal of avian HEV ORF2 was expressed in *Escherichia coli* and purified by affinity chromatography (Haqshenas et al., 2002) was kindly provided by Dr. Xiang-Jin Meng (Virginia Polytechnic Institute and State University, USA) and used as antigen for the FMIA. Fluorescent microsphere coupling was performed using a method described previously (Gimenez-Lirola et al., 2012) by addition of 25 µg of the avian HEV ORF2 recombinant protein in 2,5 × 10^6 beads. The coupling was performed at 22 ˚C according to the two step carbodiimide reaction protocol as recommended by the supplier (http://www.luminexcorp.com/prod/groups/public/documents/lmnxcorp/protein-coupling-protocol-magp.pdf). All washing steps were performed using a magnetic separator. The microspheres were resuspended by sonication in a sonicating water bath for 30 s and then vortexed vigorously for 1 min in order to disperse bead aggregates. A 200 µl aliquot containing 2.5 × 10^6 microspheres was transferred to a 1.5 ml microcentrifuge tube for activation. The beads were washed once in H_2O and resuspended in 80 µl of 100 mM sodium phosphate, pH 6.2. Then, 10 µl of N-hydroxysulfosuccinimide sodium salt (sulfo-NHS) (sulfo-NHS, 50 mg/ml; Thermo Scientific, Rockford, IL, USA) and 10 µl 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC, 50 mg/ml; Thermo Scientific), both prepared immediately before

usage, were added and the mixture was incubated for 20 min while rotating in the dark. After incubation, the activated beads were washed twice and resuspended with 50 mM 2-[N-morpholino]ethanesulfonic acid, pH 5.0 (MES). The activated beads were used for coupling with the ORF2 polypeptide which was done under rotation for 2 h. After completion of the coupling process, the beads were washed three times and resuspended in fetal bovine serum-based storage buffer (Gibco®, Life Technologies, Grand Island, NY, USA), counted, and stored in the dark at 2-8 °C.

2.3.2 FMIA

The assay was performed at 22 °C using Bio-Plex Pro™ flat bottom plates (Bio-Rad, Richmond, CA, USA) (Fig. 1). Coupled beads were diluted in the storage buffer to a final concentration of 2500 beads/well (50 beads/µl). Then, 50 µl of the bead suspension and 50 µl of serum sample (diluted 1:50) were added to each well. Plates were incubated on a shaker for 30 min at 500 rpm and washed three times with PBS containing 0.05% Tween-20 (PBST). Next, 50 µl of a 1:2,000 dilution of a biotin-conjugated goat anti-chicken IgY Fc (Gallus Immunotech Inc, Fergus, Ontario, Canada) in assay buffer was added to each well and the plate was incubated on a shaker for 30 min. After three washing steps, 50 µl of a 1:100 dilution of streptavidin R-phycoerythrin conjugate (SAPE) (MOSS, Pasadena, MD, USA) was added to each well. Finally, after 30 min of incubation on a shaker and three additional wash steps, the beads were re-suspended in 100 µl of assay buffer and were analyzed using a Luminex-100 flow cytometer (Luminex Corp., Austin, TX, USA) at default settings set by the manufacturer for routine applications. Median fluorescence intensity (MFI) of the reporter signal estimated from at least 50 beads was used for the data analysis. The MFI data was corrected for background (MFI-Bkg) levels by subtracting the negative antigen signal from the positive antigen signal. A set of internal standard controls described as high positive control, low negative control and cut off control, were selected after initial analysis and subsequently included on each plate. Results were presented as antibody index (MFI-Bkg sample/MFI-Bkg cut off serum). Samples with an index value above 1.1 were considered positive.

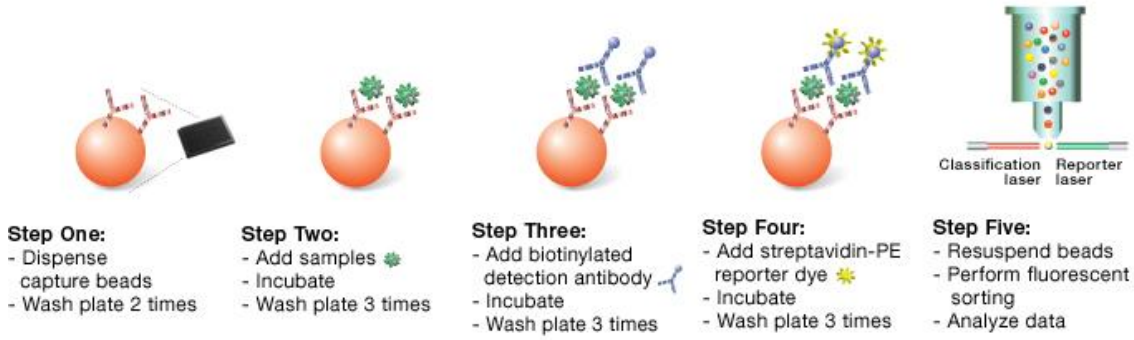

Figure. 1. Schematic representation of a FMIA workflow. Source: http://www.bioradiations.com/focus-on-applications/60-biomarker-analysis/1345-multiplex-analysis-of-inflammatory-markers-using-bio-plex-pro-human-th17-cytokine-assays.

2.4 Reproducibility

The reproducibility of the FMIA was evaluated by utilizing six different chicken sera. The coefficient of variation (CV) was used to evaluate the intra- and inter-assay variation. Each sample was tested in three different runs on different occasions to determine the inter-assay CV, and three replicates within the same run were used to calculate the intra-assay CV.

2.5 Cut-off determination

The ability of the FMIA to discriminate between positive and negative samples (sensitivity and specificity) was evaluated using receiver operator characteristic (ROC) curve analysis using both experimental and field samples. Positive samples for estimating diagnostic sensitivity included independent serum samples from experimentally infected

chickens (n = 24) collected on dpi 21 or 28 and previously confirmed as positive by ELISA (Billam et al., 2009). Negative samples for estimating diagnostic specificity included independent serum samples from experimentally inoculated chickens on dpi 0 and sham-inoculated controls (n = 36). The analysis was performed using GraphPad Prism v. 6.01 (GraphPad Software, La Jolla, CA, USA).

3. RESULTS

3.1 Evaluation of the avian HEV FMIA

3.1.1 Estimation of cut-off values for avian HEV ORF2 FMIA

The data from experimental serum samples were used to determine the optimal FMIA cut-off value by ROC analysis using the ELISA (Billam et al., 2009) as the reference method to confirm the cut-off. All 36 negative control samples were negative by FMIA and all 24 positive control samples were positive by FMIA. The cumulative area under the ROC curve (AUC) indicated that aHEV ORF2-based FMIA was 100% accurate. The optimal cut-off points were determined to be a sample MFI value ranging between 1008 and 1297 giving an overall diagnostic sensitivity and specificity of 100%. An internal cut-off control with an appropriate MFI value was selected according to these results.

3.1.2 Reproducibility of the assay

The intra-assay variation of six serum samples ranged from 0.57% to 7.83% with a median value of 5.04%; while the inter-assay variation ranged from 0.74% to 7.99% with a median value of 6.15%, indicating that the results were reproducible.

3.2 Detection of anti-aHEV antibodies in chickens experimentally infected with aHEV

The earliest detection was at 14 days after aHEV infection (7/24, 29.2%) and on dpi 21 onwards all chickens were positive for IgY anti-aHEV antibodies. Figure 2 summarizes the mean FMIA index values.

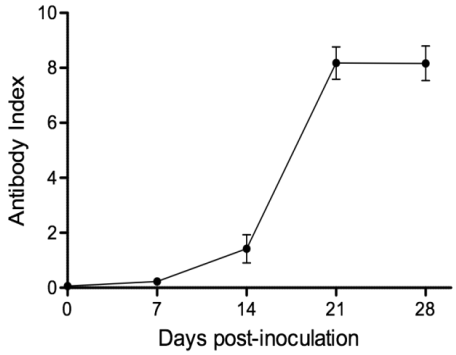

Figure 2. Mean FMIA index value (±SEM) at different days post inoculation for chickens infected experimentally with aHEV. Samples with an index value below 0.9 were considered negative and those above 1.1 were considered positive. Values from 0.9 to 1.1 were considered inconclusive.

3.3 Detection of anti-aHEV antibodies in chickens of unknown exposure status

The prevalence rates of anti-aHEV antibodies are summarized in Fig. 2. When using the aHEV antigen in FMIA, all seven analyzed chicken flocks had at least one seropositive animal. Considering all farms, the overall prevalence of aHEV antibodies was 46.8% (145/310). The proportion of positive animals ranged from 20% to 74% per farm. Considering all the examined animals by rank of age, seropositive animals were detected at any age, but chickens older than 50 weeks were found to have higher seropositivities compared to chickens from 16-26 or 31-45 weeks old (Fig. 3).

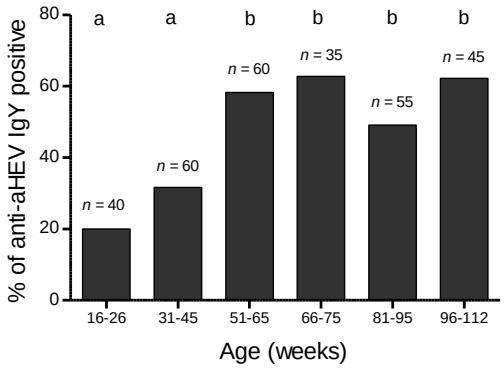

Figure 3. Detection rates of IgY anti-aHEV antibodies in serum samples from chickens of different age-groups (n = number of chickens sampled in each age-group). Different superscripts (a,b) indicate significant ($p < 0.05$) different prevalence rates among age-groups.

4. DISCUSSION

To date, ELISA remains the most widely used tool for conducting epidemiological studies of avian HEV in chickens. In this study, a novel FMIA was developed to detect avian HEV IgY antibodies in chicken populations.

The ORF2-based avian HEV FMIA developed in the present study was able to detect anti-avian antibodies in 29% (7/24) of the infected chickens as early as 14 days post-infection. This was higher than the detection rate using the same samples and the same antigen in an ELISA platform (4%, 1/24; $p = 0.02$) (Billam et al., 2009), indicating that the FMIA developed herein has a higher sensitivity.

The application of FMIA is not new to the diagnosis/detection of infectious agents in humans (Dasso et al., 2002) and its use has been increasing in veterinary serology (Chen et al., 2013b; Gimenez-Lirola et al., 2014b; Kunita et al., 2011b; Langenhorst et al., 2012a; Owolodun et al., 2013; Roerig et al., 2013b). However, to our knowledge, this present study is the first of its kind describing the use of FMIA for the detection of circulating antibodies in chicken populations. Microbead assays often have the benefit of taking advantage of multiple antigens for antibody detection for the same virus (Clavijo et al., 2006; Langenhorst et al., 2012); however, the FMIA developed in the present study was tested as a single-plex utilizing one antigen. As the FMIA has the additional advantage of utilizing considerably less amount of antigen compared to an ELISA, this assay will result in overall cost reduction and further supports that a change of detection platforms is justified especially when large numbers of chicken serum samples need to be tested.

Testing of serum samples from chickens with unknown avian HEV exposure in this study indicated that the seropositive rate of avian HEV antibodies was 46.8% (145/310) in the chickens at various ages and that all the flocks studied were seropositive for anti-avian HEV antibodies with a detection rate ranging from 20% to 74% per farm. This is in agreement with what has been previously reported in the United States (Huang et al., 2002b). Of the 76 flocks tested, 54 (71%) were positive for anti-avian HEV IgY and that within a positive farm, the percentage of positive chickens could vary from 15% to 100% (Huang et al., 2002b). The present study also reflects that the likelihood of being seropositive increases with age. This is also in accordance with previous studies (Huang et al., 2002b; Peralta et al., 2009a).

In conclusion, the FMIA platform described in this study is appropriate for detection of anti-HEV IgY antibodies in chickens. Further studies are warranted to investigate the potential of multiplexing the FMIA so that it can detect evidence of exposure to multiple chicken pathogens in one single step.

CHAPTER 4
EVIDENCE OF AVIAN HEPATITIS E VIRUS INFECTION IN BRAZILIAN CHICKEN FLOCKS

ABSTRACT

A total of 160 serum samples from chicken ranging from six to 118-weeks of age were collected on three farms in south-east Brazil and tested for avian HEV antibodies by a fluorescence microbead-based immunoassay (FMIA). Anti-avian HEV antibodies were detected on all farms investigated and in 15.6% (25/160) of the chickens. Within the farms, the proportion of seropositive animals ranged from 12% to 23%. Chickens between 64 and 69 weeks of age presented the highest rate of detection (6/20, 30%), while the youngest and oldest age groups investigated, 6-weeks-old pullets and 118 weeks-old chickens, were seronegative. Forty pooled fecal samples from eight farms were tested for avian HEV RNA by RT-PCR and three (7.5%) were positive for the helicase gene. Further genetic characterization of the PCR positive samples through sequencing was unsuccessful. This work provides evidence of circulation of avian HEV in the Brazilian chicken population.

Key words: avian hepatitis E, layer chickens, diagnosis

1. INTRODUCTION

Hepatitis E virus (HEV) is a member of the genus *Hepevirus* within the family *Hepeviridae*, which are non-enveloped, icosahedral, single-stranded, positive sense RNA viruses that encode three open reading frames (Tam et al., 1991). The taxonomy of *hepeviruses* is not fully elucidated but up to now there are at least four recognized genotypes of mammalian HEV, a separate floating species consisting of avian HEV and a number of divergent isolates infecting rats, bats, and ferrets (Bilic et al., 2009; Johne et al., 2010; Marek et al., 2010; Smith et al., 2013). Avian HEV isolates share approximately 48-60% nucleotide sequence identity with mammalian HEVs, and antigenic cross-reactivity of the capsid protein between mammalian and avian HEV isolates has been described (Haqshenas et al., 2002). Phylogenetic analysis indicates at least three different avian HEV genotypes and additional putative genotypes, suggesting a high diversity within avian HEV (Bilic et al., 2009; Marek et al., 2010; Zhao et al., 2010; Banyai et al., 2012; Kwon et al., 2012).

Avian HEV was first associated with big liver and spleen disease (BLSD) in Australia (Payne et al., 1999) and hepatitis-splenomegaly syndrome (HSS) syndrome in North America (Haqshenas et al., 2001). Subsequently, avian HEV infection has been associated with disease outbreaks in chicken flocks worldwide (Huang et al., 2002b; Agunos et al., 2006; Bilic et al., 2009; Morrow et al., 2008; Marek et al., 2010; Zhao et al., 2010; Sprygin et al., 2012). Clinical signs in diseased chickens typically are characterized by a drop in egg production and high mortality rates and on gross exam enlarged livers, enlarged spleens, and serosanguineous fluid in their abdominal cavities can be observed (Meng et al., 2008). So far, avian HEV has only been identified in chickens, although the virus is capable to cross the species barrier and under experimental conditions turkeys were infected successfully with avian HEV (Sun et al., 2004b).

Due to the lack of an efficient system to propagate HEV in cell culture (Meng et al., 2008), infection with avian HEV is primarily diagnosed by reverse transcription-PCR (RT-PCR) and enzyme-linked immunosorbent assay (ELISA) methods (Sun et al., 2004a). Molecular and serological investigations have shown that avian HEV is widespread in chicken flocks in the United States, Spain and Korea (Sun et al., 2004a; Peralta et al., 2009a; Kwon et al., 2012). However, the role of avian HEV in HSS is unclear, as the virus has been detected in flocks with and without no history of HSS (Sun et al., 2004a; Peralta et al., 2009a; Kwon et al., 2012). Differences in virus strain, virus dose, diet and age have been implicated as potential co-factors for the manifestation of the full spectrum of clinical HSS (Agunos et al., 2006; Meng et al., 2008).

The status of avian HEV infection in South American chickens is largely unknown as there is only one report of serological evidence of avian HEV circulation in Brazilian chickens based on testing of a limited number of samples

(n = 25, 5/25 [20%] positive) without information of the source or age of the sampled chickens (Vitral et al., 2005). The aims of the present study were to assess the serological profile of avian HEV in Brazilian chicken farms by a fluorescence microbead-based immunoassay (FMIA) and to determine the possible avian HEV circulation by RT-PCR.

2. MATERIAL AND METHODS

2.1 Samples

A total of 160 serum samples and 40 fecal samples randomly collected from different layer and broiler breeder farms located in the south-east region of Brazil in the Minas Gerais State were used in this study. No particular health problems had been reported in the examined flocks. Sampled farms were determined based on the agreement to participate in this study and in the availability to provide samples for the testing.

Serum samples were collected during September 2011 and were unrelated to fecal samples, collected between August and September 2012. Blood samples were collected by venipuncture of the brachial vein in 5 ml serum tubes from chickens ranging from 6 to 118 weeks of age from three farms (n = 50 to 60/farm, 10 chickens per house). Blood was centrifuged at 1500 × g for 10 min to collect serum, aliquoted into 1.5 ml microtubes and stored at -20 °C until use.

Fecal samples were collected directly from floor droppings (2 g of fresh fecal material from five sites in each house) from two to six different houses in each of eight farms, with hens' age ranging from 20 to 102 weeks. Fecal samples were homogenized and an aliquot of 0.4 g were resuspended in 4 ml PBS, vigorously vortexed and centrifuged at 1500 × g for 10 min. Samples were stored at −80 °C until use.

2.2 Fluorescent microbead-based immunoassay (FMIA) to detect anti-HEV antibodies in chicken sera

Chicken serum samples were tested by an avian-HEV specific FMIA for the presence of avian HEV antibodies as described previously on Chapter 3.

2.3 RNA extraction and avian HEV RNA detection

RNA extractions from fecal samples were performed using the QIAamp® Viral RNA Mini kit (Qiagen, Valencia, California, USA). Extracts were subsequently used for detection of the helicase and capside gene of avian HEV using primers previously described (Sun et al., 2004a) in an nested RT-PCR reaction. Briefly, for the helicase gene detection, external primer set 5′-TGTTATYACACCCACCAARACGYTG-3′, and 5′-CCTCRTGGACCGTWATCGACCC-3′; and internal primer set 5′-GCCACGGCTRTTACACCYCAYGT-3′, and 5′-GACCCRGGRTTCGACTGCTT-3′ were used. For the capside gene, external primer set 5′- TCGCCYGGTAAYACWAATGC-3′, and 5′-GCGTTSCCSACAGGYCGGCC-3′; and internal primer set 5′-ACWAATGCYAGGGTCACCCG -3′, and 5′-ATGTACTGRCCRCTSGCCGC -3′ were used. Reverse transcriptase reaction and first round PCR were performed with OneStep RT-PCR kit (Qiagen, Valencia, California, USA) according to manufacturer's instructions. The conditions for the 1st PCR reaction were as follows: 50 ˚C for 30 min; 95 ˚C for 15 min; 35 cycles of 94˚C for 30 sec, 55˚C for 30 sec, 72˚C for 1 min; final elongation step of 72 ˚C for 10 min. The second round PCR was performed with ReadyMix® Taq PCR Reaction Mix (Sigma-Aldrich, St. Louis, Missouri, USA). The conditions for the nested PCR reaction were as follows: 95 ˚C for 5 min; 35 cycles of 94 ˚C for 30 sec, 55 ˚C for 30 sec, 72 ˚C for 30 sec; final elongation step of 72˚C for 7 min. PCR products with expected size (386 and 242 bp for the helicase and capsid gen, respectively) were examined on a 1% agarose gel, excised and purified with the QIAquick Gel Extraction Kit (Qiagen, Valencia, California, USA) used for direct sequencing of both strands and cloned into the pGEM-T Vector (Promega, Madison, Wisconsin). The recombinant plasmids were transformed into TOP10 *Escherichia coli* bacteria (Invitrogen, Foster City, California, USA) and propagated following the procedures of the cloning kit manual. The identified recombined plasmids were extracted using the QIAprep Spin MiniPrep kit (Qiagen, Valencia, California, USA) according to the manufacturers' instructions and then sequenced. Sequencing of

positive clones in both directions was performed at the Iowa State University DNA Facility, Ames, IA, USA. Sequences were aligned with published data using BLAST at the national Centre for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/).

In order to detect possible RT-PCR false-negative results due to failure in the RNA extraction or to the presence of PCR inhibitors, extracted samples were also tested to the housekeeping gene GAPDH using the primers pGAPDH-F 5'-CAGGAGCGTGACCCCAGCAACAT-3' and pGAPDH-R 5'-CCCATCAGCAGCAGCCTTCACTACC-3' as previously described (Panabieres et al., 1984). PCR products with expected size (573 bp) were examined on a 1% agarose gel.

3. RESULTS

3.1 Detection of anti-avian HEV IgG antibodies in chickens

A total of 160 serum samples were tested for the presence of antibodies to avian HEV and 25 (15.6%) were found positive. All three farms had anti-avian HEV IgY positive chickens and the overall proportion of positive animals ranged from 12 to 23% per farm. The detection rates varied between age groups (Fig. 1). Chickens between 64 and 69 weeks of age, right after the end of the first egg production cycle, presented the highest rate of detection (6/20, 30%), while 6-weeks-old pullets and hens at the end of the production cycle (118-weeks-old) were seronegative (Fig. 1). Chickens within the first production cycle, between 14 and 54 weeks, presented around 17% of anti-avian HEV IgY positive animals.

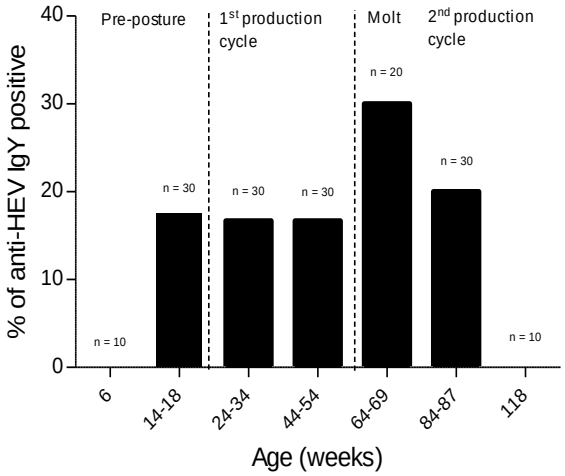

Figure 1. Percentage of positive IgY anti-avian HEV antibodies in chickens of different age-groups (n = number of animals analyzed in each week). The egg production cycle is indicated as follows: 0-18 weeks: pre-posture; 19-60 weeks: first egg production cycle; 61-70 weeks: molt; 71-120: second production cycle.

3.2 Detection of avian HEV RNA

A helicase gene- and a capsid gene-based RT-PCR assay were used to detect avian HEV RNA. Of the 40 pooled samples collected from eight farms, only three (7.5%) samples were positive for the helicase gene and none for the capsid gene. Two avian HEV helicase gene RNA positive samples were from hens (46- and 64-weeks old) belonging to the same farm and the third one was from 30-weeks old hens from another farm. Direct sequencing and cloning of those fragments was attempted on both strands at least three times and was not successful. All samples were positive for the GAPDH housekeeping gene.

4. DISCUSSION

Avian HEV has been identified in North America, Australia, Europe, and Asia (Payne et al., 1999; Haqshenas et al., 2001; Agunos et al., 2006; Bilic et al., 2009; Peralta et al., 2009a;

Marek et al., 2010; Zhao et al., 2010; Kwon et al., 2012). In Brazil, a anti-HEV antibody prevalence of 20% was identified in chickens , although this was based on testing of a small number of samples (n = 25) without information of age or source of the chickens sampled (Vitral et al., 2005). Therefore, in the present study, a larger number of serum samples from chickens from different age groups and farms were tested to establish a serological profile for avian HEV. In addition, it was attempted to detect avian HEV RNA in fecal samples.

In this study, all the three farms and 17% of sampled chickens were anti-avian HEV antibody positive. This is in agreement with previous studies which reported that 20% (5/25) of chickens in Brazil (Vitral et al., 2005), 30% (380/1276) of chickens in United States (Huang et al., 2002b), and 28% (84/297) chickens in Korea were seropositive for avian HEV antibodies (Kwon et al., 2012). Young 6-weeks-old pullets were anti-avian HEV IgY negative, with seroconversion starting around 14 weeks of age. It has been suggested that natural infection of chicken flocks with avian HEV may occur around 12 weeks of age (Sun et al., 2004a) and that the likelihood of being seropositive increases with age (Huang et al., 2002b; Peralta et al., 2009a) which could explain the lack of seropositive animals at younger ages. Chickens at the end of the second egg production cycle (118-weeks-old) were also seronegative, suggesting a decline of the antibody levels. Alternatively, the low number of serum samples tested at this age (n = 10) in addition to the overall low rates of positive chickens found in this study could have contributed to the lack of detection of positive animals in this age group. Although there is no information available on the half-life of anti-avian HEV antibodies in chickens, anti-HEV IgG antibodies seems to be long-lasting after natural infection in humans (Khuroo et al., 2010).

During an attempt to identify and characterize avian HEV RNA in pooled fecal samples from chickens from different age groups and farms, 8% of the samples tested were HEV RNA positive by RT-nested PCR targeting the helicase gene. The low avian HEV RNA detection rate in the present study could be due to the fact that the fecal samples tested in this study were obtained from animals older than 20 weeks. It has been shown that specific-pathogen-free chickens experimentally infected by the oronasal route were positive for anti-avian HEV antibodies 3 weeks after challenge and that virus shedding in feces was observed from 1 to 8 weeks post-inoculation (Billam et al., 2005). Therefore, considering a seroconversion around approximately 14 weeks of age in the present study, the fecal shedding should occur mainly from 12 to 20 weeks of age. In addition, considering the relatively low seroprevalence rate in this study and the absence of clinical signs in the farms sampled could have reduced the chance of viral detection. Previously, avian HEV RNA was detected in fecal samples from healthy chickens (Sun et al., 2004a; Peralta et al., 2009a; Kwon et al., 2012). Alternatively, the failure to detect partial fragments of helicase and capsid genes could also have been due to a high genetic heterogeneity (Sun et al., 2004a; Bilic et al., 2009; Marek et al., 2010; Banyai et al., 2012) or due to a poor quality of samples wherein viral RNA might have been degraded. However, all samples were positive to the housekeeping gene tested, indicating that the latter was not cause for the absence of amplification.

It is worth noting that none of the HEV helicase RNA positive samples could be confirmed by further sequencing. This could be explained by a low viral RNA concentration, which could have prevented both direct sequence and cloning. Alternatively, unspecific primer annealing and subsequent amplification could have been occurring which cannot be entirely ruled out. To further assess and characterize the HEV strains involved in Brazilian chicken flocks a higher number of farms and a higher number of chickens under 20 weeks must be sampled and tested.

In summary, this study provides further evidence of avian HEV circulation in 30-64 week-old chickens in Brazil although genetic characterization of viral isolates is still missing.

GENERAL CONCLUSIONS

In this thesis, a novel RT-PCR for detection of mHEV and a novel FMIA for detection of aHEV antibodies have been developed and evaluated. First, a single-plex real-time RT-PCR assay for detection of all four recognized mHEV genotypes without differentiation and a duplex real-time RT-PCR assay for detection and differentiation of HEV genotypes 3 and 4 were developed and compared to the performance of previously published assays. Single-plex assays designed to broadly detect HEV genotypes 1-4 showed a significantly better performance than duplex assays, indicating that multiplex assays for detection and differentiation of mHEV genotypes cannot be used at this point due to its low sensitivity. A previously published assay (Jothikumar et al., 2006), which is currently the most used assay for detection of human HEV in diagnostic laboratories worldwide presented the overall best performance among the tested assays.

Subsequently, Jothikumar et al. (2006) assay was used to screen pooled porcine fecal samples from different farms in Minas Gerais State, Brazil. There was a high (72.5%) positive detection of HEV RNA in all investigated farms. This result reinforces previous evidence of high circulation of HEV on pig farms and could represent a direct potential contamination source for pig handlers and an important source of environmental contamination. Phylogenetic analysis of a fragment of the ORF2 gene revealed that all yield sequences clustered in genotype 3, described previously in human and swine infection in Brazil.

A novel FMIA was developed to detect avian HEV IgY antibodies in chicken populations with an improved sensitivity when compared to an ELISA based on the same antigen used herein.

Subsenquently the novel FMIA assay was applied in the sera of chickens in Minas Gerais state, Brazil. Evidence of antibody response against avian HEV from chickens from 14- to 87-weeks old was provided although genetic characterization of viral isolates is still missing.

REFERENCES

ABRAVANEL, F., SANDRES-SAUNE, K., LHOMME, S., et al. Genotype 3 diversity and quantification of hepatitis E virus RNA. *J Clin Microbiol* 50, 897-902, 2012.

AGGARWAL, R., 2013. Diagnosis of hepatitis E. *Nat Rev Gastroenterol Hepatol.* 10, 24-33, 2012.

AGGARWAL, R. AND JAMEEL, S. Hepatitis E. *Hepatology* 54, 2218-2226, 2011.

AGGARWAL, R., KINI, D., SOFAT, S., et al. Duration of viraemia and faecal viral excretion in acute hepatitis E. *Lancet* 356, 1081-1082, 2000.

AGUNOS, A.C., YOO, D., YOUSSEF, S.A., et al. Avian hepatitis E virus in an outbreak of hepatitis--splenomegaly syndrome and fatty liver haemorrhage syndrome in two flaxseed-fed layer flocks in Ontario. *Avian Pathol* 35, 404-412, 2006.

AHN, J.M., RAYAMAJHI, N., GYUN, K.S., et al. Comparison of real-time reverse transcriptase-polymerase chain reaction and nested or commercial reverse transcriptase-polymerase chain reaction for the detection of hepatitis E virus particle in human serum. *Diagn Microbiol Infect Dis* 56, 269-274, 2006.

ANDRAUD, M., DUMAREST, M., CARIOLET, R., et al. Direct contact and environmental contaminations are responsible for HEV transmission in pigs. Vet Res 44, 102-, 2013.

ARANKALLE, V.A., PARANJAPE, S., EMERSON, S.U., et al. Phylogenetic analysis of hepatitis E virus isolates from India (1976-1993). *J Gen Virol* 80, 1691-1700, 1999.

ASSIS, S.B., SOUTO, F.J., FONTES, C.J., et al.[Prevalence of hepatitis A and E virus infection in school children of an Amazonian municipality in Mato Grosso State]. *Rev Soc Bras Med Trop.* 35, 155-158, 2002.

BALAYAN, M.S., ANDJAPARIDZE, A.G., SAVINSKAYa, S.S., et al. Evidence for a virus in non-A, non-B hepatitis transmitted via the fecal-oral route. *Intervirology* 20, 23-31, 1983.

BANYAI, K., TOTH, A.G., IVANICS, E., et al. Putative novel genotype of avian hepatitis E virus, Hungary, 2010. *Emerg Infect Dis* 18, 1365-1368, 2012.

BATTS, W., YUN, S., HEDRICK, R., ET AL. A novel member of the family Hepeviridae from cutthroat trout (Oncorhynchus clarkii). *Virus Res* 158, 116-123. 2011.

BAYLIS, S.A., BLUMEL, J., MIZUSAWA, S., et al. World Health Organization International Standard to harmonize assays for detection of hepatitis E virus RNA. *Emerg Infect Dis* 19, 729-735, 2013.

BAYLIS, S.A., HANSCHMANN, K.M., BLUMEL, J., et al. Standardization of hepatitis E virus (HEV) nucleic acid amplification technique-based assays: an initial study to evaluate a panel of HEV strains and investigate laboratory performance. *J Clin Microbiol* 49, 1234-1239, 2011.

BERTO, A., BACKER, J.A., MESQUITA, J.R., et al. Prevalence and transmission of hepatitis E virus in domestic swine populations in different European countries. *BMC Res Notes* 5, 190-, 2012.

BERTO, A., van der POEL, W.H., HAKZE-VAN der, H.R., et al. Replication of hepatitis E virus in three-dimensional cell culture. *J Virol Methods* 187, 327-332, 2013.

BILIC, I., JASKULSKA, B., BASIC, A., et al. Sequence analysis and comparison of avian hepatitis E viruses from Australia and Europe indicate the existence of different genotypes. *J Gen Virol* 90, 863-873, 2009.

BILLAM, P., HUANG, F.F., SUN, Z.F., et al. Systematic pathogenesis and replication of avian hepatitis E virus in specific-pathogen-free adult chickens. *J Virol* 79, 3429-3437, 2005.

BILLAM, P., LEROITH, T., PUDUPAKAM, R.S., et al. Comparative pathogenesis in specific-pathogen-free chickens of two strains of avian hepatitis E virus recovered from a chicken with Hepatitis-Splenomegaly syndrome and from a clinically healthy chicken. *Vet Microbiol* 139, 253-261, 2009.

BORGEN, K., HERREMANS, T., DUIZER, E., et al. Non-travel related Hepatitis E virus genotype 3 infections in the Netherlands; a case series 2. *BMC Infect Dis.* 8, 61-, 2008.

BORTOLIERO, A.L., BONAMETTI, A.M., MORIMOTO, et al. Seroprevalence for hepatitis E virus (HEV) infection among volunteer blood donors of the Regional Blood Bank of Londrina, State of Parana, Brazil. *Rev Inst Med Trop. Sao Paulo* 48, 87-92, 2006.

BOUQUET, J., CHEREL, P., PAVIO, N. Genetic characterization and codon usage bias of full-length Hepatitis E virus sequences shed new lights on genotypic distribution, host restriction and genome evolution. *Infect Genet Evol.* 12, 1842-1853, 2012.

BOUWKNEGT, M., ENGEL, B., HERREMANS, M.M., et al. Bayesian estimation of hepatitis E virus seroprevalence for populations with different exposure levels to swine in The Netherlands. *Epidemiol Infect.* 136, 567-576, 2008a.

BOUWKNEGT, M., LODDER-VERSCHOOR, F., van der POEL, W.H., et al. Hepatitis E virus RNA in commercial porcine livers in The Netherlands. *J Food Prot.* 70, 2889-2895, 2007.

BOUWKNEGT, M., FRANKENA, K., RUTJES, S.A., et al. Estimation of hepatitis E virus transmission among pigs due to contact-exposure. *Vet. Res.* 39, 40-2008b.

BRASSARD, J., GAGNE, M.J., GENEREUX, M., et al. Detection of human food-borne and zoonotic viruses on irrigated, field-grown strawberries. *Appl Environ Microbiol* 78, 3763-3766, 2012.

CACOPARDO, B., RUSSO, R., PREISER, W., et al. Acute hepatitis E in Catania (eastern Sicily) 1980-1994. The role of hepatitis E virus. Infection 25, 313-316, 1997.

CARRILHO, F.J., MENDES, C.C., SILVA, L.C. Epidemiology of hepatitis A and E virus infection in Brazil. *Gastroenterol Hepatol*. 28, 118-125, 2005.

CASAS, M., PUJOLS, J., ROSELL, R., et al. Retrospective serological study on hepatitis E infection in pigs from 1985 to 1997 in Spain. *Vet Microbiol.* 135, 248-252, 2009.

CHEN, T.H., LEE, F., LIN, Y.L., et al. Development of a Luminex assay for the detection of swine antibodies to non-structural proteins of foot-and-mouth disease virus. *J Immunol Methods* 396, 87-95, 2013.

CHRISTENSEN, P.B., ENGLE, R.E., HJORT, C., et al. Time trend of the prevalence of hepatitis E antibodies among farmers and blood donors: a potential zoonosis in Denmark. *Clin Infect Dis* 47, 1026-1031, 2008.

CLAVIJO, A., HOLE, K., LI, M., et al. Simultaneous detection of antibodies to foot-and-mouth disease non-structural proteins 3ABC, 3D, 3A and 3B by a multiplexed Luminex assay to differentiate infected from vaccinated cattle. *Vaccine* 24, 1693-1704, 2006.

CLEMENTE-CASARES, P., RODRIGUEZ-MANZANO, J., GIRONES, R. Hepatitis E virus genotype 3 and sporadically also genotype 1 circulate in the population of Catalonia, Spain. *J Water Health* 7, 664-673, 2009.

COLSON, P., BORENTAIN, P., QUEYRIAUX, B., et al. Pig liver sausage as a source of hepatitis E virus transmission to humans. *J Infect Dis* 202, 825-834, 2010.

COLSON, P., ROMANET, P., MOAL, V., et al. Autochthonous infections with hepatitis E virus genotype 4, France. *Emerg Infect Dis* 18, 1361-1364, 2012.

COOPER, K., HUANG, F.F., BATISTA, L., et al. Identification of genotype 3 hepatitis E virus (HEV) in serum and fecal samples from pigs in Thailand and Mexico, where genotype 1 and 2 HEV strains are prevalent in the respective human populations. *J Clin Microbiol.* 43, 1684-1688, 2005.

COSSABOOM, C.M., CORDOBA, L., DRYMAN, B.A., et al. Hepatitis E virus in rabbits, Virginia, USA. *Emerg Infect Dis* 17, 2047-2049, 2011.

COSSABOOM, C.M., CORDOBA, L., SANFORD, B.J., et al. Cross-species infection of pigs with a novel rabbit, but not rat, strain of hepatitis E virus isolated in the United States. *J Gen Virol* 93, 1687-1695, 2012.

CROSSAN, C., BAKER, P.J., CRAFT, J., et al. Hepatitis E virus genotype 3 in shellfish, United Kingdom. *Emerg Infect Dis* 18, 2085-2087, 2012.

DASSO, J., LEE, J., BACH, H., et al. A comparison of ELISA and flow microsphere-based assays for quantification of immunoglobulins. *J Immunol Methods* 263, 23-33, 2002.

DE DEUS, N., CASAS, M., PERALTA, B., et al. Hepatitis E virus infection dynamics and organic distribution in naturally infected pigs in a farrow-to-finish farm. *Vet Microbiol.* 132, 19-28, 2008a.

DE DEUS, N., PERALTA, B., PINA, S., et al. Epidemiological study of hepatitis E virus infection in European wild boars (Sus scrofa) in Spain. *Vet Microbiol.* 129, 163-170, 2008b.

DE SOUZA, A.J., GOMES-GOUVEA, M.S., SOARES, M.C., et al. HEV infection in swine from Eastern Brazilian Amazon: evidence of co-infection by different subtypes. *Comp Immunol Microbiol Infect Dis* 35, 477-485, 2012.

DELL'AMICO, M.C., CAVALLO, A., GONZALES, J.L., et al. Hepatitis E virus genotype 3 in humans and Swine, Bolivia. *Emerg Infect Dis* 17, 1488-1490, 2011.

DI BARTOLO, I., DIEZ-VALCARCE, M., VASICKOVA, P., et al. Hepatitis E virus in pork production chain in Czech Republic, Italy, and Spain, 2010. *Emerg Infect Dis* 18, 1282-1289, 2012.

DI BARTOLO, I., PONTERIO, E., CASTELLINI, L., et al. Viral and antibody HEV prevalence in swine at slaughterhouse in Italy. *Vet Microbiol* 149, 330-338, 2011.

DIEZ-VALCARCE, M., KOKKINOS, P., SODERBERG, K., et al. Occurrence of human enteric viruses in commercial mussels at retail level in three European countries. *Food Environ Virol* 4, 73-80, 2012.

DONG, S., ZHAO, Q., LU, M., et al. 2011. Analysis of epitopes in the capsid protein of avian hepatitis E virus by using monoclonal antibodies. J Virol Methods 171, 374-380.

DOS SANTOS, D.R., DE, P., V, DE OLIVEIRA, J.M., et al. Hepatitis E virus in swine and effluent samples from slaughterhouses in Brazil. *Vet Microbiol* 149, 236-241, 2011.

DOS SANTOS, D.R., VITRAL, C.L., DE, P., et al. Serological and molecular evidence of hepatitis E virus in swine in Brazil. *Vet J* 182, 474-480, 2009.

DREXLER, J.F., SEELEN, A., CORMAN, V.M., et al. Bats worldwide carry hepatitis E virus-related viruses that form a putative novel genus within the family Hepeviridae. *J Virol* 86, 9134-9147, 2012.

DROBENIUC, J., FAVOROV, M.O., SHAPIRO, C.N., et al. Hepatitis E virus antibody prevalence among persons who work with swine. *J Infect Dis* 184, 1594-1597, 2001.

DROBENIUC, J., MENG, J., REUTER, G., et al. Serologic assays specific to immunoglobulin M antibodies against hepatitis E virus: pangenotypic evaluation of performances. *Clin Infect Dis* 51, e24-e27, 2010.

ECHEVARRIA, J.M., GONZALEZ, J.E., LEWIS-XIMENEZ, L.L., et al. Hepatitis E virus infection in Latin America: a review. *J Med Virol* 85, 1037-1045, 2013.

EMERSON, S.U. AND PURCELL, R.H. Hepatitis E virus. *Rev Med Virol* 13, 145-154, 2003.

ENGLE, R.E., YU, C., EMERSON, S.U., et al. Hepatitis E virus (HEV) capsid antigens derived from viruses of human and swine origin are equally efficient for detecting anti-HEV by enzyme immunoassay. *J Clin Microbiol.* 40, 4576-4580, 2002.

ENOUF, V., DOS, R.G., GUTHMANN, J.P., et al. Validation of single real-time TaqMan PCR assay for the detection and quantitation of four major genotypes of hepatitis E virus in clinical specimens. *J Med Virol* 78, 1076-1082, 2006.

FAUQUET, C.M. AND STANLEY, J. Revising the way we conceive and name viruses below the species level: a review of geminivirus taxonomy calls for new standardized isolate descriptors. *Arch Virol* 150, 2151-2179, 2005.

FEAGINS, A.R., OPRIESSNIG, T., GUENETTE, D.K., et al. Inactivation of infectious hepatitis E virus present in commercial pig livers sold in local grocery stores in the United States. *Int J Food Microbiol.* 123, 32-37, 2008a.

FEAGINS, A.R., OPRIESSNIG, T., HUANG, Y.W., et al. Cross-species infection of specific-pathogen-free pigs by a genotype 4 strain of human hepatitis E virus. *J Med Virol* 80, 1379-1386, 2008b.

FERNANDEZ-BARREDO, S., GALIANA, C., GARCIA, A., et al. Detection of hepatitis E virus shedding in feces of pigs at different stages of production using reverse transcription-polymerase chain reaction. *J Vet Diagn Invest* 18, 462-465, 2006.

FISHMAN, J.A., SCOBIE, L., TAKEUCHI, Y., 2012. Xenotransplantation-associated infectious risk: a WHO consultation. Xenotransplantation. 19, 72-81.

GALIANA, C., FERNANDEZ-BARREDO, S., GARCIA, A., et al. Occupational exposure to hepatitis E virus (HEV) in swine workers. *Am J Trop Med Hyg.* 78, 1012-1015, 2008.

GARCIA, C.G., SANCHEZ, D., VILLALBA, M.C., et al. Molecular characterization of hepatitis E virus in patients with acute hepatitis in Venezuela. *J Med Virol* 84, 1025-1029, 2012.

GARDINALI, N.R., BARRY, A.F., DA SILVA, P.F., et al. Molecular detection and characterization of hepatitis E virus in naturally infected pigs from Brazilian herds. *Res Vet Sci* 93, 1515-1519, 2012.

GARKAVENKO, O., MUZINA, M., MUZINA, Z., et al. Monitoring for potentially xenozoonotic viruses in New Zealand pigs. *J Med Virol* 72, 338-344, 2004.

GARSON, J.A., FERNS, R.B., GRANT, P.R., et al. Minor groove binder modification of widely used TaqMan probe for hepatitis E virus reduces risk of false negative real-time PCR results. *J Virol Methods* 186, 157-160, 2012.

GIMENEZ-LIROLA, L.G., JIANG, Y.H., SUN, D., et al. Simultaneous Detection of Antibodies against Apx Toxins ApxI, ApxII, ApxIII, and ApxIV in Pigs with Known and Unknown Actinobacillus pleuropneumoniae Exposure Using a Multiplexing Liquid Array Platform. *Clin Vaccine Immunol* 21, 85-95, 2014.

GIMÉNEZ-LIROLA, L.G., XIAO, C.T., HALBUR, P.G., et al. Development of a novel fluorescent microbead-based immunoassay and comparison with three enzyme-linked immunoassays for detection of anti-Erysipelothrix spp. IgG antibodies in pigs with known and unknown exposure. *J Microbiol Methods* 91, 73-79, 2012.

GRAFF, J., NGUYEN, H., KASORNDORKBUA, C., et al. In vitro and in vivo mutational analysis of the 3'-terminal regions of hepatitis e virus genomes and replicons. *J Virol* 79, 1017-1026, 2005.

GYARMATI, P., MOHAMMED, N., NORDER, H., et al. Universal detection of hepatitis e virus by two real-time pcr assays: Taqman and primer-probe energy transfer. *J Virol Methods* 146, 226-235, 2007.

HAKZE-VAN DER HONING, R.W., VAN COILLIE E., Antonis, A.F., et al. First isolation of hepatitis E virus genotype 4 in Europe through swine surveillance in the Netherlands and Belgium. *PLoS One* 6, e22673-, 2011.

HALBUR, P.G., KASORNDORKBUA, C., GILBERT, C., et al. Comparative pathogenesis of infection of pigs with hepatitis E viruses recovered from a pig and a human. *J Clin. Microbiol.* 39, 918-923, 2001.

HANDLINGER, J.H. AND WILLIAMS, W. An egg drop associated with splenomegaly in broiler breeders. *Avian Dis* 32, 773-778, 1988.

HAQSHENAS, G., HUANG, F.F., FENAUX, et al. The putative capsid protein of the newly identified avian hepatitis E virus shares antigenic epitopes with that of swine and human hepatitis E viruses and chicken big liver and spleen disease virus. *J Gen Virol* 83, 2201-2209, 2002.

HAQSHENAS, G., SHIVAPRASAD, H.L., WOOLCOCK, P.R., et al. Genetic identification and characterization of a novel virus related to human hepatitis E virus from chickens with hepatitis-splenomegaly syndrome in the United States. *J Gen Virol* 82, 2449-2462, 2001.

HOFFMANN, B., HARDER, T., LANGE, E., et al. New real-time reverse transcriptase polymerase chain reactions facilitate detection and differentiation of novel A/H1N1 influenza virus in porcine and human samples. *Berl Munch Tierarztl Wochenschr* 123, 286-292, 2010.

HSU, I.W. AND TSAI, H.J. Avian hepatitis e virus in chickens, taiwan, 2013. *Emerg Infect Dis* 20, 149-151, 2014.

HUANG, F.F., HAQSHENAS, G., GUENETTE, D.K., et al. Detection by reverse transcription-PCR and genetic characterization of field isolates of swine hepatitis E virus from pigs in different geographic regions of the United States. *J Clin Microbiol.* 40, 1326-1332, 2002a.

HUANG, F.F., HAQSHENAS, G., SHIVAPRASAD, H.L., et al. Heterogeneity and seroprevalence of a newly identified avian hepatitis e virus from chickens in the United States. *J Clin Microbiol.* 40, 4197-4202, 2002b.

HUANG, F.F., SUN, Z.F., EMERSON, S.U., et al. Determination and analysis of the complete genomic sequence of avian hepatitis E virus (avian HEV) and attempts to infect rhesus monkeys with avian HEV. *J Gen Virol* 85, 1609-1618, 2004.

HUANG, S., ZHANG, X., JIANG, H., et al. Profile of acute infectious markers in sporadic hepatitis E. *PLoS One* 5, e13560-, 2010.

IBARRA, V.H., RIEDEMANN, G.S., REINHARDT, V.G., et al. [Presence of anti hepatitis E virus antibodies in swine: is it an animal reservoir for hepatitis E?]. *Rev Med Chil.* 135, 997-1001, 2007.

JOHNE, R., PLENGE-BONIG, A., HESS, M., et al. Detection of a novel hepatitis E-like virus in faeces of wild rats using a nested broad-spectrum RT-PCR. *J Gen Virol* 91, 750-758, 2010.

JOTHIKUMAR, N., APARNA, K., KAMATCHIAMMAL, S., et al. Detection of hepatitis E virus in raw and treated wastewater with the polymerase chain reaction. *Appl Environ Microbiol* 59, 2558-2562, 1993.

JOTHIKUMAR, N., CROMEANS, T.L., ROBERTSON, B.H., et al. A broadly reactive one-step real-time RT-PCR assay for rapid and sensitive detection of hepatitis E virus. *J Virol Methods* 131, 65-71, 2006.

KABA, M., DAVOUST, B., MARIE, J.L., et al. Detection of hepatitis E virus in wild boar (Sus scrofa) livers. *Vet J* 186, 259-261, 2010.

Kaba, M., Moal, V., Gerolami, R., et al. Epidemiology of mammalian hepatitis E virus infection. *Intervirology* 56, 67-83, 2013.

KACI, S., NOCKLER, K., JOHNE, R. Detection of hepatitis E virus in archived German wild boar serum samples. *Vet Microbiol.* 128, 380-385, 2008.

KAMAR, N., BENDALL, R., LEGRAND-ABRAVANEL, F., et al. Hepatitis E. *Lancet* 379, 2477-2488, 2012.

KAMAR, N., IZOPET, J., ROSTAING, L. Hepatitis E virus infection. *Curr Opin Gastroenterol.* 29, 271-278, 2013a.

KAMAR, N., ROSTAING, L., IZOPET, J. Hepatitis E virus infection in immunosuppressed patients: natural history and therapy. *Semin Liver Dis* 33, 62-70, 2013b.

KAPUR, N., THAKRAL, D., DURGAPAL, H., et al. Hepatitis E virus enters liver cells through receptor-dependent clathrin-mediated endocytosis. *J Viral Hepat.* 19, 436-448, 2012.

KARETNYI, Y.V., GILCHRIST, M.J., NAIDES, S.J. Hepatitis E virus infection prevalence among selected populations in Iowa. *J Clin Virol* 14, 51-55, 1999.

KASORNDORKBUA, C., GUENETTE, D.K., HUANG, F.F., et al. Routes of transmission of swine hepatitis E virus in pigs. *J Clin. Microbiol.* 42, 5047-5052, 2004.

KHUROO, M.S. Study of an epidemic of non-A, non-B hepatitis. Possibility of another human hepatitis virus distinct from post-transfusion non-A, non-B type. *Am J Med* 68, 818-824, 1980.

KHUROO, M.S. AND KHUROO, M.S. Seroepidemiology of a second epidemic of hepatitis E in a population that had recorded first epidemic 30 years before and has been under surveillance since then. *Hepatol. Int* 4, 494-499, 2010.

KIESSLICH, D., ROCHA JUNIOR, J.E., CRISPIM, M.A., Prevalence of hepatitis E virus antibodies among different groups in the Amazonian basin. *Trans R Soc Trop Med Hyg* 96, 215-, 2002.

KLEIBOEKER, S.B., SCHOMMER, S.K., LEE, S.M., et al. Simultaneous detection of North American and European porcine reproductive and respiratory syndrome virus using real-time quantitative reverse transcriptase-PCR. *J Vet Diagn Invest* 17, 165-170, 2005.

KOONIN, E.V., GORBALENYA, A.E., PURDY, M.A., et al. Computer-assisted assignment of functional domains in the nonstructural polyprotein of hepatitis E virus: delineation of an additional group of positive-strand RNA plant and animal viruses. *Proc Natl. Acad. Sci USA* 89, 8259-8263, 1992.

KUMAR, S., SUBHADRA, S., SINGH, B., et al. Hepatitis E virus: the current scenario. *Int J Infect Dis* 17, e228-e233, 2013.

KUNIHOLM, M.H., PURCELL, R.H., MCQUILLAN, G.M., et al. Epidemiology of hepatitis E virus in the United States: results from the Third National Health and Nutrition Examination Survey, 1988-1994. J *Infect Dis* 200, 48-56, 2009.

KUNITA, S., KATO, K., ISHIDA, M., et al. Simultaneous detection of antibodies to mouse hepatitis virus recombinant structural proteins by a microsphere-based multiplex fluorescence immunoassay. *Clin Vaccine Immunol* 18, 758-766, 2011.

KWON, H.M., SUNG, H.W., MENG, X.J. Serological prevalence, genetic identification, and characterization of the first strains of avian hepatitis E virus from chickens in Korea. *Virus Genes* 45, 237-245, 2012.

LANDIS, J. AND KOCH, G. The measurement of observer agreement for categorical data. *Biometrics* 33, 159-174, 1977.

LANGENHORST, R.J., LAWSON, S., KITTAWORNRAT, A., et al. Development of a fluorescent microsphere immunoassay for detection of antibodies against porcine reproductive and respiratory syndrome virus using oral fluid samples as an alternative to serum-based assays. *Clin Vaccine Immunol* 19, 180-189, 2012.

LEBLANC, D., WARD, P., GAGNE, M.J., et al.. Presence of hepatitis E virus in a naturally infected swine herd from nursery to slaughter. *Int J Food Microbiol.* 117, 160-166, 2007.

LEGRAND-ABRAVANEL, F., KAMAR, N., Sandres-Saune, K., et al. Characteristics of autochthonous hepatitis E virus infection in solid-organ transplant recipients in France. *J Infect Dis* 202, 835-844, 2010.

LEGRAND-ABRAVANEL, F., MANSUY, J.M., DUBOIS, M., et al. Hepatitis E virus genotype 3 diversity, France. *Emerg Infect Dis.* 15, 110-114, 2009.

LHOMME, S., DUBOIS, M., ABRAVANEL, F., et al. Risk of zoonotic transmission of HEV from rabbits. *J Clin Virol* 58, 357-362, 2013.

LI, T.C., CHIJIWA, K., SERA, N., et al. Hepatitis E virus transmission from wild boar meat. *Emerg Infect Dis.* 11, 1958-1960, 2005.

LIN, K., WANG, C., MURTAUGH, M.P., et al. Multiplex method for simultaneous serological detection of porcine reproductive and respiratory syndrome virus and porcine circovirus type 2. *J Clin Microbiol* 49, 3184-3190, 2011.

LOPES DOS SANTOS, D.R., LEWIS-XIMENEZ, L.L., da SILVA, M.F., et al. First report of a human autochthonous hepatitis E virus infection in Brazil. J Clin Virol 47, 276-279, 2010.

LU, L., LI, C., HAGEDORN, C.H. Phylogenetic analysis of global hepatitis E virus sequences: genetic diversity, subtypes and zoonosis. *Rev Med Virol.* 16, 5-36, 2006.

LYRA, A.C., PINHO, J.R., SILVA, L.K., et al. HEV, TTV and GBV-C/HGV markers in patients with acute viral hepatitis. Braz. *J Med Biol Res.* 38, 767-775, 2005.

MANSUY, J.M., LEGRAND-ABRAVANEL, F., CALOT, J.P., et al. High prevalence of anti-hepatitis E virus antibodies in blood donors from South West France. *J Med Virol* 80, 289-293, 2008.

MAREK, A., BILIC, I., PROKOFIEVA, I., et al. Phylogenetic analysis of avian hepatitis E virus samples from European and Australian chicken flocks supports the existence of a different genus within the Hepeviridae comprising at least three different genotypes. *Vet Microbiol* 145, 54-61, 2010.

Massi, P., Tosi, G., Gelmetti, D., et al. Big liver and spleen disease in broiler breeders in Italy. *It J Animal Sci.* 4, 303-305, 2005.

MASUDA, J., YANO, K., TAMADA, Y., et al. Acute hepatitis E of a man who consumed wild boar meat prior to the onset of illness in Nagasaki, Japan. *Hepatol Res.* 31, 178-183, 2005.

MATSUBAYASHI, K., KANG, J.H., SAKATA, H., et al. A case of transfusion-transmitted hepatitis E caused by blood from a donor infected with hepatitis E virus via zoonotic food-borne route. *Transfusion* 48, 1368-1375, 2008.

MCCREARY, C., MARTELLI, F., GRIERSON, S., et al. Excretion of hepatitis E virus by pigs of different ages and its presence in slurry stores in the United Kingdom. *Vet Rec.* 163, 261-265, 2008.

MENG, J., DAI, X., CHANG, J.C., et al. Identification and characterization of the neutralization epitope(s) of the hepatitis E virus. *Virology* 288, 203-211, 2001.

MENG, X.J. Hepatitis E virus: animal reservoirs and zoonotic risk. *Vet Microbiol* 140, 256-265, 2010.

MENG, X.J. From barnyard to food table: the omnipresence of hepatitis E virus and risk for zoonotic infection and food safety. *Virus Res* 161, 23-30, 2011.

MENG, X.J. Zoonotic and foodborne transmission of hepatitis E virus. *Semin. Liver Dis* 33, 41-49, 2013.

MENG, X.J., ANDERSON, D., ARANKALLE, V.A., et al., Hepeviridae. In King AMQ, Adams MJ, Carstens EB et al., (Ed), Virus Taxonomy: *9th Report of the International Committee on Taxonomy of Viruses.* London: Academic Press, 2012, 1021-1028.

MENG, X.J., PURCELL, R.H., HALBUR, P.G., et al. A novel virus in swine is closely related to the human hepatitis E virus. *Proc. Natl. Acad. Sci. U.S.A* 94, 9860-9865, 1997.

MENG, X.J., SHIVAPRASAD, H.L., PAYNE, C.. Avian Hepatitis E virus infections. In: Saif Y.M., Fadly J., Glisson L.R. et al. (Eds.) *Diseases of Poultry.* 12th ed, Ames: Blackwell Publishing Press, 2008, 443-452.

MENG, X.J., WISEMAN, B., ELVINGER, F., et al. Prevalence of antibodies to hepatitis E virus in veterinarians working with swine and in normal blood donors in the United States and other countries. *J Clin Microbiol.* 40, 117-122, 2002.

MIRAZO, S., RAMOS, N., RUSSI, J.C., et al. Genetic heterogeneity and subtyping of human Hepatitis E virus isolates from Uruguay. *Virus Res* 173, 364-370, 2013.

MIYASHITA, K., KANG, J.H., SAGA, A., et al.Three cases of acute or fulminant hepatitis E caused by ingestion of pork meat and entrails in Hokkaido, Japan: Zoonotic food-borne transmission of hepatitis E virus and public health concerns. *Hepatol Res*. 42, 870-878, 2012.

MOAL, V., LEGRIS, T., BURTEY, S., et al. Infection with hepatitis E virus in kidney transplant recipients in southeastern France. *J Med Virol.* 85, 462-471, 2013.

MOKHTARI, C., MARCHADIER, E., HAIM-BOUKOBZA, S., et al. Comparison of real-time RT-PCR assays for hepatitis E virus RNA detection. *J Clin Virol* 58, 36-40, 2013.

MORROW, C.J., SAMU, G., MATRAI, E., et al. 2008. Avian hepatitis E virus infection and possible associated clinical disease in broiler breeder flocks in Hungary. *Avian Pathol* 37, 527-535, 2008.

MUNNE, M.S., ALTABERT, N.R., VLADIMIRSKY, S.N., et al. Identifications of polyphyletic variants in acute hepatitis suggest an underdiagnosed circulation of hepatitis E virus in Argentina. *J Clin Virol* 52, 138-141, 2011.

MUNNE, M.S., VLADIMIRSKY, S., OTEGUI, L., et al.[Molecular characterization of hepatitis E virus in three acute liver failure cases in children in Argentina]. *Acta Gastroenterol Latinoam*. 36, 125-130, 2006a.

MUNNE, M.S., VLADIMIRSKY, S., OTEGUI, L., et al. Identification of the first strain of swine hepatitis E virus in South America and prevalence of anti-HEV antibodies in swine in Argentina. *J Med Virol* 78, 1579-1583, 2006b.

NAKAI, I., KATO, K., MIYAZAKI, A., et al. Different fecal shedding patterns of two common strains of hepatitis E virus at three Japanese swine farms. *Am J Trop Med Hyg*. 75, 1171-1177, 2006.

NAMSAI, A., LOUISIRIROTCHANAKUL, S., WONGCHINDA, N., et al. Surveillance of hepatitis A and E viruses contamination in shellfish in Thailand. *Lett. Appl Microbiol* 53, 608-613, 2011.

NISHIZAWA, T., TAKAHASHI, M., ENDO, K., et al. Analysis of the full-length genome of hepatitis E virus isolates obtained from wild boars in Japan. J *Gen Virol* 86, 3321-3326, 2005.

OKAMOTO, H. Genetic variability and evolution of hepatitis E virus. *Virus Re*s 127, 216-228, 2007.

OKAMOTO, H., 2013. Culture systems for hepatitis E virus. *J Gastroenterol*. 48, 147-158, 2013.

OLSEN, B., AXELSSON-OLSSON, D., THELIN, A., et al. Unexpected high prevalence of IgG-antibodies to hepatitis E virus in Swedish pig farmers and controls. *Scand J Infect Dis.* 38, 55-58, 2006.

OWOLODUN, O.A., GIMENEZ-LIROLA, L.G., GERBER, P.F., et al. Development of a fluorescent microbead-based immunoassay for the detection of hepatitis E virus IgG antibodies in pigs and comparison to an enzyme-linked immunoassay. *J Virol Methods* 193, 278-283, 2013.

PAIVA, H.H., TZANEVA, V., HADDAD, R., et al. Molecular characterization of swine hepatitis e virus from southeastern Brazil. *Braz J Microbiol.* 38, 693-698, 2007.

PANABIERES, F., PIECHACZYK, M., RAINER, B., et al. Complete nucleotide sequence of the messenger RNA coding for chicken muscle glyceraldehyde-3-phosphate dehydrogenase. *Biochem Biophys Res Commun*. 118, 767-773, 1984.

PARANA, R. AND LYRA, M. New hepatitis viruses. *Braz. J Infect Dis* 4, 214-216, 2000.

PASSOS, A.M., HERINGER, T.P., MEDINA-PESTANA, J.O., et al. First report and molecular characterization of hepatitis E virus infection in renal transplant recipients in Brazil. *J Med Virol* 85, 615-619, 2013.

PAVIO, N., MENG, X.J., RENOU, C. Zoonotic hepatitis E: animal reservoirs and emerging risks. *Vet Res* 41, 46-, 2010.

PAYNE, C.J., ELLIS, T.M., PLANT, et al. Sequence data suggests big liver and spleen disease virus (BLSV) is genetically related to hepatitis E virus. *Vet Microbiol.* 68, 119-125, 1999.

PERALTA, B., BIARNES, M., ORDONEZ, G., et al. Evidence of widespread infection of avian hepatitis E virus (avian HEV) in chickens from Spain. *Vet Microbiol.* 137, 31-36, 2009a.

PERALTA, B., CASAS, M., DE DEUS, N., et al. Anti-HEV antibodies in domestic animal species and rodents from Spain using a genotype 3-based ELISA. *Vet Microbiol.* 137, 66-73, 2009b.

PURCELL, R.H. AND EMERSON, S.U. Hepatitis E: an emerging awareness of an old disease. *J Hepatol.* 48, 494-503, 2008.

PURCELL, R.H. AND EMERSON, S.U. Hidden danger: the raw facts about hepatitis E virus. *J Infect Dis.* 202, 819-821, 2010.

PURCELL, R.H., ENGLE, R.E., ROOD, M.P., et al. Hepatitis E virus in rats, Los Angeles, California, USA. *Emerg Infect Dis*. 17, 2216-2222, 2011.

RABENAU, H.F., KESSLER, H.H., KORTENBUSCH, M., et al. Verification and validation of diagnostic laboratory tests in clinical virology. *J Clin Virol.* 40, 93-98, 2007.

RAJ, V.S., SMITS, S.L., PAS, S.D., et al. Novel hepatitis E virus in ferrets, the Netherlands. *Emerg Infect Dis.* 18, 1369-1370, 2012.

RENOU, C., CADRANEL, J.F., BOURLIERE, M., et al. Possible zoonotic transmission of hepatitis E from pet pig to its owner. *Emerg Infect Dis.* 13, 1094-1096, 2007.

RENOU, C., MOREAU, X., PARIENTE, A., et al. A national survey of acute hepatitis E in France. *Aliment Pharmacol Ther.* 27, 1086-1093, 2008.

REUTER, G., FODOR, D., FORGACH, P., et al. Characterization and zoonotic potential of endemic hepatitis E virus (HEV) strains in humans and animals in Hungary. *J Clin Virol*. 44, 277-281, 2009.

REYES, G.R., YARBOUGH, P.O., TAM, A.W., et al. Hepatitis E virus (HEV): the novel agent responsible for enterically transmitted non-A, non-B hepatitis. *Gastroenterol. Jpn. 26 Suppl 3*, 142-147, 1991.

RITCHIE, S. AND RIDDELL, J.C. 'Hepatitis-splenomegaly' syndrome in commercial laying hens. *Can Vet J.* 32, 500-501, 1991.

RIVEIRO-BARCIELA, M., RODRIGUEZ-FRIAS, F., BUTI, M. Hepatitis E virus: new faces of an old infection. *Ann Hepatol.* 12, 861-870, 2013.

ROERIG, A., CARLSON, R., TIPOLD, A. Evaluation of a microsphere-based immunofluorescence assay for the determination of Immunoglobulin A concentrations in cerebrospinal fluid of dogs. *Res Vet Sci* 94, 69-72, 2013.

ROGEE, S., TALBOT, N., CAPERNA, T., et al. New models of hepatitis E virus replication in human and porcine hepatocyte cell lines. *J Gen Virol* 94, 549-558, 2013.

ROSE, N., BOUTROUILLE, A., FABLET, C., et al. The use of Bayesian methods for evaluating the performance of a virus-like particles-based ELISA for serology of hepatitis E virus infection in swine. *J Virol Methods* 163, 329-335, 2010.

ROSSI-TAMISIER, M., MOAL, V., GEROLAMI, R., et al. Discrepancy between anti-hepatitis E virus immunoglobulin G prevalence assessed by two assays in kidney and liver transplant recipients. *J Clin Virol* 56, 62-64, 2013.

RUTJES, S.A., LODDER, W.J., BOUWKNEGT, M., et al. Increased hepatitis E virus prevalence on Dutch pig farms from 33 to 55% by using appropriate internal quality controls for RT-PCR. *J Virol Methods* 143, 112-116, 2007.

RUTJES, S.A., LODDER, W.J., LODDER-VERSCHOOR, F., et al. Sources of hepatitis E virus genotype 3 in The Netherlands. *Emerg Infect Dis.* 15, 381-387, 2009.

SAID, B., IJAZ, S., KAFATOS, G., et al. Hepatitis E outbreak on cruise ship. *Emerg Infect Dis.* 15, 1738-1744, 2009.

SATO, Y., SATO, H., NAKA, K., et al. A nationwide survey of hepatitis E virus (HEV) infection in wild boars in Japan: identification of boar HEV strains of genotypes 3 and 4 and unrecognized genotypes. *Arch Virol* 156, 1345-1358, 2011.

SCHIELKE, A., SACHS, K., LIERZ, M., et al. Detection of hepatitis E virus in wild boars of rural and urban regions in Germany and whole genome characterization of an endemic strain. *Virology J.* 6, 58-, 2009.

SCHLAUDER, G.G., DAWSON, G.J., ERKER, J.C., et al. The sequence and phylogenetic analysis of a novel hepatitis E virus isolated from a patient with acute hepatitis reported in the United States. *J Gen Virol*. 79, 447-456, 1998.

SCOBIE, L. AND DALTON, H.R. et al. Hepatitis E: source and route of infection, clinical manifestations and new developments. *J Viral Hepat*. 20, 1-11, 2013.

SILVA, S.M., OLIVEIRA, J.M., VITRAL, C.L., et al. Prevalence of hepatitis E virus antibodies in individuals exposed to swine in Mato Grosso, Brazil. *Mem Inst Oswaldo Cruz* 107, 338-341, 2012.

SMITH, D.B., PURDY, M.A., SIMMONDS, P., et al. Genetic variability and the classification of hepatitis E virus. *J Virol.* 87, 4161-4169, 2013.

SONG, Y.J., JEONG, H.J., KIM, Y.J., et al 2010. Analysis of complete genome sequences of swine hepatitis E virus and possible risk factors for transmission of HEV to humans in Korea. *J Med Virol.* 82, 583-591, 2010.

SONODA, H., ABE, M., SUGIMOTO, et al. Prevalence of hepatitis E virus (HEV) Infection in wild boars and deer and genetic identification of a genotype 3 HEV from a boar in Japan. *J Clin Microbiol.* 42, 5371-5374, 2004.

SPRYGIN, A.V., NIKONOVA, Z.B., ZINYAKOV, N.G. Avian hepatitis E virus identified in Russian chicken flocks exhibits high genetic divergence based on the ORF2 capsid gene. *Avian Pathol*. 41, 459-463, 2012.

SUN, Z.F., LARSEN, C.T., DUNLOP, A., et al. Genetic identification of avian hepatitis E virus (HEV) from healthy chicken flocks and characterization of the capsid gene of 14 avian HEV isolates from chickens with hepatitis-splenomegaly syndrome in different geographical regions of the United States. *J Gen Virol.* 85, 693-700, 2004a.

SUN, Z.F., LARSEN, C.T., HUANG, F.F., et al. Generation and infectivity titration of an infectious stock of avian hepatitis E virus (HEV) in chickens and cross-species infection of turkeys with avian HEV. *J Clin Microbiol.* 42, 2658-2662, 2004b.

SÜß, B., FLEKNA, G., WAGNER, M., et al. Studying the effect of single mismatches in primer and probe binding regions on amplification curves and quantification in real-time PCR. *J Microbiol Methods* 76, 316-319, 2009.

TAKAHASHI, K., KITAJIMA, N., ABE, N., et al. Complete or near-complete nucleotide sequences of hepatitis E virus genome recovered from a wild boar, a deer, and four patients who ate the deer. *Virology* 330, 501-505, 2004.

TAKAHASHI, M., NISHIZAWA, T., SATO, H., et al. Analysis of the full-length genome of a hepatitis E virus isolate obtained from a wild boar in Japan that is classifiable into a novel genotype. *J Gen Virol*. 92, 902-908, 2011.

TAKAHASHI, M. AND OKAMOTO, H. Features of hepatitis E virus infection in humans and animals in Japan. *Hepatol Res.* 44:43-58, 2014.

TAM, A.W., SMITH, M.M., GUERRA, M.E., et al. Hepatitis E virus (HEV): molecular cloning and sequencing of the full-length viral genome. *Virology* 185, 120-131, 1991.

TEI, S., KITAJIMA, N., TAKAHASHI, K., et al. Zoonotic transmission of hepatitis E virus from deer to human beings. *Lancet* 362, 371-373, 2003.

TESSE, S., LIOURE, B., FORNECKER, L., et al. Circulation of genotype 4 hepatitis E virus in Europe: first autochthonous hepatitis E infection in France. J *Clin Virol.* 54, 197-200, 2012.

TOYODA, K., FURUSYO, N., TAKEOKA, H., et al. Epidemiological study of hepatitis E virus infection in the general population of Okinawa, Kyushu, Japan. *J Gastroenterol Hepatol*. 23, 1885-1890, 2008.

TRINTA, K.S., LIBERTO, M.I., DE, P., et al. Hepatitis E virus infection in selected Brazilian populations. *Mem Inst Oswaldo Cruz* 96, 25-29, 2001.

TROXLER, S., MAREK, A., PROKOFIEVA, I., ET AL. TaqMan real-time reverse transcription-PCR assay for universal detection and quantification of avian hepatitis E virus from clinical samples in the presence of a heterologous internal control RNA. *J Clin Microbiol.* 49, 1339-1346, 2011.

TSAREV, S.A., EMERSON, S.U., REYES, G.R., et al. Characterization of a prototype strain of hepatitis E virus. *Proc Natl. Acad. Sci USA* 89, 559-563, 1992.

TSAREV, S.A., TSAREVA, T.S., EMERSON, S.U., et al. Infectivity titration of a prototype strain of hepatitis E virus in cynomolgus monkeys. *J Med Virol.* 43, 135-142, 1994.

VAIDYA, S.R., TILEKAR, B.N., WALIMBE, A.M., et al. Increased risk of hepatitis E in sewage workers from India. *J Occup Environ Med.* 45, 1167-1170, 2003.

van GAGELDONK, P.G., van SCHAIJK, F.G., van der KLIS, F.R., et al. Development and validation of a multiplex immunoassay for the simultaneous determination of serum antibodies to Bordetella pertussis, diphtheria and tetanus. *J Immunol Methods* 335, 79-89, 2008.

VASICKOVA, P., KRALIK, P., SLANA, I., et al. Optimisation of a triplex real time RT-PCR for detection of hepatitis E virus RNA and validation on biological samples. *J Virol Methods* 180, 38-42, 2012.

VILLALBA, M.L., LAY, L.L., CHANDRA, V., et al. Hepatitis E virus genotype 1, Cuba. *Emerg Infect Dis*.14, 1320-1322, 2008.

VITRAL, C.L., PINTO, M.A., LEWIS-XIMENEZ, L.L., et al. Serological evidence of hepatitis E virus infection in different animal species from the Southeast of Brazil. *Mem. Inst. Oswaldo Cruz* 100, 117-122, 2005.

WAGNER, B., FREER, H., ROLLINS, A., et al. A fluorescent bead-based multiplex assay for the simultaneous detection of antibodies to B. burgdorferi outer surface proteins in canine serum. *Vet Immunol Immunopathol.* 140, 190-198, 2011a.

WAGNER, B., FREER, H., ROLLINS, A., et al. Development of a multiplex assay for the detection of antibodies to *Borrelia burgdorferi* in horses and its validation using Bayesian and conventional statistical methods. *Vet Immunol Immunopathol.* 144, 374-381, 2011b.

WANG, Y., LING, R., ERKER, J.C., et al. A divergent genotype of hepatitis E virus in Chinese patients with acute hepatitis. *J Gen Virol* 80, 169-177, 1999.

WARD, P., POITRAS, E., LEBLANC, D., et al. Comparative analysis of different TaqMan real-time RT-PCR assays for the detection of swine Hepatitis E virus and integration of Feline calicivirus as internal control. *J Appl. Microbiol.* 106, 1360-1369, 2009.

WENZEL, J.J., Preiss, J., Schemmerer, M., et al. Test performance characteristics of Anti-HEV IgG assays strongly influence hepatitis E seroprevalence estimates. *J Infect Dis.* 207, 497-500, 2013.

WENZEL, J.J., PREISS, J., SCHEMMERER, M., et al. Detection of hepatitis E virus (HEV) from porcine livers in Southeastern Germany and high sequence homology to human HEV isolates. *J Clin Virol.* 52, 50-54, 2011.

WERNIKE, K., HOFFMANN, B., DAUBER, M., et al. Detection and typing of highly pathogenic porcine reproductive and respiratory syndrome virus by multiplex real-time rt-PCR. *PLoS ONE* 7, e38251-, 2012.

WICHMANN, O., SCHIMANSKI, S., KOCH, J., et al. Phylogenetic and case-control study on hepatitis E virus infection in Germany. *J Infect Dis* 198, 1732-1741, 2008.

WILHELM, B.J., RAJIC, A., GREIG, J., et al. A systematic review/meta-analysis of primary research investigating swine, pork or pork products as a source of zoonotic hepatitis E virus. *Epidemiol Infect.* 139, 1127-1144, 2011.

WORM, H.C., SCHLAUDER, G.G., BRANDSTATTER, G. Hepatitis E and its emergence in non-endemic areas. *Wien. Klin. Wochenschr.* 114, 663-670, 2002.

XING, L., KATO, K., LI, T., et al. Recombinant hepatitis E capsid protein self-assembles into a dual-domain T = 1 particle presenting native virus epitopes. *Virology* 265, 35-45, 1999.

YAO, Y., NELLAKER, C., KARLSSON, H. Evaluation of minor groove binding probe and Taqman probe PCR assays: Influence of mismatches and template complexity on quantification. *Mol. Cell Probes* 20, 311-316, 2006.

YAZAKI, Y., MIZUO, H., TAKAHASHI, M., et al. Sporadic acute or fulminant hepatitis E in Hokkaido, Japan, may be food-borne, as suggested by the presence of hepatitis E virus in pig liver as food. *J Gen Virol.* 84, 2351-2357, 2003.

YUGO, D.M. AND MENG, X.J. Hepatitis e virus: foodborne, waterborne and zoonotic transmission. *Int J Environ Res Public Health* 10, 4507-4533, 2013.

ZAFRULLAH, M., OZDENER, M.H., PANDA, S.K., et al. The ORF3 protein of hepatitis E virus is a phosphoprotein that associates with the cytoskeleton. *J Virol.* 71, 9045-9053, 1997.

ZHAI, L., DAI, X., MENG, J. Hepatitis E virus genotyping based on full-length genome and partial genomic regions. *Virus Res.* 120, 57-69, 2006.ZHANG, H., MOHN, U., PRICKETT, J.R., et al. Differences in capabilities of different enzyme immunoassays to detect anti-hepatitis E virus immunoglobulin G in pigs infected experimentally with hepatitis E virus genotype 3 or 4 and in pigs with unknown exposure. *J Virol Methods* 175, 156-162, 2011.

ZHANG, X., LI, A., SHUAI, J., et al. Validation of an internally controlled multiplex real time RT-PCR for detection and typing of HEV genotype 3 and 4. *J Virol Methods* 193, 432-438, 2013.

ZHAO, C., MA, Z., HARRISON, T.J., et al. A novel genotype of hepatitis E virus prevalent among farmed rabbits in China. *J Med Virol.* 81, 1371-1379, 2009.

ZHAO, Q., SUN, Y., ZHAO, J., et al. Development and application of an indirect ELISA for detection of antibodies against avian hepatitis E virus. *J Virol Methods* 187, 32-36, 2013.

ZHAO, Q., ZHOU, E.M., DONG, S.W., et al. Analysis of avian hepatitis E virus from chickens, China. *Emerg Infect Dis.* 16, 1469-1472, 2010.

ZHOU, X., DE MAN, R.A., DE KNEGT, R.J., et al. Epidemiology and management of chronic hepatitis E infection in solid organ transplantation: a comprehensive literature review. *Rev Med Virol.* 23, 295-304, 2013.

ZWETTLER, D., FINK, M., REVILLA-FERNANDEZ, S., et al. First detection of hepatitis E virus in Austrian pigs by RT-qPCR. *Berl Munch Tierarztl Wochenschr* 125, 281-289, 2012.