NOEMI ROVARIS GARDINALI

IDENTIFICAÇÃO MOLECULAR DO GENOTIPO 3B DO VÍRUS DA HEPATITE E EM FÍGADO, BILE E AMOSTRAS FECAIS DE SUÍNOS ASSINTOMÁTICOS DE REBANHOS BRASILEIROS

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Dissertação apresentada para a obtenção do título de Mestre em Ciência Animal (Área de Concentração: Sanidade Animal) da Universidade Estadual de Londrina.
Orientador: Prof. Dr. Amauri Alcindo Alfieri.

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Dissertação apresentada para a obtenção do título de Mestre em Ciência Animal (Área de Concentração: Sanidade Animal) da Universidade Estadual de Londrina.

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Londrina, 15 de julho de 2011.

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Muito obrigada

"Não basta ensinar ao homem uma especialidade, porque se tornará assim uma máquina utilizável e não uma personalidade. É necessário que adquira um sentimento, senso prático daquilo que vale a pena ser empreendido, daquilo que é belo, do que é moralmente correto".

Albert Einstein

GARDINALI, Noemi Rovaris. **Identificação molecular do genotipo 3b do vírus da hepatite E em fígado, bile e amostras fecais de suínos assintomáticos de rebanhos brasileiros.** 2011. 103 f. Dissertação (Mestrado em Ciência Animal) - Universidade Estadual de Londrina, Londrina, 2011.

RESUMO

A hepatite E, causada pela infecção pelo vírus da hepatite E (HEV), é um problema de saúde pública em países industrializados e em desenvolvimento. Suínos domésticos são reservatórios do vírus e alguns casos de hepatite E podem ser adquiridos pelo consumo de vísceras de suínos, contato com as fezes de animais portadores do vírus ou por contaminação ambiental. A exposição ocupacional (granjeiros, veterinários e trabalhadores de abatedouros) é considerada fator de risco para a infecção pelo HEV. O objetivo desse estudo foi avaliar a presença do HEV em fezes, bile e no fígado de suínos assintomáticos. Foram colhidas 170 amostras de fezes de suínos de diferentes categorias (reprodução, leitões lactentes e desmamados e animais de terminação) e 118 amostras de bile e fígado de animais em idade de abate. As amostras foram provenientes de 24 rebanhos suinícolas da região oeste do estado do Paraná, sendo as amostras fecais colhidas em 14 rebanhos e as amostras de bile e fígado, colhidas em um frigorífico, provenientes de 10 rebanhos. A identificação do HEV nas amostras biológicas foi realizada por *nested* PCR. Nas amostras fecais foram utilizados dois sistemas de *nested* PCR sendo um direcionado para a ORF2 do HEV e utilizado como triagem e outro direcionado para a ORF1. Esse último, também utilizado nas amostras de bile e fígado. Os produtos amplificados foram sequenciados para a identificação e realização das análises filogenéticas. O RNA do HEV foi identificado em 26/170 (15,3%) das amostras fecais de animais e em 10/14 (71,4%) rebanhos. Em animais provenientes de abatedouro o vírus foi detectado em 1 (0,84%) amostra de bile e em 2 (1,7%) amostras de fígado. Por análises filogenéticas, todas as estirpes de HEV foram identificadas como pertencentes ao genotipo 3b. Os resultados demonstram a ampla distribuição do HEV nos rebanhos avaliados e a presença da infecção em suínos assintomáticos provenientes da região oeste do estado do Paraná. O genotipo (3b) do HEV identificado nesse estudo, apresentou alta similaridade com estirpes virais de origem humana. Considerando o aspecto zoonótico da infecção, a identificação do HEV em excreções e em vísceras de suínos assintomáticos pode apresentar reflexos principalmente em saúde pública.

Palavras-chave: Virus da hepatite E (HEV). Suínos. Abatedouro. Fígado. Bile. Fezes.

GARDINALI, Noemi Rovaris. **Molecular identification of genotype 3b of hepatitis E virus in liver, bile and fecal samples from asymptomatic pigs in Brazilian herds.** 2011. 103 f. Dissertação (Mestrado em Ciência Animal) - Universidade Estadual de Londrina, Londrina, 2011.

ABSTRACT

Hepatitis E, caused by infection with hepatitis E virus (HEV), is a major public health concern in developing and industrialized countries. Domestic pigs are reservoirs of HEV, and some cases of hepatitis E can be acquired through consumption of contaminated pork offal, contact with the feces of animals carrying the virus or environmental contamination. Occupational exposure to pigs by farmers, veterinarians, and slaughterhouse workers has been linked to an increased risk of HEV infection. The aim of this study was to evaluate HEV presence in feces, bile and liver samples from asymptomatic pigs. A total of 170 porcine fecal samples from different categories production (breeder sows and boars, suckling piglets, weaned, and growing pigs) and 118 samples of bile and liver samples from pigs at slaughter age were collected. The samples were from 24 pig herds from West region of Paraná state. The fecal samples were collected from 14 herds, and bile and liver samples that were collected at a slaughterhouse were from 10 herds. The HEV identification from biological samples was tested with *nested* PCR. The fecal samples were tested with two sets of primers designed to amplify ORF2 region for the screening test, and ORF1 region. HEV RNA identification performed in bile and liver samples was done targeting ORF1 region. The amplified products were sequenced for identification and phylogenetic analyses. HEV RNA was detected in 26/170 (15.3%) of the porcine fecal samples, and from 10/14 (71.4%) farms. From the slaughtered animals, HEV RNA was identified in 1 (0.84%) and 2 (1.7%) bile and liver samples, respectively. The phylogenetic analyses allowed the identification of all HEV strains as belonging to genotype 3 subtype 3b. The results demonstrate the widespread distribution of HEV in the surveyed farms, and the infection in asymptomatic pigs from the western region of Paraná state. HEV genotype 3b, identified in the present study, shows high similarity with human HEV strains. Considering the zoonotic aspect of the infection, HEV identification in excretions and offal from asymptomatic pigs may have a reflection especially in public health.

Keywords: Hepatitis E virus (HEV). Pigs. Slaughterhouse. Live. Bile. Fecal samples.

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1 REVISÃO

1.1 INTRODUCTION

Viral hepatitis is a major public health concern worldwide and presents a high medical importance. The infection is caused by six or seven different human viruses, hepatitis A virus (HAV) through hepatitis G virus (HGV) (Ellet, 2000). They are a group of very diverse pathogens that have in common their predilection to primarily infect the liver. However, they differ from each other in viral family, presents different replication strategies, genomic organization and structure, and also epidemiology, and the ability to cause chronic disease and cancer (Mushahwar, 2008).

Of all hepatitis viruses type, HAV and hepatitis E virus (HEV) share many features. They are both transmitted mainly by the enteric route, with a single serotype and cause an acute, self-limiting infection that may vary from inapparent to fulminant hepatitis (Mushahwar, 2008). Nevertheless, they also present important differences. HAV is acquired very early in the life while HEV is acquired in the young adulthood, and also, HEV disease is more severe than HAV. The death rate associated with HEV infection (1 to 4%) is higher than that of HAV (0.1 to 2%), especially in patients with preexisting chronic liver disease (Dalton et al., 2008). Besides that, the disease is indistinguishable without highly specific serologic and molecular tests (Mushahwar, 2008; Purcell and Emerson, 2008).

Hepatitis E accounts for a significant number of enterically transmitted cases of viral hepatitis in humans (Purcell and Emerson, 2001). HEV is the causative agent of more than 50% of acute hepatitis in endemic regions with poor sanitation and hygiene in developing countries where outbreaks of acute hepatitis E are generally associated with fecal contamination of water supplies (Smith, 2001; Aggarwal and Naik, 2009; Meng, 2010a).

Until the discovery of swine HEV, the disease was seen only in industrialized countries as imported cases and was assumed to be limited to developing countries, (Mushahwar, 2008). However, recent cases of acute hepatitis E in industrialized countries demonstrated that HEV occurs sporadically (Emerson and Purcell, 2008). Several of these sporadic cases from patients who had not travelled to endemic regions strongly suggest autochthonous origin of hepatitis E

(Clemente-Casares et al., 2003). Besides, molecular analyses have shown that the strains recovered from these patients form a group of HEV isolates that are genetically divergent compared with strains from HEV-endemic countries (Schlauder and Mushahwar, 2001).

Meng et al. (1997) isolated and characterized the first swine HEV in USA, and Haqshenas et al. 2001 identified an avian HEV from chickens with hepato-splenomegaly. Of all the viral hepatitis, HEV is the unique with animal reservoirs (Pavio et al., 2010). Recent discovery of HEV strains from wild boar, deer, mongooses, rabbits, and rats accumulate evidence that hepatitis E is a zoonotic disease (Tei et al., 2004; Li et al., 2005; Nakamura et al., 2006; Zhao et al., 2009; Johne et al., 2009).

Human and swine HEV strains are genetically closely related, and transmission trough the consumption of contaminated pork food, direct exposure to pig and pig feces or environmental contamination may be the cause of autochthonous HEV in industrialized countries (Yazaki et al., 2003; Purcell and Emerson, 2010). Therefore, the nature of the virus in domestic pigs, wild boar and deer as well as in other animal species raises an important public health concern over food safety and zoonosis (Meng et al., 2009; Pavio and Mansuy, 2010).

1.2 History

Until 1980 HAV was the unique type of waterborne viral hepatitis recognized (Khuroo, 1980). In the winter of 1955-56 a large waterborne epidemic of viral hepatitis in New Delhi, India affected 29,000 people after an incident of sewage contamination of drinking water (Khuroo, 1980; Jameel, 1999). The incident was thought to be an epidemic of hepatitis A. Just when a retrospective study testing for antibody to HAV (anti-HAV) of stored sera from these patients was done in the 1980s, the new agent previously known as enterically transmitted non-A, non-B hepatitis (ET-NANBH) was first recognized (Purcell and Emerson, 2008). Another epidemic occurred in Kashmir in 1978, and ET-NANBH was also the responsible agent (Wong et al., 1980).

Balayan et al. (1983) give the first existence proof of a new hepatitis viral agent when the disease was successfully transmitted into himself by oral administration of pooled stool extracts from 9 patients from a Soviet military camp in

Afghanistan. Spherical virus-like particles were visualized in stool samples from the volunteer patient 28 days post-inoculation. Eight years after that, the entire genome of the new virus, now called hepatitis E virus, was cloned, fully sequenced and characterized as the first HEV strain (genotype 1), from Myanmar (formerly Burma) (Tam et al., 1991). The "E" can stand for "enteric", "epidemic", "endemic" and also is the fifth viral agent associate with infectious hepatitis after hepatitis A, B, C, and D (Jameel, 1999).

The second human isolate was detected in an outbreak in Mexico, and constitutes HEV genotype 2 (Huang et al, 1992). The Burmese and Mexican strains presented very similar genomic organization, but they were distinct to each other with 76% nt identity over the entire genome (Tam et al., 1991; Huang et al, 1992). After this, isolates from Pakistan, China, and India were detected and identified as closely related with the Burma strain (Okamoto, 2007). These isolates occurred in developing countries with unsafe water supplies and poor sanitation and hygiene.

The genetic variability appears when a variant hepatitis E virus was identified in a hepatitis patient from United States (HEV US-1) with no history of travelling to HEV endemic regions (Schlauder et al., 1998). HEV US-1 represented a distinct isolate, significantly divergent from the Burmese and Mexican strains, and later was classified as genotype 3 (Schlauder et al., 1998; Erker et al., 1999; Lu et al., 2006). A large number of additional isolates from non-endemic regions in North America and West Europe were identified, and all of them were most closely related to the US isolates (Schlauder et al., 1999; Zanetti et al., 1999). High genetic diversity of HEV strains from patients from Taiwan and China were also detected, and they were distinct from the original Chinese isolates (genotype 1), HEV US-1, and Mexico isolates (Wu et al., 1998; Hsieh et al., 1999; Wang et al., 1999). The Asian isolates were then grouped at genotype 4 (Lu et al., 2006).

The first evidence of HEV animal strain was reported from pigs in Nepal in 1995 by the detection of HEV antibodies and RNA, but swine HEV from these animals were not characterized (Clayson et al., 1995). Meng et al. (1997) identified in US the first HEV strain in pigs, designated swine hepatitis E virus (swine HEV). The novel virus had its genome cloned and characterized from naturally infected pigs and showed to be antigenically and genetically closely related, but distinct from human HEV (Meng et al., 1997; Schlauder et al., 1998). These data

raised the possibility of zoonotic transmission from pigs to humans. Since the discovery of swine HEV in US, HEV seems to be endemic in pig herds worldwide, and recent studies reported that pig should be considered a reservoir for HEV (Meng et al., 2010a; Lewis et al., 2010).

The first evidence of HEV direct transmission and that the virus can be transmitted zoonotically by foodborne was described in two families that shared several raw meals from a sika deer *(Cervus Nippon)* (Tei et al., 2003). Also, HEV was detected in wild boars *(Sus scrofa)* (Sonoda et al., 2004; Martelli et al., 2008; Meng et al., 2009), rats (Johne et al., 2010), and rabbits (Zhao et al., 2009). In chickens, the avian HEV is associated with hepatitis-splenomegaly syndrome and is genetically and antigenically related to mammalian HEV, but it is not involved in zoonotic transmission (Meng, 2010b).

1.3 HEPATITIS E VIRUS

1.3.1 Classification and Phylogeny

HEV was originally classified in the *Caliciviridae* family until 1998 based on its surface and genomic organization (Tam et al., 1991). HEV also shares several properties with rubella virus and alphaviruses of the *Togaviridae* family as well as to the plant furoviruses (Koonin et al., 1992). However, HEV presents enough differences, and in 2004 the International Committee for Taxonomy of Viruses (ICTV) has classified Hepatitis E Virus as the sole member of the genus *Hepevirus* in the family *Hepeviridae* (Emerson et al., 2004).

HEV genome varies significantly and consists of at least four major mammalian genotypes with one single serotype (Fig.1), which are also sudiveded inseveral subtypes (Fig.2), one avian HEV (chickens, turkeys, and other birds), and the unclassified rabbit and rat HEV (Haqshenas et al., 2001; Schlauder and Mushahwar, 2001; Lu et al., 2006; Zhai et al., 2006; Pavio et al., 2010). Each HEV mammalian genotype appears to have a specific geographical distribution. In spite of this extensive nucleotide (nt) variation between mammalian, avian and the unclassified HEV, a single serotype appears to exist (Emerson et al., 2004; Meng, 2010a).

HEV Genotypes 1 and 2 are restricted to humans from developing countries with poor sanitation, and often associated with outbreaks and epidemics (Lu et al., 2006; Meng et al., 2009). Genotype 1 HEV strains were detected in human cases from Asia and Africa, where the disease is considered endemic, although sporadic cases were also described. This genotype is classified into five subtypes namely from 1a to 1e (Lu et al., 2006). Genotype 2 was first reported from an outbreak in Mexico, and also occurred in cases from Western Africa, being subdivided into two subtypes (2a and 2b) (Lu et al., 2006; Aggarwal and Naik, 2009). HEV genotypes 3 and 4 are responsible for sporadic cases of hepatitis in developing and industrialized countries, and the virus can infect humans and swine (Dalton et al., 2008). HEV genotype 3 has also been reported in other animal species such as wild boar and deer (Aggarwal and Naik, 2009). The high diversity of these genotypes seems to be related to their zoonotic origin from a large number of animals (Lu et al., 2006). Genotype 3 isolates have been reported in human autochthonous cases in several industrialized countries in European countries, Japan, Australia, and American countries, including Argentina and Brazil (Lu et al., 2006; Dalton et al., 2008; Dos Santos et al., 2009). Genotype 3 has also a high prevalence in pig population worldwide (Meng et al., 2010a). This genotype is the most variable with 10 subtypes, classified from 3a to 3j (Lu et al., 2006). Genotype 4 is divided into seven subtypes (4a to 4g), contains strains isolated from humans in industrialized areas from Japan, China, and Taiwan, in pigs from Japan, China, and India, and in wild boar in South Korea (Dalton et al., 2008; Kim et al., 2011).

Avian HEV was initially considered as HEV genotype 5, but in the ninth ICTV report (2009) was proposed that avian HEV is a new species within *Hepeviridae* family. It presents a shorter genome with only approximately 50% sequence similarity with mammalian HEV isolates, constituting a genetically distinct group of the *Hepevirus* genus (Haqshenas et al., 2001; Pavio et al., 2010). Recently, a novel virus was isolated from rabbits in farms in China, and the new strain showed to be most closely related to human and swine HEV than to avian HEV (Zhao et al., 2009). However, the analysis of the entire genome provided evidences that rabbit HEV strain not belongs to one of the established genotypes of human and swine HEV, but represents a novel genotype. Further information should be clarified to elucidate the zoonotic potential of rabbit HEV. Also, a new hepatitis E-like virus was identified in the feces of wild rats in Germany. Based upon partial sequence

analyses, it seems that rat HEV may represent an additional new HEV genotype within the *Hepevirus* genus (Johne et al., 2010).

1.3.2 Morphology and Genome Organization

HEV is a spherical, nonenveloped, small virus with approximately 27 to 34 nm in diameter (Fig. 3A) (Balayan et al., 1983). The genome of mammalian HEV is a single-stranded, positive sense 5'capped-RNA molecule, polyadenylated at 3' with approximately 7.2 kilobases (kb) in length, except by the poly (A) tail (Fig. 4) (Purcell and Emerson, 2001). The RNA molecule contains untranslated regions (UTR) at both 5' and 3' ends that fold into stem-loop structures, and three discontinuous open reading frames (ORFs 1, 2, and 3) (Tam et al., 1991). The ORF2 overlaps ORF3, but neither overlaps ORF1 (Graff et al., 2006).

Based upon the Burmese prototype strain, ORF1 begins at the 5'end of the viral genome after a 27-base pair (bp) UTR sequence and extends 5,079 nt to the 3'end (Jameel, 1999). The ORF1 encodes a polyprotein of 1,693 amino acids that is processed into individual functional units involved in viral replication and protein processing (Koonin et al., 1992). Computer predictions and analogy with other positive strand RNA viruses reveals that several putative functional domains including methyltransferases, papain-like cysteine proteases, helicases and RNA-dependent RNA polymerases were identified in the ORF1 (Huang et al., 2007).

The ORF2 located at the 3'end of the genome, encodes the major viral capsid protein and carry out a signal peptide at its N-terminal end and three N-linked glycosylation sites (Asn 137, Asn310, and Asn562) (Jameel, 1999). The ORF2, which is approximately 2 kb, is putatively translated from a subgenomic RNA (Pavio et al., 2010). It has been shown that the capsid protein also contains immunogenic epitopes that induces neutralizing antibodies, and is the target for vaccine development (Zhang et al., 2008). The N-terminally truncated capsid protein, consisting of amino acid (aa) residues 112 to 660, when expressed in baculovirus can self-assemble into virus-like particles (VLPs) (Li et al., 2005). The crystal structure of a HEV-VLP containing a partial ORF3 protein (residues 70-123) fused to the N-terminal ORF2 protein (residues 112-608) reveals that the HEV capsid assembled into a structure with 60 subunits arranged into 30 protruding spikes. This 3D structure contains two distinct domains: the shell domain (S) and the protrusion

domain (P) that are organized in T = 1 icosahedral symmetry as showed in Fig. 3B (Wang et al., 2008).

ORF3 is the smallest reading frame, with 372 nt, and its 3'endoverlaps ORF2 by 331 nt (Okamoto, 2007). Such as ORF2, ORF3 is probably translated from a subgenomic RNA (Pavio et al., 2010). This frame encodes a small cytoskeleton-associated phosphoprotein (Zafrullah et al., 1997). The N-terminus of ORF3 has a rich region of cysteine, binds to RNA, and forms a complex with the capsid protein (Tyagi et al., 2002). The C-terminus of ORF3 protein is multifunctional domain and may be involved in virion morphogenesis and pathogenesis (Tyagi et al., 2002).

1.3.3 Virion Properties

HEV virion is sensitive by iodinate disinfectants. Survival of HEV in the intestinal tract suggests that the virus is relatively stable to acid and mild alkaline conditions (Purcell and Emerson, 2001). Emerson et al. (2005) showed that HEV is still infectious after heating at 56°C (the temperature of rare to medium-cooked meat) for 1 h. However, HEV is complete inactivated by adequate cooking such as frying or boiling the contaminate pork meat for 5 min with an internal temperature of 71°C (Feagins et al., 2008b).

The buoyant density of hepatitis E viral antigen or virus particles is 1.35-1.40 g/cm^3 in CsCl and 1.29 g/cm^3 in potassium tartrate and glycerol (Meng, 2010a; Pavio et al., 2010), and the sedimentation coefficient of HEV is 183S (Bradley et al., 1988).

The HEV is a fastidious virus, and its propagation *in vitro* has been attempted in primary and in various continuous cell lines. However the *in vitro* HEV propagation in cell culture systems did not provide high-titer of infectious particles (Okamoto, 2011, *in press).* The lack of an efficient cell culture system for HEV has hampered some analysis of HEV replication cycle as well studies on the prevention of HEV infection.

Only very recently was developed the first efficient cell culture system for HEV capable of releasing infectious HEV progenies in high titers into culture media, using PLC/PRF/5cells originating from human hepatocellular carcinoma and A549 cells from human lung cancer as hosts cells (Okamoto, 2011, *in*

press). Other transformed cell lines such as the hepatic Huh7 and HepG2 and the colon carcinoma Caco-2 cell lines are also permissive for HEV infection and virion production (Ahmad et al., 2011, *in press).*

1.3.4 Replication Cycle

Due to non-availability of efficient in vitro culture system or small animal models of HEV infection, little is known about the mechanisms of HEV replication, transcription, and translation. Reyes (1993) and Jameel (1999) proposed a model of replication based on domain homologies between HEV and other positive stranded RNA viruses. The binding and entry of HEV is poorly understood. Following attachment into a permissive cell, the genomic positive stranded RNA is uncoated and translated into nonstructural polyproteins encoded by ORF1 in the cytosol. Cellular proteases make the cleavage of ORF1 nsP probably with the help of papain-like cysteine protease. The genomic positive strand is replicated by the viral replicase into the negative strand replicative intermediates. These serve as template for the synthesis of the genomic and subgenomic positive strands. Subsequently, the subgenomic RNA can be translated into the structural proteins, leading to the assembly of viral particles and the release of progeny virions that exit the cell through an undefined pathway. Recent evidence suggests that the ORF3 protein together with lipids coats this particle during the budding process (Ahmad et al., 2011, *in press).* In experimentally infected pigs (Meng et al., 1998b) and rhesus monkeys (Nanda et al., 1994), positive and negative strands HEV RNAs were observed in the liver. In these studies were also verified that the subgenomic RNAs are not required to initiate an infection, and must be synthesized as part of replication process, since *in vitro* transcripts of full-length cDNA clones showed to be infectious for non human primates and pigs.

1.4 Epidemiology

1.4.1 General Features

The incidence of clinical hepatitis E in humans caused by genotypes 1 and 2 strains during outbreaks in endemic regions is most frequent in individuals

from 15 to 35 years old, and infrequent in children (Khuroo, 1980; Wong, 1980; Smith, 2001). On the other hand, the disease caused by genotypes 3 and 4 occurs most frequently among persons >30 years of age, and immunocompromised individuals, such as people with underlying liver disease, HIV-positive patients, and organ transplant recipients (Kamar et al., 2008; Purcell and Emerson, 2008). However, young to middle aged adults, also can be infected by HEV genotypes 3 and 4. The death rate in general population infected with HEV is about 1 to 4% in developing countries (Peron et al. 2007a; Purcell and Emerson, 2008), but it seems to be higher in industrialized countries ranging from 8% to 11% (Table 1) (Dalton et al., 2008).

During hepatitis E outbreaks and sporadic cases of hepatitis E in industrialized countries a variable incubation period of 2-10 weeks have been reported (Dalton et al., 2008; Aggarwal and Naik, 2009). In human volunteers the incubation period after oral exposure is about 4 to 5 weeks (Chauhan et al., 1993). Therefore the incubation time can range from 2 weeks to 2 months or more, with an average of 40 days (Purcell and Emerson, 2008; Pavio et al., 2010). About five days prior to the beginning of jaundice, HEV RNA can be detected in blood and stool. A few days to weeks after the onset of clinical symptoms the virus is cleared from the blood, but still continues to be shed in feces for another two or three weeks (Chauhan et al., 1993).

Swine HEV infection caused by genotypes 3 and 4 strains is widespread worldwide and infection in pigs generally occurs at 2-4 months of age given the lack of maternal antibodies (Meng et al., 1997; de Deus et al., 2008; Vitral et al., 2005). Once a pig is infected, swine HEV can be detected in feces after 2-3 weeks, and virus shedding can last up to 7 weeks (Meng et al., 1998b). Clinical disease is not observed in pigs, although microscopic evidence of hepatitis was observed in infected pigs (dos Santos et al., 2009; Halbur et al., 2001).

In chickens, hepatitis-splenomegaly (HS) syndrome is associated to avian HEV infection. The disease is considered enzootic in chicken flocks in US and has been reported in Australia and Canada with serological evidence in United Kingdom and Spain (Peralta et al., 2009; Meng, 2010b). Like human and swine HEV, the seroprevalence in chickens appears to be age-dependent and increases with age (Huang et al., 2002).

1.4.2 Routes of Transmission

In humans, HEV is mainly an enterically transmitted pathogen and there are four routes of transmission reported as: i) fecal-oral transmission by drinking contaminated water supplies (waterborne transmission); ii)consumption of raw or undercooked meat and offal of infected wild and domestic animals, such as deer, boar and swine (zoonotic foodborne transmission); iii) transfusion of infected blood products (parenteral) and, iv) vertical transmission (materno-fetal) (Mushahwar, 2008; Aggarwal and Naik, 2009). Person to person transmission of HEV can occur but it is uncommon, as opposed to other enterically transmitted infections, like HAV (Smith, 2001; Teshale et al., 2010b).

Like human HEV, the transmission route of swine HEV is mainly by the oral-fecal route. Pigs become infected when direct contact with infected pigs occur randomly or through ingestion of feces-contaminated water or feed (Bouwknegt et al., 2008). Intravenous and intrahepatic routes of inoculation can be readily reproduced in pigs, but experimental reproduction of swine HEV by the oral route of inoculation has been difficult (Kasorndorkbua et al., 2004; Bouwknegt et al., 2008). Besides that, transplacental HEV infection by genotype 3 was reported in field pig farms (Hosmillo et al., 2010).

Transmission of avian HEV is probably fecal-oral, like the other mammalian HEVs, and experimental oral-nasal inoculation has been successfully reproduced in specific-pathogen-free chickens (Billam et al., 2005). Meng. (2010a) suggested that rodents in the chicken farms might serve as a mechanical carrier to avian HEV.

In some human cases, it is not possible to establish the exact route of acquisition of infection, particularly in cases that occur in industrialized countries and sporadic cases in developing countries. For better understanding, these two distinct epidemiological patterns observed in different geographical regions will be studied separated in this review.

1.4.3 HEV in Developing Countries

Hepatitis E in humans is endemic in both developing and industrialized countries around the world. However, epidemics only occur in

developing countries. In waterborne jaundice outbreaks, HEV is considered the foremost agent. These outbreaks have been caused primarily by HEV genotype 1 and they have been reported frequently in India, China, Southeast and Central Asia, the Middle East, and Northern and Western parts of Africa (Khuroo et al., 1980; Naik et al., 1992; Teshale et al., 2010a). Also, small outbreaks had occurred in Mexico and Western Africa caused by HEV genotype 2 (Velazquez et al., 1990). Sporadic cases of acute hepatitis E in developing countries also occur, but with lower frequency (Purcell and Emerson, 2001). These developing regions are considered hyperendemic for HEV, since the hepatitis E outbreaks that occurred in the past 40 years or the HEV infections accounts for more than 25% of reported cases of sporadic acute ET-NANBH (Teo, 2010). Brazil and Latin America seems to have a different epidemiology, and probably should not be considered a hyperendemic region (Dell'Amico et al., 2011; dos Santos et al., 2009, 2011; Lopes dos Santos et al., 2010; Mirazo et al., 2011; Munne et al., 2006).

Most reported HEV outbreaks have been triggered by fecal contamination of drinking water, and can affect hundred to thousand people (Khuroo et al., 1980; Naik et al., 1992; Teshale et al., 2010a). Contamination occurs generally due the absence and inadequacy of sewage or waste water treatment systems and frequently follows heavy rainfall and floods, which create conditions that mix sources of water with human excreta. The outbreaks can vary from unimodal, lasting a few weeks, to prolonged, multi-peaked epidemics that can last more than a year (Naik et al., 1992). Although outbreaks are connected to fecal contamination, the source of sporadic hepatitis E in these countries is not clear. Some cases due to animal meat ingestion and direct contact with infected animals have been reported (Purcell and Emerson, 2001; Meng, 2003). Foodborne zoonotic infection with HEV genotype 1 and 2 has not been reported.

Anti-HEV antibodies have been found in health people in all parts of the world. Based upon anti-HEV IgG detection in developing countries, seroprevalence rates increase with age (Arankalle et al., 1993). In these areas, like India and Southeast Asia, high rates of seroprevalence were shown, ranging from 27% to 80% in general population (Favorov et al., 1992). However, in some hyperendemic areas, the anti-HEV seroprevalence in general population is usually <40%, different from other enteric diseases like HAV, which prevalence may reach close to 100% by age of five years in endemic countries (Teshale et al., 2010c). On

the other hand, in Egypt, where sporadic cases but not outbreaks have been reported, HEV seroprevalence is very high, with more than 70% of the rural population positive for IgG anti-HEV (Stoszek et al., 2006a). These differences can be explained by the employment of different anti-HEV assays.

1.4.4 HEV in Industrialized Countries

In industrialized countries, the less virulent HEV genotypes 3 and 4 are responsible for only occasional cases of acute hepatitis E. Initially, these cases were found to be related to travel to high endemic areas. Nowadays many cases of autochthonous (locally-acquired) hepatitis E in industrialized areas have been reported (Erker et al., 1999). Seroprevalence range from 2 to 7.8% in some Europeans countries (Italy, Spain, Germany, Netherlands, and Portugal), industrialized countries of Asia-Pacific to 18.2 to 20.6% in the USA, Russia, Southern France, Korea and China (Pavio and Mansuy, 2010).

Studies in different populations have been shown that people who has a professional contact with pigs, such as veterinarians, slaughterhouse workers and pig handlers presents more frequently anti-HEV antibodies (Drobeniuc et al., 2001; Meng et al., 2002; Bouwknegt et al., 2008). Bouwknegt et al. (2008) showed statistical association between exposure to pig or their environment and high HEV seroprevalence. The same was reported by Drobeniuc et al. (2001) that observed an increased prevalence of HEV infection among pig farmers (51.1%), whereas only 24.7% of control subjects with no occupational exposure to pig were seropositive.

In industrialized countries, the high prevalence of anti-HEV antibodies among people with direct contact with pigs and persons from the general population, may result from zoonotic spread of inapparent infected wild and/or domestic animals. There is support for a zoonotic source of HEV infection in industrialized countries since HEV strains from the rare autochthonous cases are genetically related to swine strains (Kwo et al., 1997; Erker et al., 1999).

The true incidence, prevalence, pathogenicity, and risk of HEV infection in developed countries are unknown, and other factors can be associated with the high seroprevalence observed in these regions. Commercial assays for anti-HEV antibodies vary in sensitivity and specificity and are still unreliable. Even the FDA in US, hepatitis E test has never been licensed (Purcell and Emerson, 2010;

Teshale et al., 2010c). In the absence of a universally accepted diagnostic test, many potential undiagnosed and uninvestigated cases of hepatitis E still remains. Besides that, most countries have no surveillance system to report hepatitis E, and an investigation of imported or autochthonous cases to confirm the diagnosis and HEV genotype is not realized, thus hindering a better understanding of HEV infection.

Based upon the serological studies, if HEV seroprevalence in human from some countries is about 20% and no outbreaks of hepatitis E have been observed, probably the majority of infections are asymptomatic. On the other hand, chronic and clinically severe HEV infections have been observed in organ transplant recipients (Kamar, 2008). Recently, the first description of chronic hepatitis E in a immunocompetent man was described in Spain, where the disease is probably transmitted by zoonotic route and genotype 3 is prevalent (González Tallón et al., 2011). These data do not allow clear conclusions about pathogenesis, transmission, nonhuman reservoirs, and interspecies transmission. Therefore, as a recently described disease, much remains to be understood about HEV infection, especially in industrialized countries.

1.4.5 HEV in Brazil and Latin America

Outbreaks of hepatitis E in Brazil have never been reported, even though some regions have favorable environmental conditions (Carrilho et al., 2005). In the Western region of the Brazilian Amazon Basin for example, the overall prevalence for HEV antibodies is 4% in riverine communities (de Paula et al., 2001). The abundance of water resources in the Amazon may play a limiting factor for HEV transmission, once epidemics have been reported only in situations of massive contamination of the restricted water supplies by sewage, or in rural areas, by contamination of water sources shared by many people (Assis et al., 2002).

Anti-HEV antibodies also have been detected in a significant proportion of healthy people in Brazilian Southeast, Northeast and Southern (Parana et al., 1997; Goncales et al., 2000; Bortoliero et al., 2006). The first human autochthonous case in Brazil was reported by Lopes dos Santos et al., 2010 and was linked to consumption of pork meat a few months before the outcome of disease. Based upon phylogenetic analysis, the Brazilian human HEV strain was classified as genotype 3, subgenotype 3b and zoonotic origin of infection was suggested.

Just a few reports describe the molecular characterization of human HEV in Latin America. Genotype 1 was reported in two outbreaks in Havana, Cuba, genotype 2 in Mexico and genotype 3 in autochthonous cases from Argentina and Brazil (Huang et al., 1992; Munne et al., 2006; Villalba et al., 2008; Lopes dos Santos et al., 2010). Seroprevalence studies without virus detection and HEV phylogenetic classification have been shown at Latin America. Antibodies against HEV have been detected in sera from Latino population with a prevalence ranging from 1.6% to 7.5%, suggesting that HEV is not highly endemic in Latin America (Pujol et al., 1994; Brahm et al., 1996; Cruells et al., 1997).

Vitral et al. (2005) conducted a retrospective study of HEV seroprevalence in several animal species in the Southeast Brazil, and demonstrated that HEV is circulating among several animal species like cows, dogs, chickens, pigs and rodents. HEV is highly prevalent in Brazilian pig commercial herds with IgG anti-HEV prevalence of 95.5% and 83% in pigs older than 20 weeks from Southeast and Central-west Brazil, respectively (Guimarães et al., 2005; Vitral et al., 2005). Recently, the first molecular evidence of HEV in Brazilian pig herds was described and was classified as genotype 3 subtype 3b (Dos Santos et al., 2009). Swine HEV strains were also detected in bile and waste samples from a slaughterhouse in Rio de Janeiro, and were classified in the same genotype and subgenotype (Dos Santos et al., 2011). All Brazilian swine HEV strains were closely related to the sample obtained from the first reported autochthonous human case in Brazil, supporting the hypothesis of zoonosis.

The presence of anti-HEV IgG has been found in Latino pig herds and the prevalence varies from 7% to 95.5% in pigs from birth to slaughter (Vitral et al., 2005; Ibarra et al., 2007). Molecular detection and characterization of HEV RNA in this swine population is scarce and involve genotype 3 strains from pigs in Brazil, Mexico, Argentina, and Costa Rica (Cooper et al, 2005; Munne et al., 2006; Kase et al, 2008; Dos Santos et al., 2009;). These data suggest that swine could be an important reservoir for virus transmission in Latin America.

In spite of detection of swine HEV, genotypes 1 and 2 are circulating in some of these Latino countries, such as Cuba and Mexico. These data provides evidence of zoonotic transmission by pigs and also transmission by fecally contaminated drinking water. Therefore the epidemiology in Latin America is much more complex, requiring more studies for better elucidation. The same applies to

Brazil, since its large extent and frontiers with many countries hamper further understanding of HEV epidemiology.

1.5 Clinical Signs and Pathological Lesions

1.5.1 Human

Clinical features of HEV in human are very similar to other enterically transmitted hepatotropic viruses with acute self-limiting and symptomatic disease. However, different of HAV, HEV disease can varies in severity from acute subclinical to fulminant hepatitis, and chronic cases have also been reported in specific situations (Emerson and Purcell., 2003; Kamar et al., 2008). Asymptomatic or inapparent HEV infection is more common than overt hepatitis E with jaundice (Smith, 2001).

The clinical presentation in patients from industrialized countries is similar to that from developing countries. Typical symptoms of acute hepatitis E are characterized by subclinical to severe cholestatic hepatitis, with jaundice, anorexia, dark urine, enlarged tender liver, elevated liver enzymes, abdominal pain, nausea, vomiting and occasionally fever (Smith, 2001). The sickness can last for a few weeks, although some patients may have a prolonged sickness that can range in severity from subclinical to fulminant with complications like cholestatic manifestations (Moucari et al., 2007; Ramachandran et al., 2008).

In endemic areas, HEV superinfection can occur in patients with pre-existing chronic liver disease of any etiology leading to patient's decompensation. Such patients may be at a higher risk of poor outcome (Kumar et al., 2004). It appears that, compared to genotypes 1 and 2, genotypes 3 and 4 are less pathogenic in humans. In low endemic regions, patients infected with genotype 4 tended to have more severe clinical manifestations than those with genotype 3 (Ohnishi et al., 2006). Besides that, autochthonous hepatitis E in industrialized countries is frequently misdiagnosed as drug-induced liver injury, a frequent problem in elderly people (Dalton et al., 2007). In these cases, most patients are elderly men who often have another coexistent disease, resulting in a worse prognosis when compared to those in hyperendemic areas.

Until recently, HEV was described as a self-limited infection with no chronic liver disease. However, chronic hepatitis E has been documented in immune-suppressed patients progressing to cirrhosis. In particular, different cases of persistent HEV infection in solid organ transplant recipients (kidney, liver, and pancreas) have been reported (Haagsma et al., 2008; Kamar et al., 2008). A recent report of a reactivation of HEV infection 3 months after complete recovery and allogenic stem cell transplantation, lead to speculation that the virus might persist in hepatocytes (le Coutre et al., 2009). In all these cases, persistent viremia with genotype 3 have been reported (Aggarwal and Naik., 2009).

Another population that might be at risk of chronic HEV infection is HIV patients with an advanced stage of immunodeficiency (Pavio et al., 2010). Therefore, chronic patients may serve as long-term carriers of HEV and in spite of infrequent, chronic hepatitis E presents clinically significant occurrence in immune-suppressed patients and also can rapidly progress into cirrhosis (Haagsma et al., 2008).

HEV pathogenesis is poorly understood and explained based on analysis of the clinical and serologic events of a typical HEV infection. Probably the primary site of replication is the liver, with hepatocytes being the most likely cell type (Ahmad et al., 2011, *in press).* HEV replicates in the cytoplasm of hepatocytes (Krawczynski and Bradley, 1989) and is released into the bile and bloodstream by mechanisms that are not understood. In pigs experimentally infected with swine HEV, the negative-sense RNA was detected primarily in the small intestine, lymph node, colon and liver, indicating a possible replication in these sites (de Deus et al., 2008; Williams et al., 2001).

Pathological features of sporadic cases of acute hepatitis E in industrialized countries are limited to patients with severe disease, since hepatic histopathology was performed only in these cases. Liver histology in acute hepatitis E is characterized by severe intralobular necrosis, polymorph and lymphocytic inflammation, and acute cholangitis with numerous neutrophils (Peron et al., 2007b; Malcolm et al., 2007). It seems that endemic HEV cases show less severity in the liver histology than in autochthonous acute cases (Malcolm et al., 2007).

1.5.2 Pregnant Women

Hepatitis E outbreaks, particularly from certain geographical regions in India, have been associated with a high disease attack rate among pregnant women, often leading to acute liver failure (ALF) and death (Naidu and Vishwanathan, 1957; Khuroo et al., 1981). HEV-related ALF, especially during the third trimester is associated with increased fetal and mother mortality (Smith, 2001). Various studies have been reported that hepatitis E mortality rate during pregnancy ranges from 15 to 20% (Naidu and Vishwanathan, 1957; Khuroo et al., 1981; Rab et al., 1997; Smith, 2001).

However, recent studies in India and elsewhere reported that the severity of viral hepatitis is similar among pregnant and non-pregnant women (Bhatia et al., 2008). In 1996, a study of fulminant hepatitis in a tropical population also showed that the mortality rate between HEV infected pregnant and non-pregnant women were similar in North India (Acharya et al., 1996). In low endemic regions only two cases of HEV-related ALF involving pregnant women have been described and both women had returned recently from India, suggesting non-autochthonous HEV infection (Hussaini et al., 1997).

It is notable the discrepancies in mortality rates and ALF HEV-related from one geographical region to another. In some regions of India, like in Kashmir the mortality rate in pregnant women is usually fairly high (Rab et al., 1997). On the other hand, in the same country, in New Delhi, there is no difference in mortality rate and disease severity between pregnant and non-pregnant women infected by HEV (Bhatia et al., 2008). A recent Egyptian study also detected no cases of fulminant HEV hepatitis among pregnant women despite the high seroprevalence (84.3%) of HEV in this endemic country (Stoszek et al., 2006b). The reasons for the geographical discrepancies regarding the mortality rate HEV-related during pregnancy are still unknown. However, recent studies suggested that the HEV-related ALF among pregnant patients with a large number of deaths could be because HEV infection causes more severe hepatitis, often leading to ALF in endemic areas (Bhatia et al., 2008). Besides that, various factors such as HEV genotypes or subtypes, viral load, other co-infections, socio-economic status of the patients, hormonal changes during pregnancy, the immunological status of the patients, and the public health care in the region could play an important role in the

observed geographical difference (Meng., 2010b; Pavio et al., 2010). Therefore, more studies are needed to elucidate these discrepancies.

1.5.3 Swine

Swine HEV infection is present in all swine-producing regions worldwide and pigs are asymptomatic (Meng, 2003). Meng et al. (1997) reported that in early stages of naturally infected pigs with HEV, gross pathological lesions were not observed in the liver. However, the same pigs had microscopic evidence of hepatitis with mild to moderate multifocal and periportal lymphoplasmacytic hepatitis with mild focal hepatocellular necrosis. In Brazil, dos Santos et al. (2009) also reported histopathological alterations in liver tissues in pigs at slaughter age.

In experimental infections, the results were similar. Halbur et al. (2001) compared the pathogenesis of infected pigs with hepatitis E recovered from a pig and from a human. The authors observed no clinical signs from pigs, although these animals had midly to moderately enlarge hepatic and mesenteric lymph nodes. Microscopic lesions were also observed and characterized by multifocal lymphoplasmacytic hepatitis and focal hepatocellular necrosis. Based on microscopic evaluation, the human HEV strains induced more severe and persistent hepatic lesions in pigs than swine HEV (Halbur et al., 2001).

1.6 HEPATITIS E AS A ZOONOSE

At first, hepatitis E was considered an anthropozoonotic infection. Nowadays HEV is recognized as a zoonotic disease in industrialized countries and pigs, wild boars, and deer are the reservoirs (Meng, 2010a). Autochthonous cases and high anti-HEV IgG seroprevalence in non endemic areas supported evidence of zoonosis. Principal modes of infection are through contaminated food, direct exposure to pig and pig feces, or environmental contamination. However, zoonotic transmission in industrialized countries is not fully understood (Purcell and Emerson, 2010).

HEV can replicate in liver, colon, and small intestine, and the infected pigs and other animals shed the viral particles in feces (Meng 2010b; Williams et al., 2001). Taken together, these data raise concerns for environmental and food safety.

The zoonotic nature of HEV was showed in two cases from Japan, in which the virus was transmitted to people after the consumption of contaminated Sika deer (sushi) and wild boar (grilled) meat. In both cases, viral sequences recovered from the patients and the leftover frozen meats were identical or near identical (99.95% identity) and belong to genotype 3. The patients presented clinical symptoms 40 to 60 days after consumption of contaminated meat (Tei et al., 2003; Li et al., 2005).

About 2% of pig livers sold in Japanese grocery stores and 11% in US tested positive for swine HEV (Yazaki et al., 2003; Feagins et al., 2007). More important, in the study from US, the contaminating virus present in pig livers remained fully infectious when inoculated in SPF pigs (Feagins et al., 2007). The Japanese HEV sequences recovered from pig livers were identical or closely related to some viral strains detected in human hepatitis E patients (Yazaki et al., 2003). Other Japanese studies described that human cases of hepatitis E were linked to consumption of raw or undercooked pork products like wild boar barbecue, raw wild boar liver, grilled or uncooked pork liver (Matsuda et al., 2003; Tei et al., 2004; Colson et al., 2010). The possibility of HEV transmission through fresh pork sausages also has been strongly suggested, since processes such as salting, air-drying and smoking are not efficient in inactivating pathogens like HEV (Emerson and Purcel, 2010).

Contaminated water or water supplies are considered route of transmission for HEV infection, since fecal-oral route is the predominant one (Meng, 2010a). HEV genotype 3 has been detected in swine manure and wastewater associated with swine operations (Meng, 2011 *in* press). Genotype 3 HEV recovered from sewage water of pig slaughterhouses in Brazil are genetically closely related to Brazilian human HEV strain described recently (Dos Santos et al, 2011). Similar results were described in other countries, and HEV detected in pig manure collected from concrete holding pits and from lagoons on Iowa farms, remains infectious when inoculated into naive pigs (Kasorndorkbua et al, 2005). Thus, pig manure land applications could be source for contamination of drinking and irrigation water in artesian wells, rivers, ponds or costal water, and consequently could lead to potential transmission of HEV to humans. Considering this information, vegans and vegetarians might not be completely free to become infected with HEV and falling ill from it.

1.7 Cross-Specie Infection

It has been demonstrated that under experimental conditions, HEV genotypes 3 and 4 can infect across species barrier. Inoculation of rhesus monkeys and chimpanzee with genotypes 3 and 4 of swine HEV led to productive infections (Meng et al., 1998a; Arankalle et al., 2006). Although both animals species remained asymptomatic, viremia, virus shedding in feces, and seroconversation were observed (Meng et al., 1998a). Moderate increases in hepatic enzyme and minor hepatic pathological lesions were occasionally observed (Aggarwal et al., 2001).

HEV strains genotypes 3 and 4 from humans rapidly became infectious when inoculated in SPF pigs. The animals showed viremia and seroconversation within 2 weeks post-inoculation (Halbur et al., 2001; Feagins et al., 2008a). It seems that human HEV genotypes 3 and 4 have already adapted to replicate in pigs, and a recent phylogenetic study strongly suggested that natural host species of these genotypes is the swine (Xia et al., 2011). In spite of swine be naturally infected by HEV genotypes 3 or 4, they are not susceptible to infections by a genotype 1 (Sar-55) nor to the Mexican strain of genotype 2 (Meng et al., 1998b).

Avian HEV can also infect across species barriers: Avian HEV from a chicken successfully infected turkeys (Sun et al., 2004). However, avian HEV strains were inefficient to infect rhesus monkeys, suggesting a limited risk for human infection through avian HEV (Huang et al., 2004). Lambs and Wistar rats were reportedly infected with HEV isolates (Meng, 2011 *in press),* however independent confirmation of these data is still lacking.

1.8 Diagnosis

Human viral hepatitis E could not be distinguished clinically from others types of acute viral hepatitis. Acute hepatitis E diagnosis is based on ALT elevation and anti-HEV IgG and/or anti-HEV IgM detection and/or HEV RNA presence in serum and/or stool samples (Pavio et al., 2010). Enzyme-linked immunosorbent assay (ELISA) kits for antibodies to all four HEV genotypes are commercially available, and are based on detection of antibodies against ORF2 (Khuroo and Khuroo, 2008). Anti-HEV-specific IgM detection without IgG indicates very recent infection, whereas anti-HEV IgG presence without IgM indicates past

infection (Bihl and Negro, 2010). Different laboratories developed RT-PCR and real time PCR assays, but no commercial kits are available (Schlauder et al., 1998; Pavio et al., 2010).

The diagnosis in pigs of HEV infection is not performed routinely, since swine HEV infection is subclinical in pig herds. The recombinant human HEV capsid antigen and the capsid protein of swine HEV genotype 3 have been used in an ELISA to detected anti-HEV in pigs (Meng et al., 1997, 1998a, 2002). Genotypes 3 and 4 of human and swine origin are indistinguishable, thus a differential diagnosis assay for swine HEV is not possible or necessary (Cooper et al., 2005; Gyarmati et al., 2007). Specific and sensitive RT-PCR assays have been developed for the detection of swine HEV, and fecal samples are the preferred for this kind of diagnosis (Meng et al., 1997).

The majority of chickens infected with avian HEV present subclinical signs, consequently avian HEV diagnosis is primarily based on detection of RNA virus by RT-PCR or antibodies by ELISA, such as in swine and human. Recently, a *nested* broad-spectrum RT-PCR capable of detecting different HEV types, including the four mammalian genotypes, chickens, and the new HEV type of rats was reported (Johne et al., 2009).

1.9 Prevention and Control

As fecal-oral transmission is the predominant route for HEV infection, in developing countries the main measures are based on improvement of personal and public hygiene (proper treatment of water and sewage). Measures to improve the quality of water, like boiling and chlorination may be useful in neutralizing the virus. Safe disposal of human excreta is also important (Meng et al., 2010a).

In industrialized countries, the routes and sources of autochthonous hepatitis E are multiple and remain uncertain. Zoonotic transmission is probably the main route of hepatitis E infection, but even that, the determination of specific measures of preventing is almost impossible. Epidemiological studies in this region have been done, and measures like avoid eating raw or undercooked pork, deer, and wild boar meat (especially liver) or avoid having contact with infected pigs can be useful for preventing HEV infection. French health authorities published in May 2009

recommendations to cook pork liver sausages prior to consumption in the figatellis package (Colson et al., 2010).

The measurements mentioned above can help avoiding HEV infection. However, in developing and in industrialized countries the disease generally persists, and a vaccine can provide a second and more efficient mode of prevention. Unfortunately, a vaccine against HEV in humans is not available yet, but experimental vaccines appear to be very promising (Shrestha et al., 2007). Two separate subunit vaccines have been tested in clinical trials, and they showed to be safe and effective (Shrestha et al., 2007; Zhang et al., 2009).

1.10 Final Considerations

As we could see in this review, HEV infection is geographically widespread and several studies have been reported leading to a better understanding of virus biology, host-pathogen interaction, epidemiology, immune responses during infection and clinical course of HEV. The recent successes in cell culture of the virus probably will help to elucidate many doubts in this regard.

Ten years ago HEV was believed to be restricted to the developing countries. However, hepatitis E in industrialized countries is more common that previously recognized, may have a zoonotic source, and chronic infection with possible progression to cirrhosis have been demonstrated. HEV is now considered a zoonotic disease, and pig, wild boar and deer are the reservoirs. There are other animal species seropositive for IgG anti-HEV, suggesting that these animals can be infected by HEV. Additional research on the natural history and ecology of HEV will help understand the significance and magnitude of the HEV zoonotic risk.

Studies with particular attention on HEV genetic variability and recombination in animals must be done to prevent the potential emergence of more pathogenic strains of HEV. In addition, developments in prevention of HEV infection and strategies for the use of HEV vaccines have already been done. These vaccines maybe of particular use in some high risk groups or certain situations, for example populations displaced due to floods and war, organ transplanted recipients and HIV patients.

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Figure 1 – Phylogenetic tree representing 39 sequences of full length HEV genome retrieved from GenBank (accession numbers are indicated). Alignment was performed using ClustalW in MEGA 4.1. The tree was reconstructed using the neighbor-joining method with 1,000 bootstrap replicates. Source: Pavio et al., 2010 modified

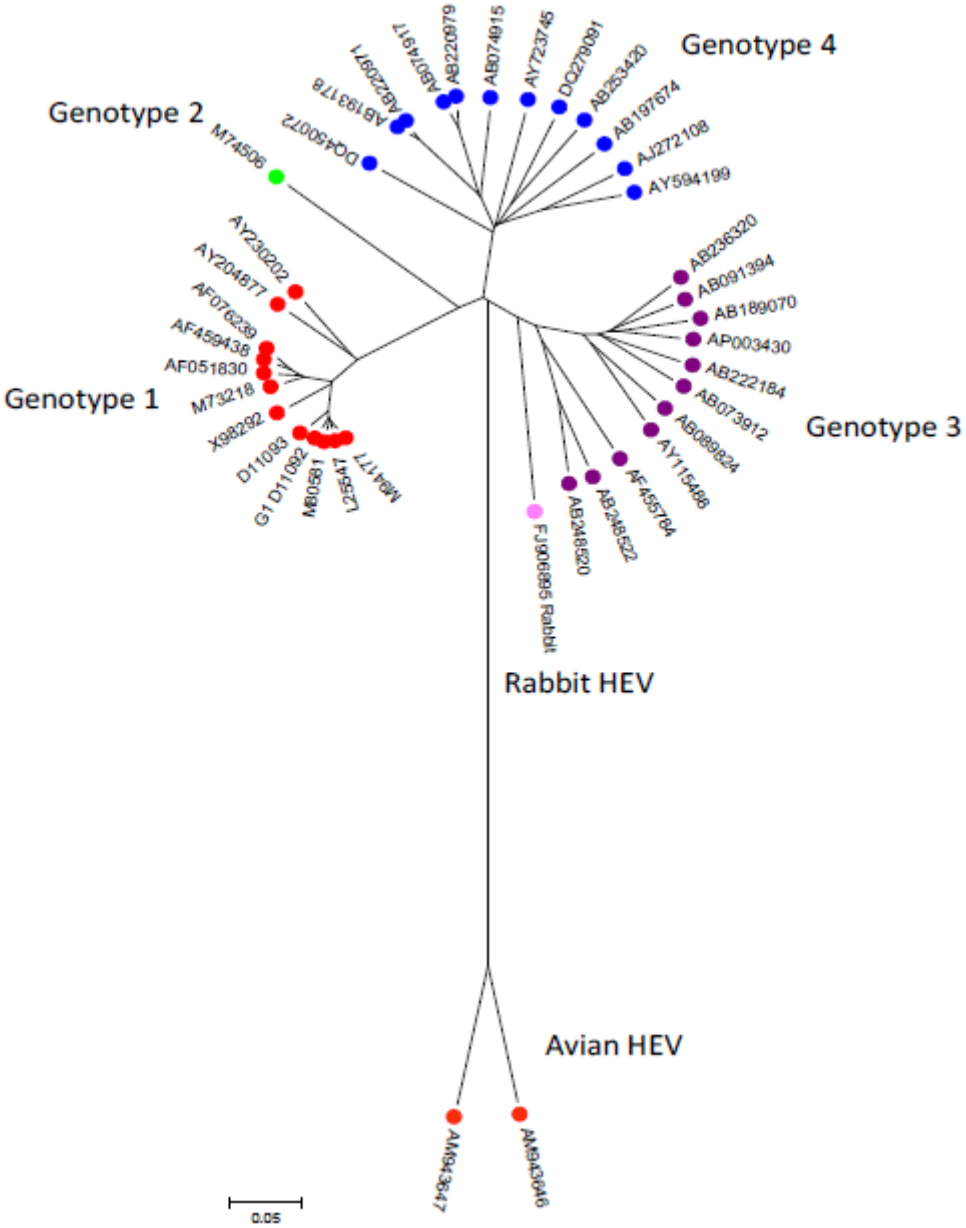

Figure 2 – Phylogenetic tree representing 37 sequences of ORF1 HEV genome retrieved from GenBank (accession numbers are indicated), indicates the four mammalian genotypes and subtypes. Alignment was performed using ClustalW in MEGA 4.1. The tree was reconstructed using the neighbor-joining method with 1,000 bootstrap replicates. Classification was performed according to Lu et al. (2006)

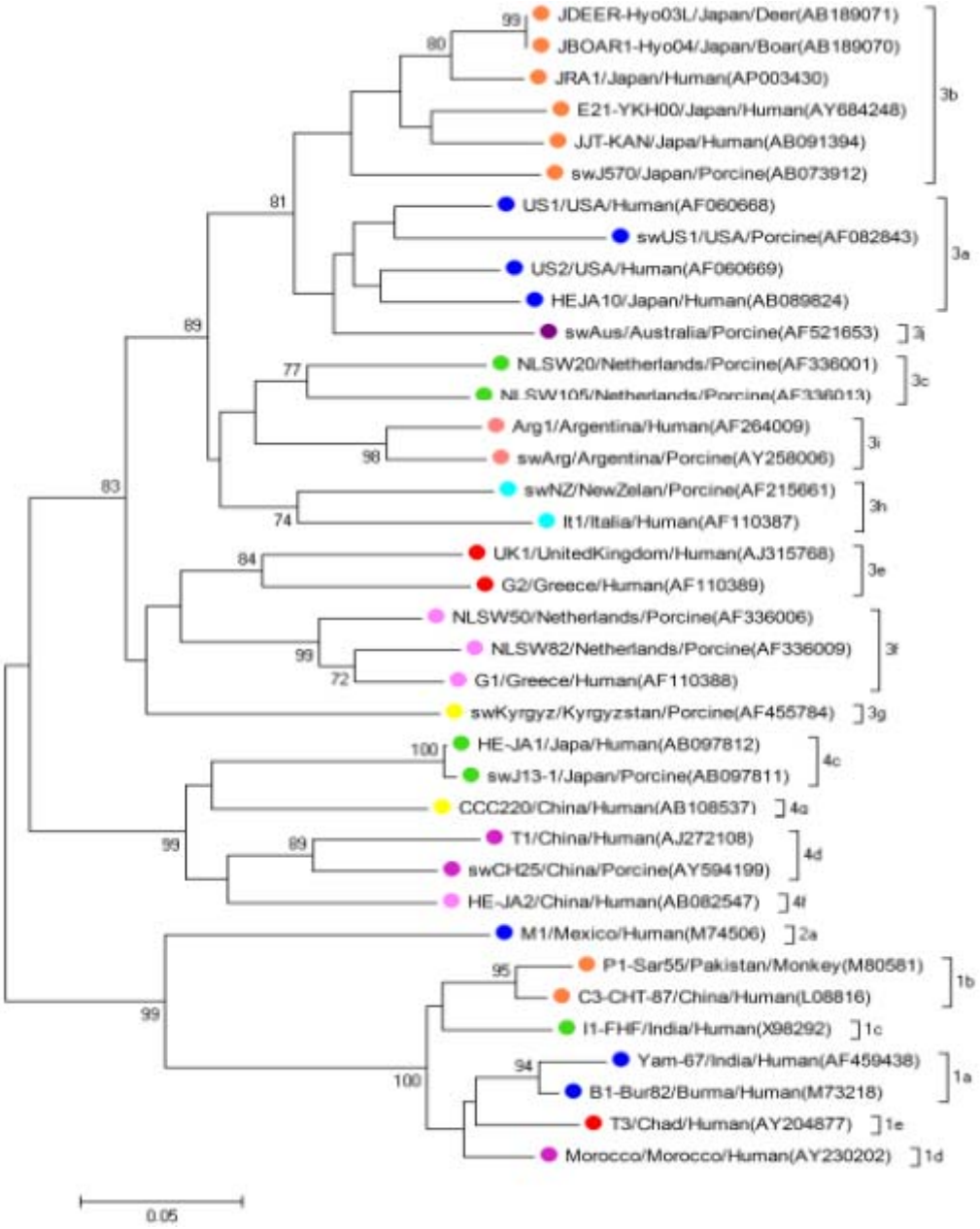

Figure 3 (A) – Imunoelectron microscopy (Â-223,00) of antibody-coated hepatitis E virus recovered from the stool of a patient with hepatitis E in Mexico. **(B)** Three dimensional structure of recombinant HEV-VLP ORF3/ORF2 at a resolution of 24 A° determined by cryo-EM and image reconstruction. The particle is colorcoded to differentiate two distinct domains: the shell domain (white) and the protrusion domain (green). The scale bar represents 100 A°. Source: **(A)** Emerson and Purcell, 2007; **(B)** Wang et al., 2008

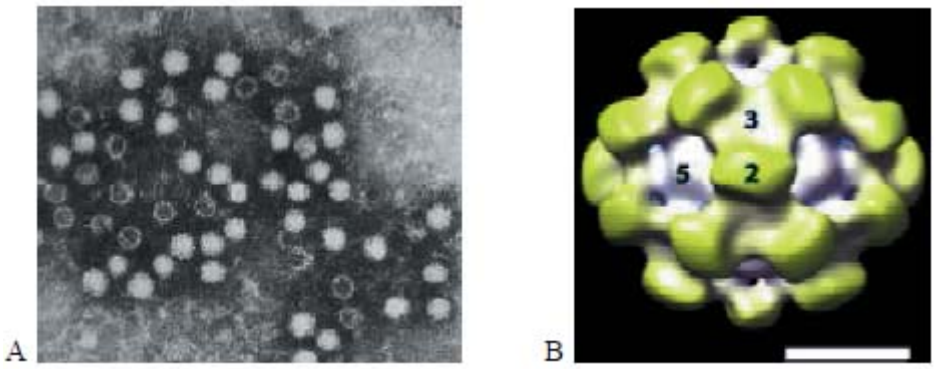

Figure 4 – Genome organization and proteins of HEV. **(A)** Representation of the ~7.2 kb positive strand RNA genome of HEV and its structures. **(B)** The three open reading frames (ORFs) are shown. ORF1 with various functional units - methyltransferase (MeT), papain-like cysteine protease (PCP), RNA helicase (Hel) and RNA dependent RNA polymerase (RdRp). ORF2 encodes the viral capsid protein; the N-terminal signal sequence (blue box) and glycosylation sites are indicated. ORF3 encodes a small regulatory phosphoprotein. Details of the ORF3 proteins are shown, including two N-terminal hydrophobic domains (blue boxes) and two C-terminal proline-rich regions (red boxes). Source: Chandra et al., 2008

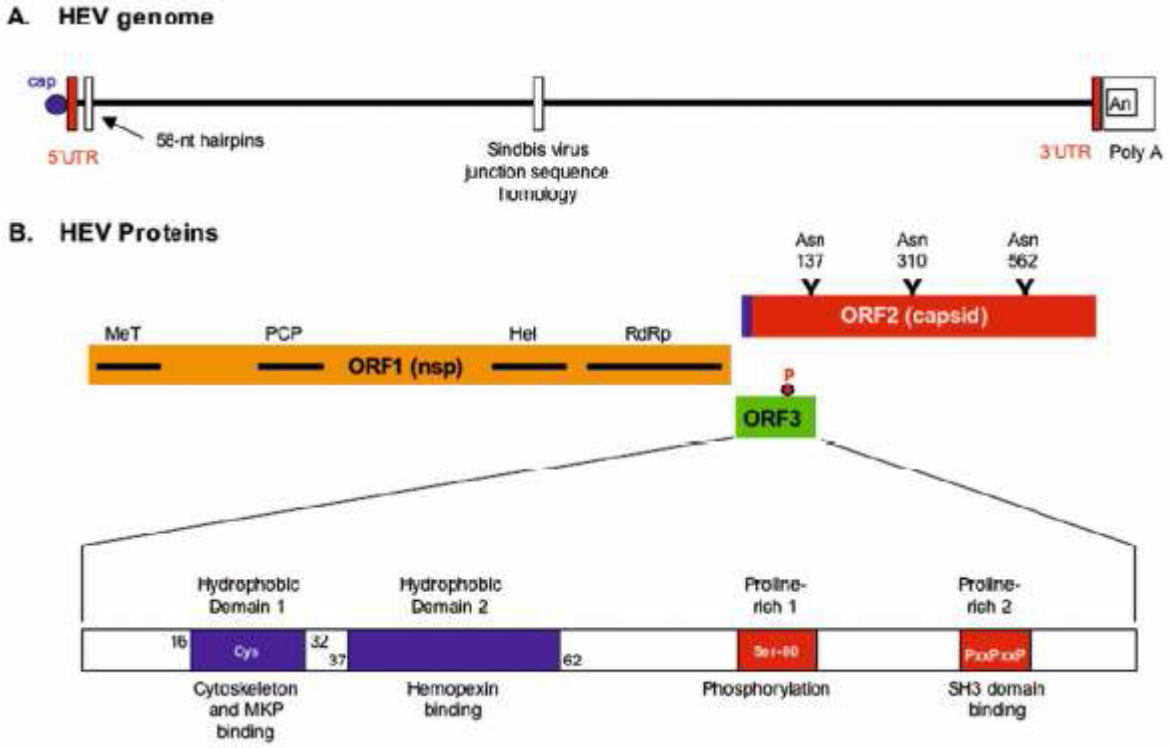

Table 1 – HEV strains and clinical epidemiological characteristics HEVNatural host Disease pattern GeographicalTransmission Age distribution Disease Chronicity Death rate Strainsdistributionseverity

HEV Strains	Natural host	Disease pattern	Geographical distribution	Transmission	Age distribution	Disease severity	Chronicity	Death rate
Genotype 1	Humans	Hyperendemic* Endemic	Asia and Africa Africa	Waterborne Person-to-person?	Young adults (15 to 35 years)	Mild Mild	No No	1-4% 1-4%
Genotype 2	Humans	Hyperendemic*	Central America And Africa	Waterborne	Young adults (15 to 35 years)	Mild	No	1-4%
Genotype 3	Humans, pigs, deer, mongoose	Sporadic	Asia, America, Europe, Oceania	Foodborne zoonotic	Older males (>30 years)	High	Yes**	8%
Genotype 4	Humans, pigs	Sporadic and small outbreaks	Asia	Foodborne zoonotic	Older males (>30 years)	Very high	No	11%

2 OBJETIVOS

2.1 OBJETIVO GERAL

- Detectar a presença do HEV em amostra de fezes, fígado e bile de suínos assintomáticos provenientes de rebanhos da região oeste do estado do Paraná e realizar a filogenia das estirpes virais identificadas.

2.2 OBJETIVOS ESPECÍFICOS

- Detectar, por meio da *nested* PCR com *primers* específicos para as ORF1/ORF2, a presença do HEV em amostras de fezes de suínos assintomáticos de diversas categorias (reprodutores, leitões lactentes e desmamados e em animais de terminação) de rebanhos da região oeste do estado do Paraná;
- Detectar a presença do HEV, por meio da *nested* PCR com *primers* específicos para a ORF1, em amostras de fígado e bile de suínos colhidas em um abatedouro da região oeste do Paraná;
- Identificar a frequência de infecção do HEV nas diferentes categorias de produção de suíno;
- Sequenciar os fragmentos amplificados das ORF1/ORF2 obtidos pela *nested* PCR para a identificação do vírus e determinar os genotipos de HEV circulantes nos rebanhos suínos avaliados;
- Realizar análises moleculares comparativas entre as ORF1/ORF2 das estirpes de HEV identificadas nesse estudo com aquelas pertencentes aos quatro genotipos de origem humana e suína disponíveis em bases públicas de dados.

3 ARTIGO PARA PUBLICAÇÃO

3.1 Molecular Detection and Characterization of Hepatitis E Virus in Naturally Infected Pigs from Brazilian Herds[1]

MOLECULAR DETECTION AND CHARACTERIZATION OF HEPATITIS E VIRUS IN NATURALLY INFECTED PIGS FROM BRAZILIAN HERDS

Abstract: Hepatitis E virus (HEV), the causative agent of hepatitis E, is an important public health concern in many developing and industrialized countries. Transmission of HEV occurs predominantly by fecal-oral route and domestic pigs are reservoirs of the virus. Swine and human HEV strains are genetically related, suggesting the occurrence of zoonotic transmission through contaminated pork, direct exposure to pigs and pig feces, or environmental contamination. The aim of this study was to investigate the presence of swine HEV from pigs of different production categories, from different pig farms in one of the greatest pork production regions in South Brazil. A total of 170 porcine fecal samples from breeder sows, boars, suckling piglets, weaned, and growing pigs were collected from 14 pig farms located in Paraná state. The fecal samples were screened by *nested* PCR with primers targeting ORF2 region, and the positive samples from screening were submitted to a *nested* PCR targeting the ORF1 region. The screening of the fecal samples detected HEV RNA in 62.5% of the pig farms and in 15.3% of the fecal samples. HEV RNA was detected in all production categories, excepted by breeder sows and boars, and the highest (23/71; 32.4%) virus shedding was in growing pigs. In 15 fecal samples was possible to amplify the HEV RNA with both ORF1 and ORF2 regions. The phylogenetic analyses obtained for both ORFs confirmed that all the Brazilian swine HEV isolates clustered with genotype 3, subtype 3b. Considering the prevalence of HEV infection in Brazilian pig herds and the high similarity of the Brazilian swine and human HEV strains, healthy pigs are probably a source for human HEV infection in Brazil, such as reported in other countries, raising public health concerns about the zoonotic aspect of HEV infection.

Keywords: Hepatitis E virus (HEV). Swine. Fecal samples. *Nested* PCR. Phylogeny.

Introduction

Hepatitis E virus (HEV), the causative agent of hepatitis E, is an important public health concern in many developing and industrialized countries. HEV belongs to the *Hepeviridae* family, genus *Hepevirus,* and is a small, non-enveloped, single-stranded RNA, positive-sense with approximately 7.2 kb in length (Purcell and Emerson, 2001). The RNA molecule contains three discontinuous open reading

[1] Artigo editado de acordo com as normas de publicação do periódico Veterinary Microbiology, disponível em:

frames (ORFs 1, 2 and 3) (Tam et al., 1991). The ORF1 of HEV encodes a protein with nonstructural functions that are involved in viral replication and protein processing (Koonin et al., 1992; Huang et al., 2007). The ORF2 encodes the major viral capsid protein that contains immunogenic epitopes that induces neutralizing antibodies, and is the target for vaccine development (Zhang et al., 2008). The remain ORF3 encodes a small cytoskeleton-associated phosphoprotein (Zafrullah et al., 1997).

HEV genome varies significantly and consists of at least four major mammalian genotypes with a single serotype, one avian HEV, and the unclassified rabbit and rat HEV (Lu et al., 2006; Pavio et al., 2010). Mammalian HEV genotypes 1 and 2 are restricted to humans from developing countries with poor sanitation and are often associated with outbreaks and epidemics. HEV genotypes 3 and 4 are responsible for sporadic cases of hepatitis E in developing and industrialized countries and infect humans, swine, and other animal species such as wild boar and deer (Aggarwal and Naik, 2009; Dalton et al., 2008).

Genotype 3 isolates have been reported in human autochthonous cases in several industrialized countries from Europe and Asia (Dalton et al., 2008). This genotype was also detected in America, including Brazil (Dos Santos et al., 2009; Lu et al., 2006). Besides, genotype 3 also has a high prevalence in pig populations worldwide, except for countries in Africa, and presents the most variable genotype (Lu et al., 2006). Genotype 4 contains strains isolated from humans in industrialized areas in Japan, China, and Taiwan, in pigs from Asia, and in wild boar from South Korea (Dalton et al., 2008; Kim et al., 2011).

Human being is the natural host for HEV, while domestic pigs, wild boar, and deer are considered natural reservoirs of HEV genotypes 3 and 4 and, nowadays, HEV is considered a zoonotic disease (Khuroo, 2011, *in press;* Meng, 2010). Increasing evidence of autochthonous cases and high anti-HEV IgG seroprevalence in non-endemic areas support the zoonotic transmission of HEV from pigs to humans (Drobeniuc et al., 2001; Lu et al., 2006). Besides, swine HEV strains are genetically closely related to human genotypes 3 and 4 (Clemente-Casares et al., 2003; Emerson and Purcell, 2003; Lu et al., 2006).

<http://www.elsevier.com/wps/find/journaldescription.cws_home/503320/authorinstructions>.

Seroepidemiological studies reported that pig handlers such as veterinarians and butchers have an increased risk of HEV infection (Meng et al., 2002). Under experimental conditions, HEV genotypes 3 and 4 from pigs could infect non-human primates (Meng et al., 1998a). Principal routes of HEV transmission are the ingestion of contaminated pork, deer, and wild boar meat or offal, direct exposure to pigs and pig feces, or environmental contamination such as water sources. Recent studies confirmed that pig is the source for human HEV infection in industrialized countries (Colson et al., 2010), but zoonotic transmission in these areas is not fully understood (Purcell and Emerson, 2010).

Considering the large territory extension of Brazil, and since only strains from two different Brazilian regions (Rio de Janeiro and Mato Grosso States) have been identified, the nature of HEV infection and the genetic variability among swine HEV isolates from Brazil are not known. The aim of this study was to evaluate the presence of swine HEV from pigs of different production categories, from different pig farms in one of the greatest pork production region in South Brazil, and perform the phylogenetic analysis of the HEV isolates with others human and swine HEV strains.

Material and Methods

Animal and samples

A total of 170 pig fecal samples were collected in June 2009 from 14 pig farms located at West region of Paraná state, Brazil. The farms were from Toledo district (24° 42' 49'' S; 53° 44' 35'' W), one of the greatest Brazilian pork producing areas. Farms were of the following types: 5 farrow-to-weaning farms (named F1 to F5) that keep breeder sows and boars, suckling piglets, and weaned pigs until 8-week-old; and 9 grower-to-finish farms (named F6 to F14) where 9-week-old pigs are housed and fed until they reach 24-week-old. In each farm, fresh fecal samples were collected from animals of all categories. A total of 99 fecal samples were obtained from farrow-to-weaning farms and 71 samples were collected from grower-to-finish farms. The housing conditions of animals included a multi-site production system, with sanitation and overall management generally of a higher standard. All the fecal

samples were obtained from asymptomatic pigs. The samples were transported on ice and then stored at -20°C until use.

RNA extraction

The viral RNA was extracted from fecal suspensions 10-20% (w/v) diluted in Tris/Ca^{++} buffer. A combination of phenol/chloroform/isoamyl alcohol and silica/guanidine isothiocyanate methods was performed (Alfieri et al., 2006). Fractions from each sample were treated with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) (Sambrook and Russell, 2001). After that, the aqueous phase was processed according to silica/guanidine isothiocyanate method (Boom et al., 1990). RNA was eluted in diethyl pyrocarbonate (DEPC) treated water (Invitrogen Life Technology, Carlsbad, CA, USA) and kept at -20 °C until use. Aliquots of Tris/Ca^{++} were included as negative control in each round of extraction.

RT-PCR and nested PCR

Oligonucleotide primers targeting HEV ORF2 (table 1) were used for screening test in all fecal samples. Reverse transcription (RT) reaction was carried out with 5 uL of the extracted RNA, 1 uL (20 uM) of primer set 3517N-reverse and 4 uL of ultrapure water in an initial denaturation step at 97°C for 5 min. The samples were placed on ice for 5 min, and 10 uL of RT mix containing 1x RT buffer (50 mM Tris-HCl pH 8.3; 3 mM MgCb; 75 mM KCl), 0.1 mM each dNTP (Invitrogen Life Technology, Carlsbad, CA, USA), 10 mM DTT, 100 units of M-MLV reverse transcriptase (Invitrogen Life Technology, Carlsbad, CA, USA), and ultrapure sterile water were added to a final volume of 20 uL. The solution was incubated at 42°C for 30 min followed by the enzyme inactivation at 94°C for 5 min.

The tubes were placed on ice and 8 uL cDNA was added to 42 uL PCR mix containing 1.5x PCR buffer (30 mM Tris-HCl pH 8.4; 75 mM KCl), 2 mM $MgCl_2$, 0.2 mM of each primer (3516N/forward and 3517/reverse), 2.5 units of Platinum Taq DNA polymerase (Invitrogen™ Life Technology, São Paulo, SP, BR) and ultrapure sterile water to a final volume of 50 uL. The amplification was performed in a thermocycler (PTC - 200, MJ Research Co, Water Town, MA, USA)

with the following cycling profile: an initial denaturation step of 2 min at 94°C, followed by 40 cycles of 1 min at 94°C, 1 min at 42°C for, and 1 min at 72°C, and a final extension step of 7 min at 72°C.

For the second round PCR 3uL of DNA was added to 47 uL PCR mix, that was similar to first round PCR solution, except that primers set 3518N-forward/3519N-reverse were used, followed by 30 cycles of 1 min at 94°C, 1 min at 42°C, and 1 min at 72°C, and a final extension step of 7 min at 72°C.

The *nested* PCR amplification of the methyltransferase (MeT) region of ORF1, showed in table 1, was used in all positive samples obtained from screening test (ORF2). The RT-PCR protocol for ORF1 region were identical to that done for screening test with ORF2, excepted for the primers set, and the annealing temperature that was 50°C for ORF1 primers set.

Aliquots of 5 uL of the RT-PCR products were analyzed by 2% agarose gel electrophoresis in TBE buffer (89 mM Tris; 89 mM boric acid; 2 mM EDTA; pH 8.4) at constant voltage (90V) for approximately 45 min. The agarose gel was stained with ethidium bromide (0.5 mg/mL), and visualized under UV light. The expected amplicons size was 348 bp for ORF2 and 287 bp for ORF1.

Sequence analysis

Amplicons were purified directly from the PCR products or extracted from the agarose gel with GFX™ PCR DNA and the Gel Band Purification Kit (GE Healthcare, Little Chalfont, UK) and quantified in a Qubit™ fluorometer using Quant-iT™ dsDNA BR Assay Kit (Invitrogen Life Technologies, Eugene, OR, USA). MegaBACE^{TM} 1000 and the DYEnamicTM ET Dye Terminator Kit (GE Healthcare, Little Chalfont, UK) were used for sequencing, which was carried out with primers forward and reverse in both directions. Electropherogram quality analysis was performed using Phred (http://asparagin.cenargen.embrapa.br/phph/). CAP3 software determines the consensus sequence and similarity searches were conducted with sequences deposited in GenBank using BLAST (Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A multiple alignment were performed in CLUSTAL W (version1.4) using MEGA package version 4.1 software (http://www.megasoftware.net/) and sequence identity matrix using BioEdit software version 7.0.8.0 (http://www.mbio.ncsu.edu/bioedit/bioedit.html). The

sequence alignment was performed with representative strains from the four known genotypes, with human and swine HEV strains.

The geographic origins and GenBank accession numbers are as follows: genotype 1: Chad (AY204877, Chad), Madras (X99441, India), P1-Sar-55 (M80581, Pakistan), Morocco (AY230202, Morocco), B1-Bur-82 (M73218, Burma), C4 (D11093, China), X98292 (X98292, India); genotype 2: Ml (M74506, Mexico); genotype 3: JRA1 (AP003430, Japan), VH1 (AF195061, Spain), VH2 (AF195062, Spain), swUSl (AF082843, United States), US1 (AF060668, United States), US2 (AF060669, United States), Arg1 (AF264009, Argentina), SWArg (AY258006, Argentina), Gr2 (AF110392, Greece), Itl (AF110387, Italy), NLSW105 (AF336013, Netherlands), NLSW50 (AF336006, Netherlands), NLSW36 (AF336004, Netherlands), swJ570 (AB073912, Japan), PG05-03 (AB362373, Japan), JJT-Kan (AB091394, Japan), hl (GQ421465, Brazil), El (HM154537, Brazil), B24 (HM154540, Brazil), B51 (HM154539, Brazil), JYK-Sap03 (AB189931, Japan), E21-YKH00 (AY684248, Japan), SW1 (EF591852, Brazil), SW2 (EF591853 and EF591854, Brazil), SW3 (EF591855, Brazil), SW4 (EF591856, Brazil), TLS39 (EU495190, France), SWJC1990 (AB096756, Japan), SWJ8-2 (AB094227, Japan), UK8734 (AY362357, United Kingdom), Sendai (AB093535, Japan); genotype 4: swJ13-1 (AB097811, Japan), HE-JA1 (AB097812, Japan), JAK-Sai (AB074915, Japan), Tl (AJ272108, China), swCH25 (AY594199, China), TW11SW (AF302068, Taiwan) and TW8E-2 (AF032917, Taiwan). The HEV strains identified in this study were designated as BRAsw-13, BRAsw-107, BRAsw-112 (ORF2/ORF1), BRAsw-114 and BRAsw-123 (ORF2/ORF1) (GenBank accession numbers: JN190065, JN190066, JN190067/ JN190071, JN190068, JN190069, JN190070/ JN190072).

Phylogenetic trees were obtained by the neighbor-joining method with Kimura 2-parameter model (Kimura, 1980), using MEGA version 4.1 program. Statistical analysis of phylogenetic trees was determined by bootstrap method on 1000 replicates.

Results

To determine whether HEV is present in Brazilian pig herds, *nested* PCR targeting ORF2 (screening test) and ORF1 regions was performed. The ORF2 primers set (Huang et al., 2002) used in this study target the most conserved region

among all HEV isolates and were used for screening test of all the porcine fecal samples collected. The ORF1 primers set targeting MeT region (Wang et al., 1999) were used for better molecular analyses of the swine HEV strains. Isolates that are analyzed in both ORF1 and ORF2 regions, usually segregated into the same cluster giving greater reliability of the results.

The screening test of the porcine fecal samples with ORF2 primers set detected HEV RNA in 26 (15.3%) of the 170 samples, and in 10 (71.4%) of the 14 pig farms included in this study. In 15 (57.7%) of the 26 positive samples obtained in the screening test was possible to amplify the HEV RNA with ORF1 primers set as showed in table 2. ORF1 primers set also detected HEV RNA in 70% (7/10) of the positive farms.

In the screening test, no HEV RNA was detected in the farms F2, F4, F5, and F12. Of the suckling piglets, the two positive samples were from the same farm (F3), from one animal with 1-week-old and the other with 3-week-old. The only positive fecal sample from weaned pig was from farm F1, from a 8-week-old animal. The highest virus shedding was detected at growing category, in 23 (32.4%) of the 71 pigs, and in 8 (88.9%) of the 9 growing to finish farms as showed in table 2. There was significant difference between growing pigs and farrow-to-weaning categories (P=0,0001). ORF1 primers set did not detect RNA in suckling piglets and in growing pigs of 19 and 23-week-old.

The amplified products with 348 nt from ORF2 region were sequenced for a total of 10 swine HEV isolates, selected from each farm. A single representative of the samples that showed more than 99% nt identity had its sequence submitted to GenBank database.

Before performing molecular and phylogenetic analyses, primers sequence from ORF2 region were excluded at both ends, resulting in 304 nt length. The Brazilian swine HEV strains showed a nucleotide (nt) identity lower than 78% with genotypes 1, 2, and 4 and between 78% and 92.2% with genotype 3. All the swine HEV sequences identified showed the highest nt identity (90.1% to 92.2%) to TLS39 (Legrand-Abravanel et al., 2009), a French human HEV strain (figure 1). The Brazilian swine HEV strains were also closely related to the Japanese HEV strains JRA1, swJC1990, and JJT-Kan (Takahashi et al., 2001; Takahashi et al., 2003), all of them classified into genotype 3 subtype 3b (Lu et al., 2006). When compared with Brazilian swine HEV strains SW1, SW2 and SW3 previously described by Dos

Santos et al. (2009) from other Brazilian regions (Rio de Janeiro and Mato Grosso states), the nt identity ranged between 78% and 83.5%.

Among the Brazilian swine HEV sequences obtained with ORF2 primers set, nt difference in the same herd was obtained in farms F6 (BRAsw-97 and BRAsw-99 strains) and F8 (BRAsw-109, BRAsw-112 and BRAsw-114), and ranged from 97.3% to 99.6%. However, sequences from samples BRAsw-99 and BRAsw-112, that belong to different farms (F6 and F8) were identical to each other.

The amplified products of ORF1 (228 nt excluding primers) from five swine HEV strains, from five different farms were closely related to the only Brazilian human isolate h1 strain (88.5% to 89% nt identity). They also were closely related to the other known Brazilian swine HEV strains SW1 and SW2 (82.4% to 82.8% nt identity) from other herds (Mato Grosso and Rio de Janeiro states), and to Brazilian swine strains B24, B51 and E1 (84.2-86.8% nt identity) from slaughterhouses located at Rio de Janeiro state (Dos Santos et al., 2009, 2011; Lopes dos Santos et al., 2010). The ORF1 sequences clustered together to human and swine strains from Japan with high nt identity ranging from 91.6% to 93.8% (figure 2). Of these, the strains JRA1, JJT-Kan, PG05-03 and SWJ570 are classified as genotype 3 subtype 3b (Lu et al., 2006).

The topology of the phylogenetic tree obtained for the ORF1 region was similar to that of ORF2. The sequences from both regions confirmed that all the Brazilian swine HEV isolates identified in this study clustered with genotype 3 and they were similar to the subtype 3b HEV strains.

Discussion

The present study represents the first survey conducted for HEV detection among different production categories on pig herds in Brazil. The overall (15.3%) of virus shedding observed in this study is similar to that reported by Fernandez-Barredo et al. (2007) in a Spanish study, where 16% of the pigs from different production categories were positive. However, our data is considered lower when compared to other studies reported in Netherlands (22%), USA (35%) and Canada (34%) (Huang et al., 2002; Van der Poel et al., 2001; Ward et al., 2008). This can be explained, at least partially, due to the diversity of ages examined in the present study *versus* the production category examined in these other studies, where

the majority of the pigs were between two and four months old, when HEV RNA is more frequently detected in porcine fecal samples (Meng et al., 1997). In the present study, 32.4% of the porcine fecal samples from growing category were positive, when animals present more than two months of age. This result is in agreement with previous reports such as mentioned before. Viremic stages probably occur by the age of two months because it is approximately when anti-HEV immunoglobulin G antibodies acquired passively from colostrum declines (Kasorndorkbua et al., 2003; Vitral et al., 2005). It was possible to detect HEV in suckling and weaned pigs from the present study, showing that HEV could circulate among younger pigs, even that in lower frequency.

Furthermore, at 24-week-old when most of pigs are slaughtered in Brazil, HEV fecal shedding was still detected in 4 of the 17 pigs (23.5%), raising concerns about the risk of HEV transmission to slaughterhouse workers. Feces as a source of HEV infection for butchers and slaughterhouse workers have been associated to sporadic cases of hepatitis E in non endemic regions (Jary, 2005; Perez-Garcia et al., 2007). The real impact of swine HEV infection for the subjects that works in the Brazilian swine industry is unknown. Therefore, the results of present study in association with recent studies performed in Brazil reinforces the possibility that healthy pigs are probably a source for human HEV infection and raises concern about the necessity of studies with human population who are in close contact to pig (Dos Santos et al., 2009, 2011; Lopes dos Santos et al., 2010).

An interesting observation in our study is that none of the breeder sows (n=32) was shedding HEV in feces. Some authors suggested that contact with sows shedding virus is probably the cause of HEV infection in pigs, since most of them became infected at 3 to 6 weeks of age (Fernandez-Barredo et al., 2006). Considering that incubation period varies between two and three weeks, viral shedding last up to seven weeks and that most of the viremic pigs found in the majority of the studies were concentrated around 13 weeks (Meng et al., 1998b), it is possibly that animals became infected when they were in contact with their mothers. Therefore, if no sow were positive in our study, probably other routes of transmission could determine HEV infection in the analyzed pig herds. Meng et al. (2010) suggested that rodents in the chicken farms might serve as a mechanical carrier in the transmission of avian HEV. Considering this information, it is reasonable to extrapolate that the same can occur at pig herds, with rodents playing a role of

mechanical carrier or as a possible reservoir of swine HEV, and possibly maintaining HEV circulation in pig herds during the cleaning period. Besides that, fomites, water supplies, pig workers, and aerosols could also act as a transmission route.

The results reported in this study and the previously reported trials (Dos Santos et al., 2009, 2011) demonstrate that HEV is quite prevalent and widespread in Brazilian territory. In this study we could determinate that swine HEV is widespread in Brazilian pig herds and in different categories. Furthermore, molecular analysis from ORF1 and ORF2 regions revealed that all the Brazilian swine wild-type HEV strains was classified as genotype 3, subtype 3b and are closely related to human HEV strains, especially from Japan and including the Brazilian human HEV h1 strain.

Analyzing the HEV genome based on ORF2 region from 6 growing to finish farms, we found a similar nt identity between the swine HEV isolates. Intra-farming nt difference was detected between strains from farm F6 with minor variation (0.4%) and between strains from farm F8 with 2.7% nt difference. Inter-farm nt variation was higher, ranging from 0.4% to 4.5%. Two swine HEV isolates, BRAsw-99 and BRAsw-112, from two different farms, F6 and F8, were identical to each other. In Japan, Nakai et al. (2006) reported similar results with higher virus shedding at the finishing production age and the detection of identical isolates in different farms. In this way, inter-farm transmission was detected in the present study and probably because pig herds were next to each other. Some factors can be responsible for inter-farming transmission of HEV strains, like shared water supplies, food, fomites and workers, a common animal source that can circulate among the farms like rodents or other animal species and pigs from the same origin that are intended to different farms.

Molecular analysis targeting ORF1 region of HEV strains from porcine fecal samples evaluated in the present study showed they are closely related to each other but different from HEV strains described previously in Brazil and Argentina (Dos Santos et al., 2009, 2011; Lopes dos Santos et al., 2010; Schlauder et al., 2000). Argentine swine HEV strain (AY528006) showed a nt sequence difference of 13.6-16.7% with Brazilian swine wild-types (Schlauder et al., 2000). Similar results were observed with Brazilian HEV strains SW1/SW2 characterized by Dos Santos et al. (2009) in swine herds (17.6% nt diference) and from Brazilian HEV strains E1, B24, 51 reported in slaughterhouses (15.8% nt diference) (Dos Santos et

al., 2011). In spite of the nt difference observed between the Brazilian swine HEV strains described before and the strains related in this study, they are classified in the same genotype and subtype, 3b. Therefore, the results of our study provide evidence of a genomic heterogeneity of swine HEV strains in Brazil, even they present a similar classification. Since strains from different states resulted in evident nt difference and given the great number of pig producers widespread in the Brazilian territory, more studies must be performed for better understanding about HEV epidemiology on Brazilian pig herds.

In conclusion, the present study indicates that swine HEV is widespread in several production categories, especially in the growing pigs, in Brazilian pig herds. The genomic heterogeneity observed between Brazilian swine HEV strains described before and then described in this study give information about the nt diversity of the circulating HEV strains. This kind of information can help in the future, in order to plan adequate measures for prevention. Given the prevalence of HEV infection in pig herds and the high similarity of the swine HEV strains with human strains, Brazilian public health authorities may raise concerns about HEV zoonosis.

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Figure 1 – Phylogenetic tree based on the nucleotide sequences of a 304 bp region within the ORF2 gene of the HEV genome. Bootstrap values are indicated when >50% as a percentage was obtained from 1,000 replications. Avian HEV was included as an outgroup. The four major genotype (G1-G4), including human and swine strains are indicated. HEV sequences isolated in this study are highlighted. HEV sequence names are labeled as follows: BLAST identification; country of origin and host. The scale bar represents a genetic distance of 0.05 substitutions per site

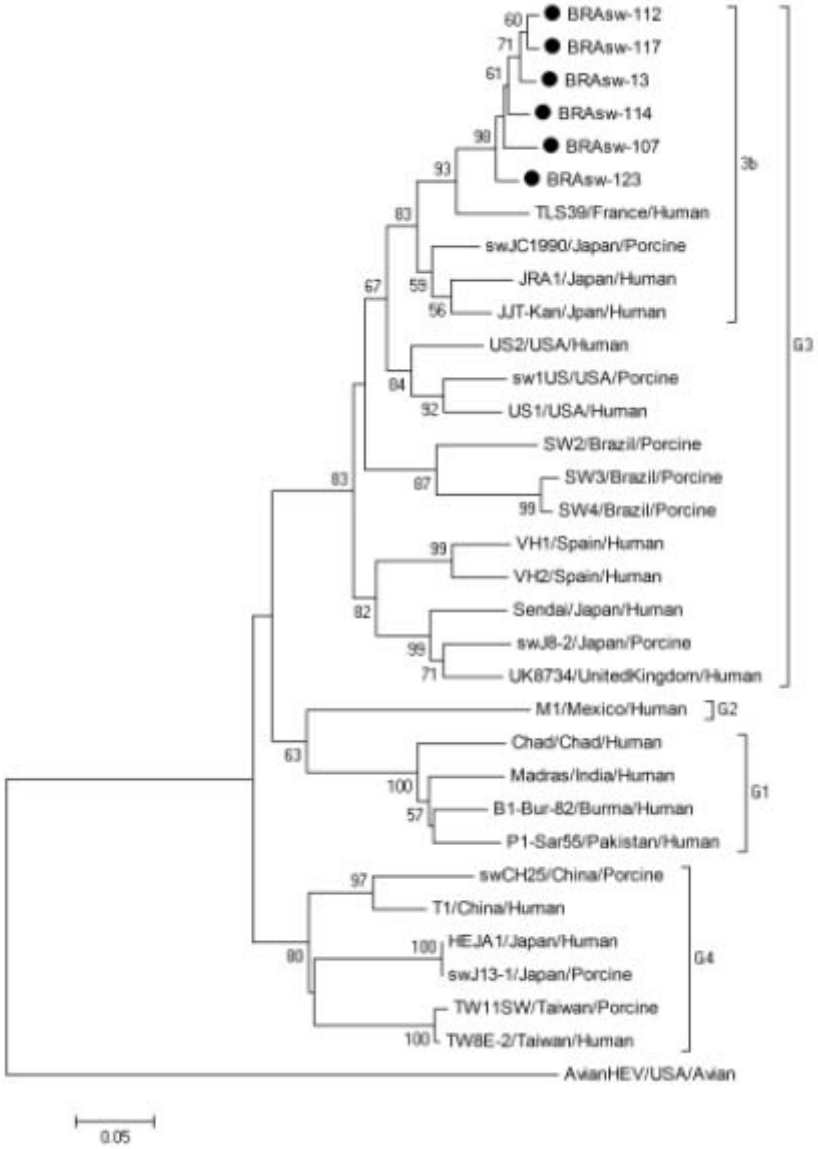

Figure 2 – Phylogenetic tree based on the nucleotide sequences of a 228 bp region within the ORF1 gene of the HEV genome. Bootstrap values are indicated when >50% as a percentage was obtained from 1,000 replications. Avian HEV was included as an outgroup. The four major genotype (G1-G4), including human and swine strains are indicated. The five HEV sequences isolated in this study are highlighted. HEV sequence names are labeled as follows: BLAST identification; country of origin and host. The scale bar represents a genetic distance of 0.05 substitutions per site

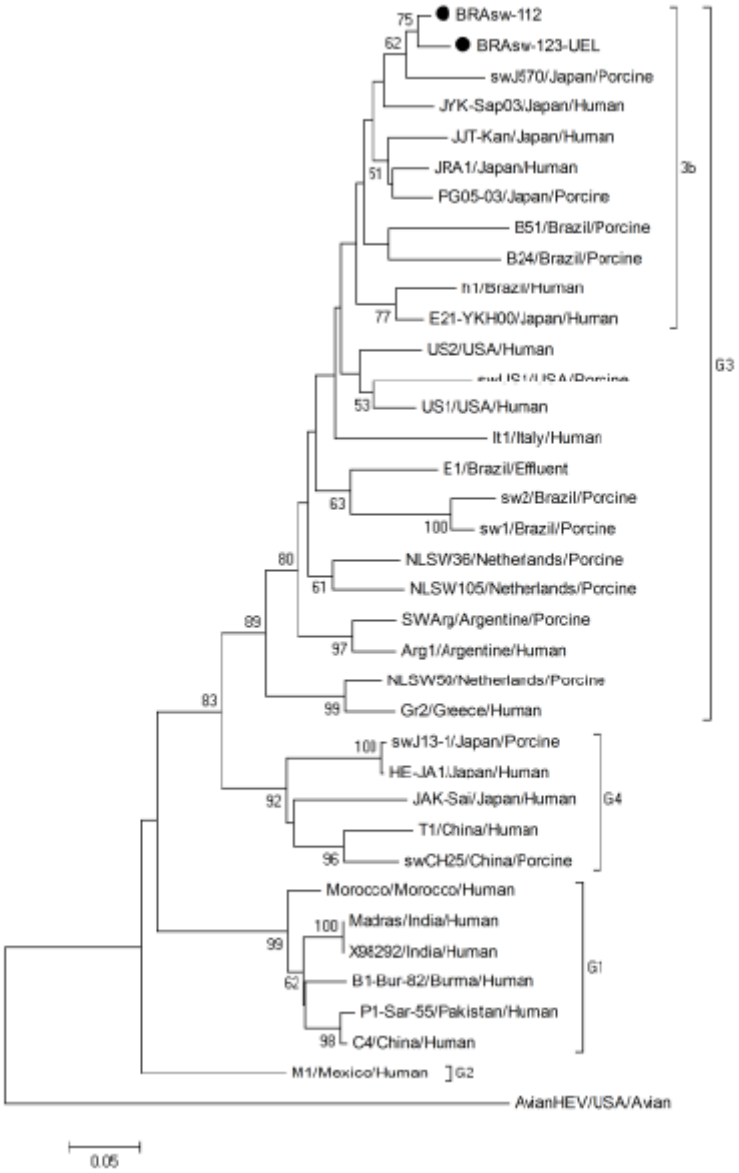

Table 1 – Oligonucleotide primers employed for *nested* PCR amplification of HEV RNA in porcine fecal samples

Primer	Sense	Sequence (5' to 3')	Position in genome[c]	Product Lenght
ORF1-s1[a]	Forward	CTGGCATYACTACTGCYATTGAGC	56-79	
ORF1-a1	Reverse	CCATCRARRCAGTAAGTGCGGTC	451-473	418 bp
ORF1-s2	Forward	CTGCCYTKGCGAATGCTGTGG	104-124	
ORF1-a2	Reverse	GGCAGWRTACCARCGCTGAACATC	367-389	286 bp
3516N[b]	Forward	AATTATGCC(T)CAGTAC(T)CGG(A)GTTG	5687-5708	
3517N	Reverse	CCCTTA(G)TCC(T)TGCTGA(C)GCATTCTC	6395-6417	731 bp
3158N	Forward	GTT(A)ATGCTT(C)TGCATA(T)CATGGCT	5972-5993	
3519N	Reverse	AGCCGACGAAATCAATTCTGTC	6298-6319	348 bp

[a]Huang et al. (2002)
[b]Wang et al. (1999)
[c]Nucleotide positions are according to the HEV Burmese strain.

Table 2 – HEV RNA detection in fecal samples of asymptomatic pigs by *nested* PCR assay with primers set targeting ORF2 and ORF1 regions

Pig Category	Age	Farm	HEV RNA		HEV isolates from screening test
			ORF2[a]	ORF1[b]	
Suckling piglets	1 to 4-week-old	F1 to F5	2/25	0/2	BRAsw-43/47
Weaned pigs	5 to 8-week-old	F1 to F5	1/33	1/1	BRAsw-13
Sows	> 1 year	F1 to F5	0/32	-	
Boars	> 1 year	F1 to F5	0/9	-	
Subtotal farrow-to-weaning			3/99 (3%)*	1/3 (33.4%)	
Growing pigs	9-week-old	F6	4/7	4/4	BRAsw-97 to 100
	12-week-old	F7	1/7	1/1	BRAsw-107
	13-week-old	F8	8/9	6/8	BRAsw-109 to 116
	15-week-old	F9	1/5	1/1	BRAsw-117
	17-week-old	F10	2/5	1/2	BRAsw-123/124
	19-week-old	F11	1/11	0/1	BRAsw-135
	21-week-old	F12	0/6	-	
	23-week-old	F13	2/4	0/2	BRAsw-144/146
	24-week-old	F14	4/17	1/4	BRAsw-149/151/163/164
Subtotal growing			23/71 (32.4%)*	14/23 (60.9%)	
Total			26/170 (15.3%)	15/26(57.7%)	

*There was significant statistical difference between this two categories (*P=0,0001*)
[a]Screening test; bEvaluated only in positive samples from screening test with ORF2

3.2 HEPATITIS E VIRUS IN LIVER AND BILE SAMPLES FROM SLAUGHTERED PIGS FROM BRAZIL[2]

HEPATITIS E VIRUS IN LIVER AND BILE SAMPLES FROM SLAUGHTERED PIGS FROM BRAZIL

Abstract: Hepatitis E, caused by infection with hepatitis E virus (HEV), is a major public health concern in developing and industrialized countries. Domestic pigs are reservoirs of HEV, and some cases of hepatitis E can be zoonotically acquired. Occupational exposure to pigs by farmers, veterinarians, butchers, and slaughterhouse workers has been linked to an increased risk of HEV infection. The aim of this study was to evaluate the presence of HEV in pig liver and bile samples, and identify the HEV isolates. Liver and bile samples were collected from 118 asymptomatic adult pigs at a slaughterhouse situated in the major Brazilian pork production area. The samples were assayed by *nested* PCR with primers set targeting ORF1 region of HEV genome. HEV RNA was detected in 2 (1.7%) and in 1 (0.84%) liver and bile samples, respectively. The phylogenetic analyses showed that the three porcine HEV strains detected clustered together with strains characterized as genotype 3 subtype 3b, which is the same genotype and closely related to the pig and human strains reported previously in Brazil. The result obtained in this study provides evidence that HEV can be detected in slaughtered pigs and suggests that healthy pigs may be a source of infection for pig liver consumers and slaughterhouse workers in Brazil.

Keywords: Hepatitis e virus. Swine. Slaughterhouse. Zoonosis.

Introduction

Hepatitis E, caused by the infection of hepatitis E virus (HEV) is a major public health concern in developing and industrialized countries. The member of *Hepevirus* genus in the family *Hepeviridae* is a non-enveloped, single-stranded, positive-sense RNA virus with a 7.2 kb genome that consists of three discontinuous open reading frames (ORF) (Tam et al., 1991; Emerson and Purcell, 2006). The mammalian HEV is classified in four genotypes and one single serotype (Lu et al., 2006). In developing countries, HEV outbreaks are mainly caused by genotypes 1 and 2 infection. Hepatitis E is recognized as a zoonotic disease with sporadic cases in industrialized countries caused by genotypes 3 and 4 and pigs are probably the main reservoirs of these HEV genotypes (Meng, 2010). Autochthonous hepatitis E in developed countries is often misdiagnosed as drug induced liver injury (Dalton et al.,

[2] Artigo editado de acordo com as normas de publicação do periódico Transboundary and Emerging Diseases,

2007). In general population, the disease is often asymptomatic, but people with underlying chronic liver disease are at risk of developing and dying from fulminant hepatic failure in approximately 70% of the cases (Dalton et al., 2008).

Occupational exposure to pigs by farmers, veterinarians, butchers, and slaughterhouse workers has been linked to an increased risk of HEV infection (Galiana et al., 2008). The potential of pig as a vehicle of HEV transmission has been linked to the consumption of raw or undercooked pork meat or viscera (Tei et al., 2003; Li et al., 2005). Other routes of zoonotic infection such as direct exposure to pig and pig feces, or environmental contamination should not be excluded. In Brazil, the incidence of HEV infection in human has been of minor concern. Previous report showed that the overall HEV seroprevalence in health population is about 4% (Goncales et al., 2000; de Paula et al., 2001). In pigs, HEV genotype 3 has been reported from pig farms and slaughterhouses in other Brazilian region (Mato Grosso and Rio de Janeiro states) (dos Santos et al., 2009, 2011).

Brazil is one of the most important pork producers and exporter in the world. The South region, including states of Paraná, Santa Catarina, and Rio Grande do Sul, represents the major pig producers in the country. Therefore, the aim of this study was to evaluate the HEV presence in porcine liver and bile samples at a slaughterhouse from one of the great Brazilian pork producing areas, and perform the phylogenetic analysis with swine and human HEV strains circulating in Brazil other countries.

Materials and Methods

Porcine liver and bile paired samples were collected from 118 adult pigs at one slaughterhouse under control of Federal Inspection located in Paraná state (Brazil). The samples were obtained in July 2010, from health animals with estimated age over 25 weeks, and raised in ten different herds situated in West region of Paraná. Roughly 10 to 15 samples from each herd were obtained. Each bile sample was withdrawn with sterile disposable syringes by needle puncture through the gall-bladder and one part from inner liver was excised with new sterile scalpel blades for each specimen. The samples were stored at -20°C until use. Bile samples

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disponível em: <http://www.wiley.com/bw/submit.asp?ref=1865-1674&site=1>.

were suspended in 10% (w/v) diethyl pyrocarbonate (DEPC) water (Invitrogen™Life Technology, Carlsbad, CA, USA). Liver tissue was disrupted manually and subsequently homogenized in 10% (w/v) phosphate buffered saline (PBS) pH 7.2, then clarified by centrifugation for 10 min at 3000 x g.

Viral RNA was extracted from aliquots of 400 uL of liver and bile suspension in a combination of phenol/chloroform/isoamyl alcohol and silica/guanidine isothiocyanate methods (Alfieri et al., 2006). Fractions from each sample were treated with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), homogenized and heated at 56 °C for 15 min (Sambrook and Russell, 2001). After centrifugation at 10,000 x g for 10 min, the aqueous phase was processed according to Boom et al. (1990). Both, liver and bile samples were assayed by *nested* PCR with primers targeting the methyltransferase (MeT) region of ORF1 of the HEV genome. Primers set ORF1-s1/ORF-1a1 were used in the first round and ORF-1s2/ORF-1a2 in the second round (Wang et al., 1999).

Reverse transcription (RT) was carried out with 5 uL of the extracted RNA, 1 uL (20 uM) of primer set ORF1-a1, and 4 uL of ultrapure water in an initial denaturation step at 97°C for 5 min. The samples were placed on ice for 5 min, and 10 uL of RT mix containing 1x RT buffer (50 mM Tris-HCl pH 8.3; 3 mM MgCb; 75 mM KCl), 0.1 mM of each dNTP (Invitrogen Life Technology, Carlsbad, CA, USA), 10 mM DTT, 100 units of M-MLV reverse transcriptase (Invitrogen™ Life Technology, Carlsbad, CA, USA), and ultrapure sterile water were added to a final volume of 20 uL. The solution was incubated at 42°C for 30 min followed by the enzyme inactivation at 94°C for 5 min.

The tubes were placed on ice and 8 uL cDNA was added to 42 uL PCR mix containing 1.5x PCR buffer (30 mM Tris-HCl pH 8.4; 75 mM KCl), 2 mM $MgCl_2$, 0.2 mM of each primer (ORF1-s1-forward and ORF1-a1-reverse), 2.5 units of Platinum Taq DNA polymerase (Invitrogen™ Life Technology, São Paulo, SP, BR) and ultrapure sterile water to a final volume of 50 uL. The amplification was performed in a thermocycler (PTC - 200, MJ Research Co, Water Town, MA, USA) with the following cycling profile: an initial denaturation step of 2 min at 94°C, followed by 40 cycles of 1 min at 94°C, 1 min at 50°C for, and 1 min at 72°C, and a final extension step of 7 min at 72°C. For the second round PCR 3 uL of DNA was added to 47 uL PCR mix, that was similar to first round PCR MIX, except that primers

set ORF1-s2-forward/ORF1-a2-reverse were used, followed by 30 cycles of 1 min at 94°C, 1 min at 42°C, 1 min at 72°C, and a final extension step of 7 min at 72°C.

Aliquots of 5 uL of the RT-PCR products, with the expected size of 287 bp were analyzed by 2% agarose gel electrophoresis in TBE buffer (89 mM Tris; 89 mM boric acid; 2 mM EDTA; pH 8.4) at constant voltage (90 V) for aproximately 45 min. The agarose gel was stained with ethidium bromide (0.5 mg/mL), and visualized under UV light.

In the positive samples, the amplicons were extracted from the agarose gel with GFX™ PCR DNA and the Gel Band Purification Kit (GE Healthcare, Little Chalfont, UK), and quantified in Qubit TM Fluorometer (Invitrogen, Molecular Probes, Eugene, OR, USA). The sequences were obtained with the DYEnamic ET Dye Terminator Kit (GE Healthcare, Pittsburgh, PA, USA) in a MegaBACE 1000/Automated 96 Capillary DNA sequencer, Thermo Sequenase™ II DNA Polymerase and the DYEnamic™ ET Dye Terminator Kit (GE Healthcare, Little Chalfont, UK) using the primers of *nested* PCR in both directions. Electropherogram quality analysis was performed using Phred and CAP3 softwares (http://asparagin.cenargen.embrapa.br/phph/) and the sequences were accepted if base quality was > 20. Similarity searches were conducted with sequences deposited in GenBank using BLAST software (Basic Local Alignment Search Tool - http://blast.ncbi.nlm.nih.gov/Blast.cgi). A multiple and pairwise alignments were performed in CLUSTAL W (version1.4) using MEGA package version 4.1 software (http://www.megasoftware.net/) and sequence identity matrix using BioEdit software version 7.0.8.0 (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Phylogenetic tree was constructed with MEGA software using Kimura 2-parameter distance correction. The HEV strains identified in this study were designated as BRsw-A and BRsw-B (GenBank accession numbers: JN166093 and JN166094).

Results

The *nested* PCR assay amplified a fragment of 287 bp in 2/118 (1.7%) livers and in 1/118 (0.84%) bile samples. HEV RNA was detected in liver and bile from one pig, and only in the liver from another one. The two animals were from different pig herds.

HEV sequences obtained from bile and liver specimen from the same animal (A) shared a nucleotide identity of 100%. The HEV sequence from the liver of animal B shared 99.5% nucleotide identity with sequences from liver and bile of animal A, and both of them shared 82-94.2% identity with genotype 3 HEV strains. The three sequences showed maximum identity to JYK-Sap03 (Mishiro et al., 2005) and E21-YKH00 (Tamada et al., 2004, *unpublished)* human HEV strains detected in Japan, with 94.2% and 93.4 % nt identity, respectively. Likewise, the sequences were closely related (89.4% identity) to HEV strain h1, the only human autochthonous case of hepatitis E reported in Brazil (Lopes dos Santos et al, 2010). They also shared 82.883.3% nt identity to porcine strains (SW1 and SW2) previously detected by dos Santos et al. (2009) in pig herds from Rio de Janeiro state, and 85.5-87.2% nt identity to strains (E1, B24 and B51) from a pig slaughterhouse from the same region (dos Santos et el., 2011). The phylogenetic analyses revealed that the three wild-type Brazilian porcine HEV strains clustered together with strains characterized as genotype 3 subtype 3b (Fig.1).

Discussion

The *nested* PCR assay used in this study reveals that the presence of HEV genome in 1.7% and 0.84% of the slaughtered pig livers and bile, respectively is not infrequently, since just few samples from each herd were collected (10 to 15 samples from each herd). The results reported here also complement information from previous investigations conducted in Brazil, evidencing HEV circulation in porcine breeding farms and slaughterhouses from different regions of Brazil. Similar findings have been reported in Japan in which 2% of pig livers obtained from groceries stores were positive for HEV (Yazaki et al., 2003), and in the USA, where 11% of retail liver packages from groceries shops were positive and the strains remains fully infectious (Feagins et al., 2007). Virus sequences recovered from commercial pig livers in Japan were closely related or almost identical to the viruses recovered from some human hepatitits E patients supporting the zoonotic transmission (Yazaki et al., 2003). Our results also show this genetic relationship between Brazilian swine HEV and human HEV, suggesting that pigs are possible the main reservoirs of HEV in Brazil.

It is known that people who have professional contact with pigs or their environment presents high anti-HEV IgG seropositivity. Studies have been suggested that this specifically population may be at higher risk of HEV infection than general population (Meng et al., 2002). Zoonotic transmission through contaminated pork food has already been confirmed, but proper cooking makes it safe to consumption (Li et al., 2005; Feagins et al., 2007). Consumption of offal including smoked but uncooked pig liver and blood from grocery has been related to HEV infections in Europe, supporting HEV transmission through fresh pork sausages, known as figatellu (Colson et al., 2010). In the present study HEV was found in pig livers that could be consumed in sausages, especially the homemade one which could also includes blood and intestinal tracts (offal that are also infected with HEV). Therefore, Brazilian subjects who consume this contaminated pork products, especially pig liver raw or undercooked, or even subjects who come into close contact with infected pigs could be at potential risk to become HEV infected.

HEV detection is higher when pigs are between 2 and 4 months of age comparing to the finishing phase of production (about 6 months), and the virus is expected to be cleared in the majority of the slaughtered animals (de Deus et al., 2008). Even that, the results of present study provides evidence that HEV can be detected in slaughtered pigs and suggests that healthy pigs may be a source of infection for pig liver consumers and slaughterhouse workers in Brazil. A recent study reported in a pig slaughterhouse from Southeast Brazilian region, Rio de Janeiro state, detected HEV genotype 3b in 9.6% of bile samples, however liver sample was not evaluated in this study (dos Santos et al., 2011). Despite the large size of the country, it appears that HEV strains from swine and human origin closely related to each other are circulating in Brazil, enhancing the possibility of zoonotic transmission.

The results obtained here raise public health concerns in Brazil, since HEV could be detected in health pigs under control of Federal Inspection at slaughter time. These contaminated animals products are going to be in close contact with slaughterhouse workers and after that, the offal may be commercialized as food. The prevalence of HEV infection in human in Brazil is unknown, and only one autochthonous case has been documented (Lopes dos Santos et al., 2010). However, serological studies suggested that subclinical or undiagnosed HEV infection can be possible (Carrilho et al., 2005). The only human autochthonous case reported in Brazil suggested a zoonotic origin of infection through consumption of

infected pork meat, and the phylogenetic analysis classified this human HEV strain within genotype 3b, which is the same genotype and closely related to the pig strains that we found in this study. This result reinforces the possibility that persons who work at slaughterhouse became accidentally infected with swine HEV.

In conclusion, this study provides evidence that slaughterhouse workers are potentially exposed to HEV from health pigs in Brazil. Besides that, pig liver and offal could act as HEV vehicles to consumers that eat them raw or undercooked. Considering HEV circulation in Brazil and that clinical presentation of hepatitis E is similar to any other acute hepatitis, HEV should be included in differential diagnosis in patients with unexplained hepatitis. Therefore, more studies must be performed to determine to what extend pig liver and offal contaminated with HEV represent a public health concern, not only in Paraná and Rio de Janeiro state as reported recently, but also from other Brazilian regions.

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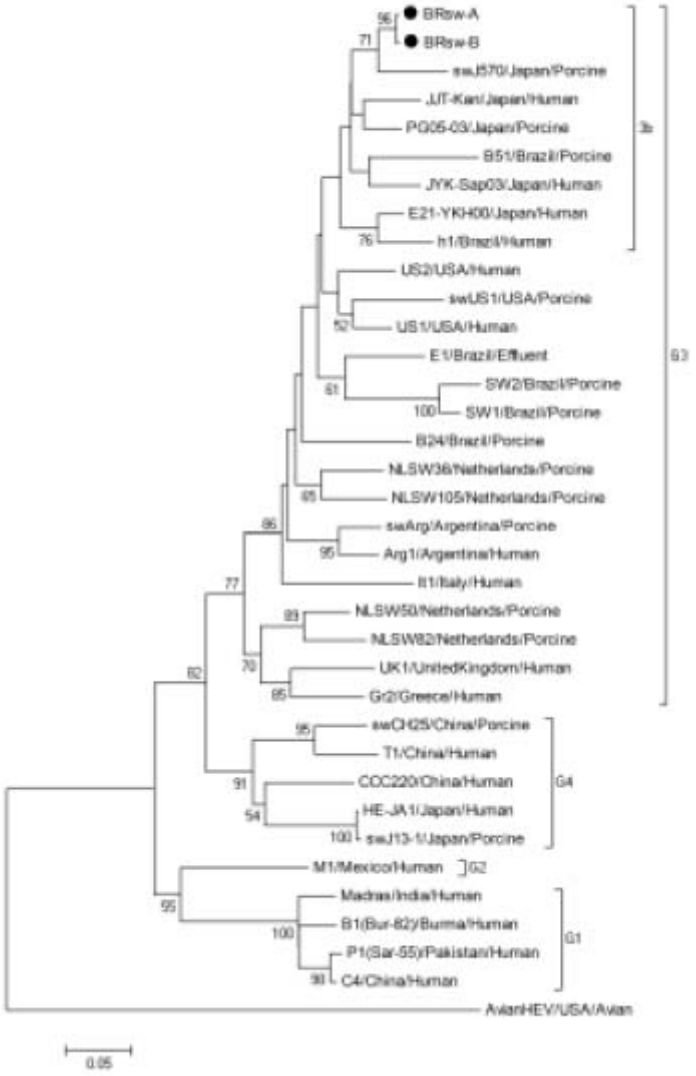
Figure 1 – Phylogenetic tree obtained by the neighbor-joining method based on the nucleotide sequences of a 228 bp region within HEV ORF1. Bootstrap values are indicated when >50% as a percentage was obtained from 1,000 replications. Avian HEV was included as outgroup. The four major genotype (G1-G4), including human and swine strains are indicated. The two sequences isolated in this study are marked with circles. HEV sequence names are labeled as follows: BLAST identification; country of origin, and host: human and porcine. The scale bar represents a genetic distance of 0.05 substitutions per site. GenBank accession number of the 37 sequences are: genotype 1: Madras (X99441), B1-Bur-82 (M73218), P1-Sar-55 (M80581), and C4 (D11093); genotype 2: M1(M74506); genotype 3: swUS1 (AF082843), US1 (AF060668), US2 (AF060669); swJ570 (AB073912), PG05-03 (AB362373), JJT-Kan (AB091394), h1 (GQ421465), E1 (HM154537), B24 (HM154540), B51 (HM154539), JYK-Sap03 (AB189931), E21-YK00 (AY684248), SW1 (EF591852, Brazil), SW2 (EF591853), NLSW105 (AF336013), NLSW36 (AF336004), G2 (AF110389), UK1 (AJ315768), NLSW50 (AF336006), NLSW82 (AF336009), It1 (AF110387), Arg1 (AF264009), and swArg (AY258006); genotype 4: SWJ-13 (AB097811), HE-JA1 (AB097812), T1 (AJ272108), swCH25 (AY594199), and CCC220 (AB108537); Avian HEV (AY535004)



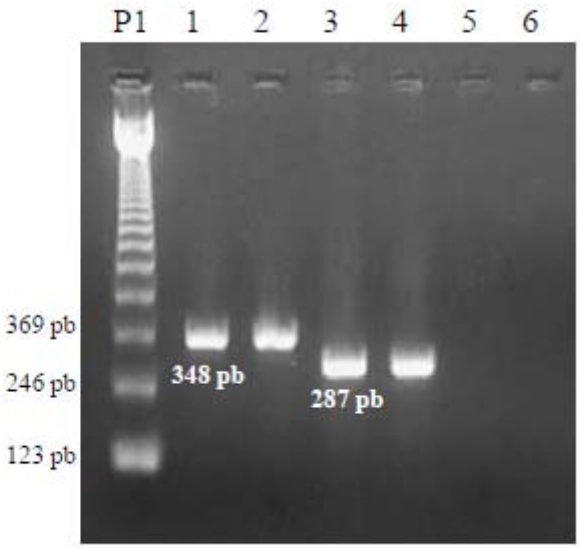
4 CONCLUSÕES

- O HEV suíno pode ser encontrado nas diversas categorias de produção, especialmente na fase de terminação, na maioria dos rebanhos suinícolas avaliados;
- A presença do genoma do HEV no fígado e na bile de suínos evidencia que o vírus pode ser detectado em animais de abatedouro e sugere que suínos assintomáticos podem ser fonte de exposição para consumidores de fígado de suíno, trabalhadores de abatedouros e de granjas de suinocultura;
- Os isolados de HEV suínos obtidos no presente estudo agruparam com estirpes de HEV classificadas como genótipo 3 e subtipo 3b;
- A análise molecular demonstrou que os isolados de HEV suínos obtidos no presente estudo apresentam alta similaridade com estirpes de HEV de origem suína e humana, especialmente as provenientes do Japão a estirpe humana brasileira denominada hl;
- Os resultados obtidos no presente estudo suscitam preocupações de saúde pública, uma vez que o HEV, atualmente considerado uma zoonose, pode ser detectado em excreções de suínos assintomáticos em diversas categorias de criação e em vísceras de animais no momento do abate.

APÊNDICE

APÊNDICE A

Gel de agarose a 2% corado com brometo de etideo submetido a eletroforese contendo os produtos da *nested* PCR que amplificam parcialmente a ORF2 (348 pb) e a ORF1(287) do HEV suino presente em amostras de fezes e de figado. Canaletas: P1 (padrao de tamanho molecular de 123 pb); 1 (amostra fecal positiva para a ORF2); 2 (amostra de figado positiva para a ORF2); 3 (amostra fecal positiva para a ORF1); 4 (amostra de figado positiva para a ORF1); 5 e 6 (controle negativo para as ORF2 e 1, respectivamente).



ANEXOS

ANEXO A

Lista de Reagentes

1. 100 mM dNTP Set, 4 x 250 uL; 25umol each (100 mM dATP Solution, 100 mM dCTP Solution, 100 mM dGTP Solution, 100 mM dTTP Solution) (Invitrogen Life Technologies®)
2. 10 x PCR-Buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl) (Invitrogen Life Technologies®)
3. 5x First-Strand Buffer (250 mM Tris-HCl, pH 8,3, 375mM KCl, 15 mM MgCh) (Invitrogen Life Technologies®)
4. 123 bp DNA Ladder (Invitrogen Life Technologies®)
5. Acetona, P.A. (CH3COCH3) P.M. 58,08 (Dinâmica®)
6. Ácido acetico glacial, P.A. (CH3COOH) P.M. 60,05 (Nuclear®)
7. Ácido borico (H3BO3) P.M. 61,83
8. Ácido cloridrico (HCl) P.M. 36,46 (Reagen®)
9. Ácido etilenodiaminotetraacido Sal di-sodico-EDTA, P.A.(C10H14N2O8Na22H2O) P.M. 372,24 (Reagen®)
10. Agarose (Gibco BRL®)
11. Água DEPC (Dietil pirocarbonato) (Invitrogen Life Technologies®)
12. Álcool etilico absoluto (C2H2OH) P.M. 46,07 (Nuclear®)
13. Álcool isoamilico (CH3)2CHCH2CH2OH) P.M. 88,15 (Synth®)
14. Azul de bromofenol (Sigma®)
15. Brometo de etídeo (C21H20N3Br) P.M. 394,3 (Sigma®)
16. Cloreto de Magnésio 50 mM ($MgCl_2$) (Invitrogen Life Technologies®)
17. Cloreto de Potássio, P.A. (KCl) P.M. 74,56 (Reagen®)
18. Cloreto de Sódio, P.A. (NaCl) P.M. 58,45 (Reagen®)
19. Clorofórmio, P.A. (CHC13) P.M. 119,38 (Dinámica®)
20. Dithiothreitol - 10 mM DTT (Invitrogen Life Technologies®)
21. Dimetilsulfóxido - DMSO (Invitrogen Life Technologies®)
22. Dodecil Sulfato de Sódio - Lauril Sulfato de Sódio - SDS (C12H25NaO4S) P.M. 288,38 (Synth®)
23. Fosfato de sódio dibásico anidro (Na2HPO4) P.M. 141,96 (Synth®)
24. Fosfato de sódio monobásico (NaH2PO4 . 2H2O) P.M. 155,99 (Reagen®)
25. Isotiocianato de guanidina P.M. 118,16 (Gibco BRL®)

26. Hidróxido de Sódio, P.A. (NaOH) P.M. 40,00 (Mallinckrodt Chemicals®)
27. Hidroximetil amino metano - Tris 99% P.M. 121,14 (Inlab®)
28. Metanol, P.A. (CH3OH) P.M. 32,04 (Allkimia®)
29. M-MLV Reverse Transcriptase - 200 units/tL (Invitrogen Life Technologies®)
30. Oligonucleotídeos iniciadores (primers) externos: 3516N/forward, 3517/reverse; internos: 3518/forward, 3519/reverse - 200 pmol direcionados para ORF2 (Invitrogen Life Technologies®)
31. Oligonucleotídeos iniciadores (primers) externos: ORF1-s1/ forward, ORF1-a1/reverse; internos: ORF1-s2/ forward, ORF1-a2/reverse - 200 pmol direcionados para ORF1 (Invitrogen Life Technologies®)
32. Platinum Taq DNA Polymerase recombinant 500 units (Invitrogen Life Technologies®)
33. Quant-iT™ dsDNA BR Assay Kit (Invitrogen Life Technologies®)
34. Sacarose, P.A. - sucrose (C12H22O11) p.m. 342,31 (Reagen®)
35. Silicon dioxide (SiO2) P.M. 60,08 (Sigma®)
36. Triton x-100

ANEXO B

Soluções e Tampões

- **Diluição dos primers destinados para ORF2**

- Primer 3516N/forward

Sequência: 5'- AATTATGCC(T)CAGTAC(T)CGG(A)GTTG -3'

Posição: 5687-5708

Concentração: 19,3 nmol

Data de fabricação: fevereiro, 2010

19,3 x 1000 = 19300 pmol

19300 / 200 = 96,5

Primer mãe (200 pmol/uL): ressuspender em 96,5 ul de água ultrapura autoclavada para obtenção de solução 10 x [ ]

Primer 1 x [ ] (20 pmol/uL): diluir 1:10 o primer mãe em água ultrapura autoclavada

- Primer 3517N/reverse

Sequência: 5' - CCCTTA(G)TCC(T)TGCTGA(C)GCATTCTC-3' Posição: 6395-6417 Concentração: 20,7 nmol Data de fabricação: fevereiro, 2010 20,7 x 1000 = 20700 pmol 20700 / 200 = 103,5

Primer mãe (200 pmol/ uL): ressuspender em 103,5 ul de água ultrapura autoclavada para obtenção de solução 10 x [ ]

Primer 1 x [ ] (20 pmol/ uL): diluir 1:10 o primer mãe em água ultrapura autoclavada

- Primer 3518N/forward

Sequência: 5' - GTT(A)ATGCTT(C)TGCATA(T)CATGGCT -3'

Posição: 5972-5993

Concentração: 27,9 nmol

Data de fabricação: fevereiro, 2010

29,7 x 1000 = 27900 pmol

27900 / 200 = 139,5

Primer mãe (200 pmol/uL): ressuspender em 139,5 ul de água ultrapura autoclavada para obtenção de solução 10 x [ ]

Primer 1 x [ ] (20 pmol/uL): diluir 1:10 o primer mãe em água ultrapura autoclavada

- Primer 3519N/reverse

Sequência: 5'- AGCCGACGAAATCAATTCTGTC -3'

Posição: 6298-6319

Concentração: 17,1 nmol

Data de fabricação: fevereiro, 2010

17,1 x 1000 = 17100 pmol

17100 / 200 = 85,5

Primer mãe (200 pmol/uL): ressuspender em 85,5 ul de água ultrapura autoclavada para obtenção de solução 10 x [ ]

Primer 1 x [ ] (20 pmol/uL): diluir 1:10 o primer mãe em água ultrapura autoclavada

- **Diluição dos primers destinados para ORF1**

- Primer ORF1-s1/forward

Sequência: 5' - CTGGCATYACTACTGCYATTGAGC-3'

Posição: 56-79

Concentração: 20,0 nmol

Data de fabricação: setembro, 2010

19.3x 1000 = 20000 pmol

20000 / 200 = 100

Primer mãe (200 pmol/uL): ressuspender em 100 ul de água ultrapura autoclavada para obtenção de solução 10 x [ ]

Primer 1 x [ ] (20 pmol/uL): diluir 1:10 o primer mãe em água ultrapura autoclavada

- Primer ORF1-a1/reverse

Sequência: 5' - CCATCRARRCAGTAAGTGCGGTC -3'

Posição: 451-473

Concentração: 18,4 nmol

Data de fabricação: setembro, 2010

18.4x 1000 = 18400 pmol

18400 / 200 = 92

Primer mãe (200 pmol/uL): ressuspender em 92 ul de água ultrapura autoclavada para obtenção de solução 10 x [ ]

Primer 1 x [ ] (20 pmol/uL): diluir 1:10 o primer mãe em água ultrapura autoclavada

- Primer ORF1-s2/forward

Sequência: 5' - CTGCCYTKGCGAATGCTGTGG -3'

Posição: 104-124

Concentração: 22,8 nmol

Data de fabricação: setembro, 2010

22,8 x 1000 = 22800 pmol

22800 / 200 = 114

Primer mãe (200 pmol/uL): ressuspender em 114 ul de água ultrapura autoclavada para obtenção de solução 10 x [ ]

Primer 1 x [ ] (20 pmol/uL): diluir 1:10 o primer mãe em água ultrapura autoclavada

- Primer ORF1-a2/reverse

Sequência: 5' - GGCAGWRTACCARCGCTGAACATC -3'

Posição: 367-389

Concentração: 18,2 nmol Data de fabricação: setembro, 2010 18,2 x 1000 = 18200 pmol 18200 / 200 = 91

Primer mãe (200 pmol/uL): ressuspender em 91 ul de água ultrapura autoclavada para obtenção de solução 10 x [ ]

Primer 1 x [ ] (20 pmol/uL): diluir 1:10 o primer mãe em água ultrapura autoclavada

- **Hidratação da sílica**

- 60 g de silica (SIGMA®)
- Adicionar 500 mL de água ultrapura autoclavada
- Agitar lentamente e manter em repouso durante 24 hs
- Por sucção, desprezar 430 mL do sobrenadante
- Ressuspender a sílica em 500 mL de água bidestilada
- Manter em repouso durante 5 hs para sedimentar
- Desprezar 440 mL do sobrenadante
- Adicionar 600 uL de HCl (32% w/v) para ajustar o pH (pH=2,0)
- Aliquotar e autoclavar

- **Fenol / clorofórmio - álcool isoamílico (25:24:1)**

- 25 mL fenol saturado em água
- 24 mL clorofórmio
- 1 mL álcool isoamílico

- **SDS 10%**

- 5 g dodecil sulfato de sódio - Lauril sulfato de sódio - SDS ($C_{12}H_{25}NaC_4S$)
- água bidestilada q.s.p 50 mL

- **Solução L6**

- 120 g de isotiocianato de guanidina (GUSCN)
- 100 mL de Tris-HCl 0,1 M pH 6,4
- 22 mL de EDTA 0,2 M pH 8,0
- 2,6 mL de Triton x 100
- Água bidestilada qsp. 1 L

- **Solução L2**

- 120 g de isotiocianato de guanidina (GUSCN)
- 100 mL de Tris-HCl 0,1 M pH 6,4
- Água bidestilada qsp. 1 L

- **Tampão de amostra para eletroforese em gel de agarose**

- azul de bromofenol 0,25%
- sacarose - sucrose ($C_{12}H_{22}C_{11}$) 45%
- Água bidestilada q.s.p. 100 mL

- **Tampão de corrida: TBE (Tris - Ácido bórico - EDTA) 10 x [ ]**

- 0,89 M Tris
- 0,89 M ácido bórico
- 0,02 M EDTA
- Agua bidestilada qsp. 1 L pH = 8,4

- **Tampão de estabilização para o rotavirus (TRIS/Ca^{++}) 10 x - pH 7,2**

- 12,12 g TRIS (0,89mM)
- 2,2 g cloreto de cálcio (1,5mM)
- Agua MilliQ autoclavada q.s.p. 1L

- **Tampão fosfato salina (PBS)**

- 137 mM cloreto de sódio (NaCl)
- 3 mM cloreto de potássio (KCl)
- 8 mM fosfato de sódio dibásico anidro (Na_2HPO_4)
- 15 mM potássio fosfato monobásico ($KH_2\ PO_4$)
- água ultrapura autoclavada q.s.p. 500 mL
-

ANEXO C

Protocolo de Técnicas

• Extração do ácido nucleico pela associação das técnicas fenol /clorofórmio/álcool isoamílico e sílica/isotiocianato de guanidina

A1) Preparo da suspensão fecal (10 a 20%)
- 100 uL ou 100 mg de fezes
- 500 uL de TERV
- Vortexar
- Centrifugar a 500 x g / 5 min
- Utilizar 400 uL do sobrenadante para a extração

A2) Preparo da suspensão de órgãos (fígado)
- 1,5 g de fígado
- Triturar
- 15 mL PBS
- Vortexar
- Centrifugar a 3000 x g / 10 min
- Utilizar 400 uL do sobrenadante para a extração

A3) Diluição da bile
- diluir em 10% (w/v) de água DEPC

B) Extração

Fase 1-Fenol
- Adicionar 50 uL de SDS 10%
- Homogeneizar em vórtex
- Banho-maria 56 °C /20 min
- Spin (Centrifugar 10.000 x g 30/s)
- Adicionar 400 uL de fenol ácido clorofórmio álcool isoamílico
- Incubar em banho-maria a 56°C/15 min
- Homogeneizar manualmente por 15 s

- Centrifugar 10.000 x g /10 min
- Recolher sobrenadante em outro tubo

-Fase 2- Sílica/isotiocianato de guanidina
- Adicionar 500 uL de solução L6
- Adicionar 25 uL de sílica hidratada
- Homogeneizar em vórtex
- Agitar durante 30 min em temperatura ambiente
- Spin
- Desprezar o sobrenadante em solução contendo NaOH 10 M
- Adicionar 500 uL de solução L2
- Homogeneizar em vórtex
- Spin
- Desprezar o sobrenadante em solução contendo NaOH 10M
- Adicionar 500 uL de solução L2
- Homogeneizar em vórtex
- Spin
- Desprezar o sobrenadante em solução contendo NaOH 10M
- Adicionar 1.000 uL de etanol 70% gelado
- Homogeneizar em vórtex
- Spin
- Desprezar o sobrenadante
- Adicionar 1.000 uL de etanol 70% gelado
- Homogeneizar em vórtex
- Spin
- Desprezar o sobrenadante
- Adicionar 1.000 uL de acetona PA gelada
- Homogeneizar em vórtex
- Spin
- Desprezar o sobrenadante
- Secar o pellet em banho-maria a 56 °C /15 min
- Adicionar 100ul de água DEPC
- Homogeneizar em vórtex
- Descansar em banho-maria a 56 °C /15 min

- Homogeneizar em vórtex
- Centrifugar a 10.000 x g/4 min
- Recolher o sobrenadante
- Estocar a -20°C

• Transcrição reversa (RT) seguida da reação em cadeia pela polimerase (PCR) para amplificação parcial do gene que codifica a ORF2 do HEV suíno

A) Desnaturação
- Em banho de gelo, adicionar 5 uL do RNA a 5 uL de primer mix (1 uL de 3157/reverse e 4uL de água ultrapura autoclavada) em um tubo para microcentrífuga
- Homogeneizar em vórtex
- Spin
- Colocar os tubos em termociclador a temperatura 97°C /5 min
- Colocar os tubos em banho de gelo por 5 min

B) RT
- Em banho de gelo, adicionar 10 uL de mix RT (4 uL de 5x First-Strand Buffer, 2 uL de DTT, 1 uL de dNTP, 2,5 uL de agua ultrapura autoclavada e 0,5 uL M-MLV) em 10 uL do desnaturado
- Homogeneizar em vórtex
- Spin
- Colocar os tubos em termociclador a temperatura 42 °C /30 min e 94 °C /5 min

C) PCR
- Em banho de gelo, adicionar 8 uL do cDNA a 42 uL de mix PCR (26 uL de água ultrapura autoclavada, 7,5 uL de 10 x PCR-Buffer, 4 uL de dNTP, 2 uL de MgCh, 1 uL de cada primer 3516/forward e 3517/reverse e 0,5 uL de Platinum Taq DNA Polymerase)
- Homogeneizar em vórtex
- Spin
- Colocar os tubos em termociclador a temperatura 94 °C /2 min, seguidos de 40 ciclos de 1 min a 94°C, 1 min a 42°C e 1 min a 72 °C. Para extensão final, são necessários 7 min a 72°C

- Estocar o produto a -20°C C) *Nested*
- Em banho de gelo, adicionar 3 uL do DNA a 47 uL de mix *Nested* (33,5 uL de água ultrapura autoclavada, 5 uL de 10 x PCR-Buffer, 4 uL de dNTP, 2 uL de $MgCl_2$, 1 uL de cada primer 3518/forward e 3519/reverse e 0,5 uL de Platinum Taq DNA Polymerase)
- Homogeneizar em vórtex
- Spin
- Colocar os tubos em termociclador a temperatura 94 °C /2 min, seguidos de 30 ciclos de 1 min a 94°C, 1 min a 42°C e 1 min a 72 °C. Para extensão final, são necessários 7
min a 72°C
- Estocar o produto a -20°C

- **Transcrição reversa (RT) seguida da reação em cadeia da polimerase (PCR) para amplificação parcial do gene que codifica a ORF2 do HEV suíno**

A) Desnaturação
- Em banho de gelo, adicionar 5 uL do RNA a 5 uL de primer mix (1 uL de ORF1-a1/reverse e 4 uL de água ultrapura autoclavada) em um tubo para microcentrífuga
- Homogeneizar em vórtex
- Spin
- Colocar os tubos em termociclador a temperatura 97°C /5 min
- Colocar os tubos em banho de gelo por 5 min

B) RT
- Em banho de gelo, adicionar 10 uL de mix RT (4 uL de 5x First-Strand Buffer, 2 uL de DTT, 1 uL de dNTP, 2,5 uL de agua ultrapura autoclavada e 0,5 uL M-MLV) em 10 uL do desnaturado
- Homogeneizar em vórtex
- Spin
- Colocar os tubos em termociclador a temperatura 42 °C /30 min e 94 °C /5 min

C) PCR
- Em banho de gelo, adicionar 8 uL do cDNA a 42 uL de mix PCR (26 uL de água ultrapura autoclavada, 7,5 uL de 10 x PCR-Buffer, 4 uL de dNTP, 2 uL de MgCb, 1

uL de cada primer ORF1-s1/forward e ORF1-a1/reverse e 0,5 uL de Platinum Taq DNA Polymerase)
- Homogeneizar em vórtex
- Spin
- Colocar os tubos em termociclador a temperatura 94 °C /2 min, seguidos de 40 ciclos de 1 min a 94°C, 1 min a 50°C e 1 min a 72 °C. Para extensão final, são necessários 7
min a 72°C
- Estocar o produto a -20°C

C) *Nested*
- Em banho de gelo, adicionar 3 uL do DNA a 47 uL de mix *Nested* (33,5 uL de água ultrapura autoclavada, 5 uL de 10 x PCR-Buffer, 4 uL de dNTP, 2 uL de $MgCl_2$, 1 uL de cada primer ORF1-s2/forward e ORF1-a2/reverse e 0,5 uL de Platinum Taq DNA Polymerase)
- Homogeneizar em vórtex
- Spin
- Colocar os tubos em termociclador a temperatura 94 °C /2 min, seguidos de 30 ciclos de 1 min a 94°C, 1 min a 50°C e 1 min a 72 °C. Para extensão final, são necessários 7
min a 72°C
- Estocar o produto a -20°C

- **Gel de agarose a 2%**

- 1 g de agarose
- 50 mL TEB 1 x
- 30 uL brometo de etídio

- **Purificação (GFX PCR DNA and Gel Band Purification Kit, GE Healhtcare®)**

- Pesar o fragmento excisado do gel em microtubo de 1,5 mL.
- Adicionar 10 uL do *Capture buffer type* 2 para cada 10 mg de gel.
- Incubar o tubo a 60°C / 15 min, homogeneizando a cada 3 min.
- Homogeneizar a cada três min.
- Centrifugar a 16.000 x g/30s

- Transferir 600 uL da amostra com o *Capture buffer type* 2 em um tubo coletor com coluna
- Incubar em temperatura ambiente por 1 min
- Centrifugar a 16.000 x g/30s
- Descartar o filtrado e recolocar a coluna no mesmo tubo.
- Adicionar 500 uL do *Wash buffer type 1* na coluna com tubo coletor
- Centrifugar a 16.000 x g / 30s
- Descartar o filtrado e transferir a coluna para um microtubo de 1,5 mL.
- Adicionar 30 uL do *Elution buffer type 6*
- Incubar a temperatura ambiente por 1 min.
- Centrifugar a 16.000 x g / 1 min.
- Estocar o DNA purificado em -20°C.

- **Quantificação (Quant-iT™ dsDNA BR Assay Kit, Invitrogen Life Technologies®)**

- Certifique-se de que todos os reagentes estejam em temperatura ambiente
- Solução de trabalho: adicionar Quant-iT Reagent (1x n uL) ao tampão Quanti-iT (199x n uL), onde n e o número de amostras mais o número de padrões
- Prepare os padrões em dois tubos diferentes, adicionando 190 uL da solução de trabalho e 10 uL de cada padrão (0 e 100) em cada tubo (volume final de 200 uL)
- Prepare as amostras, adicionando de 180-199 uL de reagente de trabalho e 1-20 uL do purificado (volume final de 200 uL)
- Homogeneizar em vórtex
- Incube por 2 min a temperatura ambiente
- Fazer a leitura no fluorômetro Qubit™

ANEXO D

Lista de *Software*

- Eletropherogram quality analysis - Phred e CAP3 (http://asparagin.cenargen.embrapa.br/phph/)
- BLAST The Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi)
- MEGA package software version 4.1 (http://www.megasoftware.net/mega4/mega41.html)
- B i oEdit software version 7.0.9.0 (http://www.mbio.ncsu.edu/bioedit.html)