SEROLOGICAL AND MOLECULAR EPIDEMIOLOGY OF HEPATITIS E VIRUS IN PIGS, PLATEAU STATE, NIGERIA

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DEPARTMENT OF VETERINARY PUBLIC HEALTH AND PREVENTIVE MEDICNE, AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA

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DECLARATION

I declare that the work in this thesis titled **"Serological and Molecular Epidemiology of Hepatitis E Virus in Pigs, Plateau State"** has been performed by me in the Department of Veterinary Public Health and Preventive Medicine, Ahmadu Bello University, Zaria, Nigeria under the supervision of Professors J.K.P. Kwaga, J.U. Umoh and Dr. D. Shamaki. The information derived from the literature has been duly acknowledged in the list of references provided. No part of this thesis was previously presented at this or any other institution.

Olajide Adewale OWOLODUN ....
Name of student Signature Date

CERTIFICATION

This thesisentitled, **“Serological and Molecular Epidemiology of Hepatitis E Virus in Pigs, Plateau State”**by Olajide Adewale OWOLODUN, meets the regulations governing the award of the Degree of Doctor of Philosophy in Veterinary Public Health and Preventive Medicine of the Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

To **Bolaji Owolodun**, for what and who you were to the family. We lost you before the completion of this research work

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ABSTRACT

Globally, there are increasing concerns for the public health implications of Hepatitis E virus (HEV) infection. The disease which was thought to be endemic only in African and Asian countries is now being established to be endemic in developed nations of Europe, America, Australia and Oceania. Animal reservoirs are incriminated in the maintenance and spread of HEV; pigs being the major reservoir.The occurrence of HEV, dynamics of viral shedding and the circulating genotype(s) were examined in this research study. Genomic sequences of Open Reading Frame 2 (ORF2) were analyzed for unique characteristics. Serum and fresh faecal samples from domestic pigs in 10 Local Government Areas (LGAs) were analyzed for the presence of anti-HEV antibodies and viral RNA. In total 500 serum samples were collected from slaughter age pigs at three separate slaughter houses in Plateau state. Anti-HEV antibodies (IgM and IgG) were assessed. Fifty faecal samples each were collected from the following LGAs: Bassa, Barkin-Ladi, Bokkos, Jos-North, Jos-South, Mangu, Shendam, Pankshin, Langtang-North and Langtang-South. In Jos abattoir, 89.1% of the pigs were anti-HEV IgM positive while only 45.3% were IgG seropositive. When stratified by age, pigs <9 months of age were more likely to be IgM seropositive ($P>0.05$) and but less likely to be IgG positive ($P>0.05$). By sex, female pigs were less likely to be IgM positive ($P>0.05$) and there was no association between sex and detection of IgG ($P>0.05$). By breed, indigenous breeds were less likely to be IgM positive and also less likely to be IgG positive ($P>0.05$). In Mangu slaughter area, 93.9% of pigs were IgM seropositive while only 51.5% were IgG seropositive. Pigs <9 months old were more likely to be IgM positive ($P>0.05$) but less likely to be IgG positive. Based on sex, female pigs were more likely to be IgM positive ($P>0.05$) but less likely to be IgG positive.In Langtang area, 91% of the pigs tested were positive for IgM and 55% positive for IgG. However, pigs <9 months were less

likely to be both IgM and IgG positive (P>0.05). Based on sex, female pigs were more likely to be IgM and IgG positive (P>0.05). A comparison between breeds could not be performed for IgM, but indigenous breeds were found to be more likely to be IgG positive.HEV RNA was detected in pigs from all LGAs sampled by qPCR, the highest being Langtang-South with 62.8% and Jos-South the least with 2.4%. Quantitatively, Ct ranged between 23.35 and 44.65. In all, 114 faecal samples were HEV RNA positive (25.7%); sequel to nested PCR, only 22 samples displayed clear discreet bands of expected size. Of the 22 samples, 19 partial sequences of the ORF2 were returned; three could not be sequenced. Blast homology searches confirmed the 19 sequences to be HEV and were 92% homologous to a Japanese strain swJ8-5 and 91% with Hungarian strain HEV072/sw/HUN-05. Intra-country HEV sequence diversity was observed among isolates recovered from this study, but all viral isolates were confirmed to be genotype 3 clustering closely to genotype 3 reference strains from the GenBank. Further characterization grouped all sequences from this study to belong to subgenotype 3e together with French, Hungarian, Japanese and UK strains. Amino acid sequence analysis revealed only a few conserved residues and 2 conserved motifs; functional implications of the amino acid residues are unknown as this was not assayed for. The evolutionary divergence of the sequences from Plateau state was estimated and discovered that maximum distance among the strains from Plateau state was 0.8, being the distance between strains from Langtang-South and Bokkos LGAs. The study concludes that HEV is widely spread in Plateau state and the strains identified are the zoonotic genotype 3 strains. It is also concluded that the HEV strains circulating in Plateau state may have been imported from Europe or Asia.

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ABBREVIATIONS

aHEV	Avian Hepatitis E virus
Bp	Basepair
cDNA	Complimentary DNA
CMV	Cytomegalovirus
CTV	Cutthroat Virus
DcHEV	Dromedary Camel Hepatitis E virus
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
ELISA	Enzyme Linked Immunosorbent assay
FHF	Fulminant hepatic failure
FMIA	Fluorescent microbead-immune assay
FRhK	Female Rhesus Kidney
HAV	Hepatitis A virus
HEV	Hepatitis E virus
HIV	Human immunodeficiency virus
ICTV	International Committee on the Taxonomy of Viruses
IEM	Immune electron microscopy
IFE	Immune fluorescence microscopy
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IPNV	Infectious pancreatic necrotic virus

NS	Non-structural
NTP	Nucleotise Triphosphate
ORF	Open Reading Frame
PHA	Phytohaemagglutinin
RdRp	RNA-Dependent-RNA-Polymerase
RT-PCR	Reverse-Transcriptase Polymerase Chain Reaction
RNA	Ribonucleic acid
RWV	Rotating Wall Vessel
sHEV	Swine Hepatitis E virus
SOT	Solid Organ Transplant
UTR	Untranslated Region
VLP	Virus-like particles

CHAPTER ONE

1.0 INTRODUCTION

1.1Hepatitis E Virus Background Information

Viral hepatitis is a global health problem especially hepatitis E virus (HEV) the causative agent of hepatitis E (Kamar *et al.*, 2012). The condition was first recognized in India in 1978 (Khuroo, 1980). It has been identified in many other countries and continents and its epidemiology has been described (Mirazo *et al.*, 2014). The disease is hyperendemic in many developing countries of the world like India, Bangladesh, China, Sudan and many other African countries where large waterborne outbreaks are known to have occurred (Kim *et al*., 2014). In industrialized countries, hepatitis E is considered an emerging disease of immense global importance and has been reported in many developed countries (Navaneethan *et al.*, 2008). Similarly, in developing countries it is also still an emerging disease that requires greater attention (Stoszek *et al.*, 2006a). HEV infection develops into a chronic infection in immunocompromised post-transplant patients. Apart from this occurrence, the infection also displays an interesting and important course in pregnant women in certain poorer regions of the world (Navaneethan *et al*., 2008).

Autochthonous HEV infections are equally emerging concerns in some countries. In Africa and other third world countries, HEV is considered endemic in both human and swine populations (Smith *et al*., 2013). However, it has not been described in many developing countries due to research neglect. HEV has been found to be endemic in Japan, Australia, the United States and Europe (Colson *et al.*, 2010). Seroprevalence rate as high as 36% has been reported in healthy populations recently, while an increasing number of autochthonous cases have also been documented (Dalton *et al.*, 2008).

Until now, the general HEV transmission routes are not fully resolved globally, but it is agreed that the faeco-oral route is the most common route of transmission. In human settlements, the virus has been identified in human sewage and mollusks without evidence of transmission to humans. On the other hand, a huge body of evidence indicates that HEV infection is a porcine related zoonosis, being that pigs and wild boars are freely and commonly infected (Casas *et al.*, 2009, Rutjes *et al.*, 2010). Furthermore, occupational exposure to pigs and hunting activities are events that have been linked to high HEV seroprevalence rates and clinical disease (Adjei *et al.*, 2009;Meng *et al.*, 2002).

The very high anti-HEV antibodies in a significant proportion of healthy individuals in the United States (Meng *et al*., 2002) suggested that animal reservoirs of HEV may be involved in its transmission and maintenance in the environment. Therefore, the existence of animal reservoir was proposed.

In Africa, the occurrence of swine HEV is not popularly reported. This scenario may not due to absence of the virus in African pigs but due to poor surveillance activities (Owolodun *et al.*, 2014). In the same vein, animal HEV is poorly reported in Africa. There are few reports of genetic detection and characterization of HEV in animals (Kaba *et al.*, 2010;Owolodun *et al*., 2014;S. de Paula *et al.*, 2013;Saad *et al.*, 2007). From these reports, it is clear that every set of faecal samples analyzed yielded at least one positive sample. The samples analyzed from Nigeria, Cameroon and Democratic Republic of Congo were from pigs but the animal samples from Egypt were from horses. These results indicate that HEV may be widespread in many other animal species in Africa. The presence of HEV in indigenous pigs in Africa may lead to major public health challenges especially in the resource limited rural communities where portable drinking water and toilet facilities are lacking.

Reports of human HEV infections in Africa abound (Adesina *et al.*, 2009;Bonney *et al.*, 2012;Delarocque-Astagneau *et al.*, 2012) and most of the large outbreaks are water borne infections due to faecal contamination. It is known that pigs are natural reservoirs of zoonotic HEV and that the farming system for small-holder practice in rural parts of the developing world is free-roaming. This encourages environmental contamination with faeces and other enteric viruses but the real contribution of the environment to HEV infections in humans and other susceptible animals is not clear. For instance, early outbreaks in India were traced to water contamination and this affected thousands of individuals. Similarly, an outbreak in Dafur, Sudan (Guthmann *et al.*, 2006) was suspected to have been caused by water contamination. This outbreak occurred in an internally displaced peoples' camp as a result of the conflict in western Sudan. Within 6 months of investigations, 2,621 hepatitis E cases were recorded with an attack rate of 3.3% and case-fatality rate of 1.7%. The major risk factors identified in this outbreak were age of patients (15-45 years) and drinking of surface water. In another outbreak in humans in displaced peoples' camp, in Chad, where heterogeneous strains of HEV were identified, water was also indirectly incriminated (Nicand *et al.*, 2005). Though HEV RNA has never been identified from water sources in Africa, risk factor analyses from studies incriminate water sources especially in camps which are characterized by over-population, absence of sewer systems, presence of human excreta and also excreta of domestic animals close to human habitation and sources of water (Caron and Kazanji, 2008;Nicand *et al*., 2005).

Even though pigs and other animals, both domestic and wild are considered reservoirs of zoonotic HEV, zoonotic strains of HEV (genotypes 3 and 4) are yet to be identified in humans in Africa. Across the continent, only anthropophilic strains have been identified so far (Blackard *et al.*, 2009;Buisson *et al.*, 2000;He *et al.*, 2000;van Cuyck *et al.*, 2003). This epidemiological picture does not preclude occurrence of zoonotic HEV in humans in Africa but calls for wider surveillance program in rural, peri-urban and urban human settlements. In the Indian sub-continent and continental Asia, zoonotic HEV is common in humans, especially those with a history of consumption of raw or undercooked pig or wild boar liver and other pork products (Choi *et al.*, 2003;Vivek and Kang, 2011). Also in Asia, HEV RNA has been identified in flowing water bodies from where drinking water and for other domestic uses is collected (Li *et al.*, 2014).

The Nigerian situation is not different from India or some of the other Asian countries mentioned above. Rural dwellers obtain drinking water from flowing streams which may contain traces of human HEV strains or maybe contaminated with faecal material from other humans. These living conditions and the associated system of animal husbandry predispose humans to risk factors for HEV infection. Therefore, it is very important to develop an algorithm for detection of HEV in domestic animals commonly found in human settlements and also in the human subjects.

World over, swine HEV has been established to be widespread. Though at the moment, the same cannot be concluded about Nigerian pigs, studies are required to identify HEV in Nigerian pigs and characterize the circulating strains. It is not known whether inter-species transmission occurs in Nigeria between pigs and humans as have been reported elsewhere. The gap in knowledge which exists on swine HEV first of all needs to be filled. The study embarked upon will serve to provide knowledge on existence of the virus in pigs in Plateau state, circulating genotypes and subtypes and dynamics of transmission.

1.2Statement of Research Problem

Hepatitis E virus (HEV) is a widespread faeco-orally transmitted agent. It is responsible for at least 50% of hepatitis cases in many developing countries and is considered to be endemic in many regions of Africa, Asia and Latin America (Buisson *et al*., 2000). Hepatitis E manifests both as epidemic and sporadic hepatitis in endemic areas. Case fatality rates during epidemics were found to be between 0.2 % and 4% (Kumar *et al.*, 2013). The clinical outcomes associated with infections of HEV are diverse. It manifests as a self-limiting acute icteric hepatitis and this is indistinguishable from acute hepatitis resulting from other hepatotrophic viruses. The infection may be severe in a fraction of patients and thus present as fulminant hepatitis (acute liver failure) (Kumar *et al*., 2013). HEV infection may become chronic especially in immune-compromised patients who have received solid organ transplants (Gerolami *et al.*, 2008).In industrialized nations, infection rates with Hepatitis E are now on the rise but still with negligible morbidity (Purcell and Emerson, 2008). Hepatitis E causes high maternal mortality during third trimester of pregnancy and case-fatality rates are in the range of 20-30%(Navaneethan *et al*., 2008;Vasickova *et al.*, 2007). Poor individual and community sanitation standards due to poor environmental hygiene have been largely implicated in the high rates of HEV infections in developing countries. Probably due to this fact, Africa, Asia and parts of Latin America are known to be endemic for HEV. The spread and maintenance of HEV in these regions is now being complicated by the occurrence/carriage of the virus in domestic and wild pig populations. In Asia especially, wild boar meat is routinely consumed with resultant spread of swine HEV (sHEV) into humans(Masuda *et al.*, 2005), thereby highlighting the zoonotic potential of the virus. Other evidences of possible zoonotic transmission of sHEV have been documented to be associated with consumption of raw or under cooked HEV infected meat products and or consumption of faecally contaminated water(Li *et al.*, 2005b;Meng, 2011).

The major animal reservoir for HEV is the pig. Workers have reported that anti-HEV antibodies are acquired naturally in primates and swine (Purcell, 1996), suggesting prior exposure to HEV or a related pathogen. Natural swine HEV infection usually presents as an asymptomatic disease. It has been established that gross pathological lesions are often absent in naturally infected pigs (Meng *et al.*, 1997). However, microscopic evidence of hepatitis often characterized by moderate multifocal and periportal lymphoplasmacytic hepatitis with mild focal hepatocellular necrosis are observed (Meng *et al.*, 1997). In some cases, mild to moderate enlargement of hepatic and mesenteric lymph nodes are observed 7 to 55 days post-infection(Halbur *et al.*, 2001). In chickens, the morbidity and mortality of hepatomegaly-splenomegaly (HS) syndrome associated with avian HEV infections in field conditions are comparatively low, although subclinical infections of avian HEV are very common (Huang *et al.*, 2002;Sun *et al.*, 2004). HS syndrome is characterized by above normal mortality in broiler breeder hens and laying hens of 30-72 weeks of age, with peak incidence between 40-50 weeks of age. Preceding death, clinical signs are not observable in chickens with HS. However, in some cases, a monumental drop in egg production of up to about 20% may occur. Dead chickens associated with avian HEV infection often have regressive ovaries, sero-sanguinous fluid in abdominal cavity and enlarged liver and spleen (Payne *et al*., 1999).

Hepatitis E is now considered a zoonotic disease. Pig farmers, swine veterinarians and other pig handlers in both developing and industrialized countries have been shown to be at risk of HEV infection . About 25% of swine veterinarians and 18% normal blood donors are HEV sero-positive in the US (Meng *et al*., 2002). The zoonotic potential of sHEV is further seen in a study in Hungary, where a high number of infected domestic and wild pigs were detected. In that study a single HEV genotype was found in both animals and man and human strains of the virus were closely related to domestic pig strains and identical sequences were found in both human and deer(Reuter *et al.*, 2009).

The evolutionary genetic relationship among human and swine HEVs points back to a common ancestor and dates back to between 536 and 1,344 years ago (Purdy and Khudyakov, 2010). The viruses may have evolved into anthropotrophic and enzootic viruses which gave rise to genotypes 1 & 2 and genotypes 3 & 4 respectively (Purdy and Khudyakov, 2010). In recent time, genetic recombination events among HEV strains have been reported (Wang *et al.*, 2010); and could be as a result of co-infection or super-infection of the mammalian host. Dual infection of a single host by HEV of two different genotypes and two different subtypes has been documented in humans and super-infection has been documented in pigs (Moal *et al.*, 2012).

In Nigeria, human cases of HEV have not been widely reported. In most circumstances, only individuals with symptoms of hepatitis get to be investigated thereby leading to a dearth of information about HEV in Nigeria. In a recent study, 13.4% seroprevalence rate was detected in Ekiti state(Adesina *et al*., 2009). Swine HEV RNA is yet to be reported in Nigeria, and there are no reports of on-going surveillance study programs in the country. The non-reporting of sHEV in Nigeria calls for immediate commencement of surveillance activities especially in high pig producing and consumption areasspanning from the north central to the southern parts of Nigeria.

In many pig producing communities in Nigeria, the semi-intensive husbandry system is mostly practiced(Ajala *et al.*, 2007). Here, pigs are allowed to scavenge for food on kitchen wastes, refuse dumps and are occasionally given supplements. This free roaming nature allows close contact between domestic pigs and humans, increases contact between humans and pig excreta and promotes faecal contamination of water through free wallowing during the warmer months of the year. Equally, pigs can also get infected with human HEV strains if they ingest human faeces or feeds contaminated with human faeces. In rural areas, there is a huge problem of shortage of potable water; therefore village dwellers rely on free flowing streams and other stagnant water bodies as sources of water. This association with pigs and their waste products suggest a possible route of cross-species infection of HEV. Cross-species infection of HEV has been documented; swine HEV in non-human primates, human HEV in pigs(Meng *et al.*, 1998)and avian HEV in turkeys(Sun *et al*., 2004).

1.3 Justification of the Research

HEV infection is a "newly" identified threat to world public health and the African continent may be placed among the most severely affected regions in the world. A thorough understanding of HEV epidemiology in Africa and its countries will provide the needed leverage for prevention and control and also enhance the implementation of evidence-based control policies geared towards abating the spread of HEV. The option of introduction of vaccines for prevention also rests on availability of field and laboratory data (geographic spread and circulating strains).

Although the main sources of animal protein in urban areas in Nigeria are beef, poultry, fish, milk and small ruminants (Ajala *et al*., 2007). Pork has always been a major meat and protein source for the rural poor. In many urban areas, pork consumption is becoming more popular, because of the ever increasing cost of beef and other meat types. Nowadays, pigs are not only sources of protein, but also form a major part of investment plans and alternative sources of family income (Ajala *et al*., 2007).

In Nigeria and several other African countries, HEV is increasingly being diagnosed in human patients (Kim *et al.*, 2014). A prominent draw back from these reports is the total absence of the source of these infections and lack of consideration of the possible animal reservoirs of the virus in Nigeria. Reports from some countries which have carried out studies on HEV attribute domestic pigs and other wild animals as possible reservoirs. The detection of sHEV in a farm in Kinshasa, Congo DR (Kaba *et al*., 2010) calls for increased surveillance and genetic typing of sHEV in Africa. HEV infection in pigs is ubiquitous globally. In some pig herds in USA, about 60-100% are infected (Meng *et al*., 1997). In an isolated report, HEV has been detected and genetically typed in work horses in old Cairo, Egypt (Saad *et al*., 2007), and this infection was not traceable to pigs. The Egyptian viruses clustered together distinct from other Genotype 1 virus sequences. The Congolese HEV belonged to Genotype 3. In neighbouring Cameroon, HEV genotype 3 was detected in domestic pigs (S. de Paula *et al*., 2013). Cameroon shares similar husbandry practices with many pig rearing areas in Nigeria in which pigs are locally raised, close association with humans exist and extensive cross-breeding takes place.

The public health importance of HEV is under-represented in Africa, unlike in the US, Europe, Asia and Australasia. In Africa today, Hepatitis E morbidity and mortality in pregnant women have increased the awareness not only for HEV infection but also for other hepatitis causing viruses like Hepatitis A, B, C and D viruses.

The need to carry out a survey and characterize hepatitis-causing viruses in domestic pigs cannot be overemphasized (due to its zoonotic nature). This is because HEV has been detected in pork and other commercially sold pig livers in the United States (Feagins *et al*., 2007) and Japan (Yazaki *et al*., 2003); of which belong to genotype 3 (zoonotic strain). More so, HEV has been detected in most major pig producing areas in the world even in intensively raised pigs where confinement is 100% and disease burden is relatively low. The detection of HEV strains in humans is a strong indication of the possibility of circulation of HEV in both human and animal populations.

The emergence of mixed HEV genotypes in Africa from both humans and animals underlies the need to carry out a survey on the main reservoir of the virus in Nigeria and establish the dominant genotype(s). The significantly close association between domestic pigs and humans and the peculiar feeding pattern (feeding on human waste) adopted in some parts of Nigeria where evidence of HEV has been identified also necessitates a survey on HEV. The changing or emerging ecology of zoonotic viruses worldwide has been partly traced to changing dynamics in human-domestic and wild animal interactions (Woolhouse and Gowtage-Sequiria, 2005). Therefore, this study is aimed at partly elucidating the epidemiology of HEV in Nigeria and filling the knowledge gap which exists on this neglected pathogen and disease.

1.4 Aim

The main objective of this project is to study the epidemiology of Hepatitis E virus in pigs of various ages in Plateau state, Nigeria.

1.5 Specific Objectives

The objectives of this study are to:

i. Determine the HEV serological status of slaughter pigs in Plateau state.

ii. Establish HEV shedding in faeces of pigs by Real-Time and nested RT-PCR.

iii. Determine the genotypes and subtypes of field strains of swine HEV in Plateau state.

iv. Analyze both nucleotide and amino acid sequences of circulating HEV strains in Plateau state.

1.6 Research Questions

i. Is Hepatitis E virus present in serum and faeces of domestic pigs in Plateau state?

ii. What are the circulating genotype(s) of HEV in swine in Plateau state and are they different from previously identified HEV in humans?

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Hepatitis E Virus

Evidence for a previously unrecognized hepatitis virus was provided in 1980 (Wong *et al.*, 1980) and this arose in part, from retrospective testing of archived serum samples from epidemics of water-borne hepatitis which occurred in Delhi, India from 1955-1956 (Ramalingaswami and Purcell, 1988). In the same vein, Khuroo (1980), supplied evidence that a previously unknown hepatitis agent was the etiologic agent of epidemic hepatitis in Kashmir valley, India in 1978. Both reports were anchored on elimination of hepatitis A virus (HAV) and were made possible by use of sensitive and specific sero-assays for HAV, which was the only known water-borne hepatitis virus at that time. Subsequently, virus-like-particles (VLP) were successfully identified by immune electron microscopy when Balayan transmitted the disease to himself (Balayan *et al.*, 1983).

Until recently, HEV taxonomic status remained ambiguous (Emerson *et al.*, 2005a). Initially, the virus was thought to belong to the family *Picornaviridae*(Balayan *et al*., 1983), but later studies provided evidence that it did not belong to this group. It was then subsequently classified into the family *Caliciviridae*between 1988 and 1998; this was based on virion morphology and chemico-physical properties. Thereafter, it was re-classified as an independent HEV-*like-virus genus*, unassigned to any family. The genomic structure of HEV was found to be significantly different from caliciviruses and any other virus; it is now currently classified under a new family *Hepeviridae*, genus *Hepevirus*(Emerson *et al*., 2005a).

HEV is a small icosahedral shaped non-enveloped virus approximately 27-34nm in diameter (Balayan *et al*., 1983;Emerson *et al*., 2005a). It has a positive sense single-stranded RNA genome about 7.2kb in length containing a short 5´ untranslated region (UTR), three discontinuous partially overlapped open reading frames (ORF) and a short 3´ UTR terminated by a poly (A) tail (Tam *et al.*, 1991). ORF1 extends about 5kb from the 5´ end of the genome and forms bulk of the variable region of the genome, it encodes non-structural polyproteins (methyltransferase, papain-like cysteine protease, RNA helicase and RNA-dependent RNA polymerase) (Koonin *et al.*, 1992). ORF2 is located at the 3´ end of the viral genome. It is about 2kb in length and codes for the viral capsid protein involved in virion assembly, interaction with host cells and immunogenicity (Jameel *et al.*, 1996;Tam et al., 1991). ORF3 is the smallest (about 369 bp) overlaps ORF1 by one nucleotide at the 5' end and jointly shares most of the remaining sequences with ORF2. The structure of HEV is depicted in Figure 2.1. ORF3 encodes for the immunogenic phosphoprotein associated with cytoskeleton and release (Jameel *et al*., 1996;Zafrullah *et al.*, 1997). The variability in HEV particle size reported by different laboratories may not be far from its proteolytic digestion during passage via the gut, labile nature to repeated freeze-thaw actions or storage of faecal specimens.

HEV particles are labile because they get degraded following high-speed pelleting in sucrose (Ticehurst, 1991). The virus is resistant to alterations in pH between mildly alkaline and acidic, thus, its ability to survive in gastrointestinal tract and cause infection. It does not, however, tolerate exposure to high salt concentration and cesium chloride (Panda *et al.*, 2007).

The biology of HEV has been difficult to study due to lack of a reliable cell culture system. The virus has, however, been propagated via cell culture in some laboratories, but reproducibility is still in question. In-vitro replication of HEV has been successfully carried out in FRhK (Female Rhesus Kidney) cells, 2BS diploid human embryonic lung cells and A549 human lung carcinoma cells. A cell culture system has been developed to propagate HEV in vitro (Tam *et al.*, 1996). More recently a more sustainable and reproducible culture system has evolved, the 3D cell culture system (Berto *et al.*, 2013b). This system utilizes a rotating wall vessel (RWV) which facilitates studies into the viability of virions and efficiently replicates HEV in human hepatoblastoma PLC/PRF/5 cells for up to 5 months.

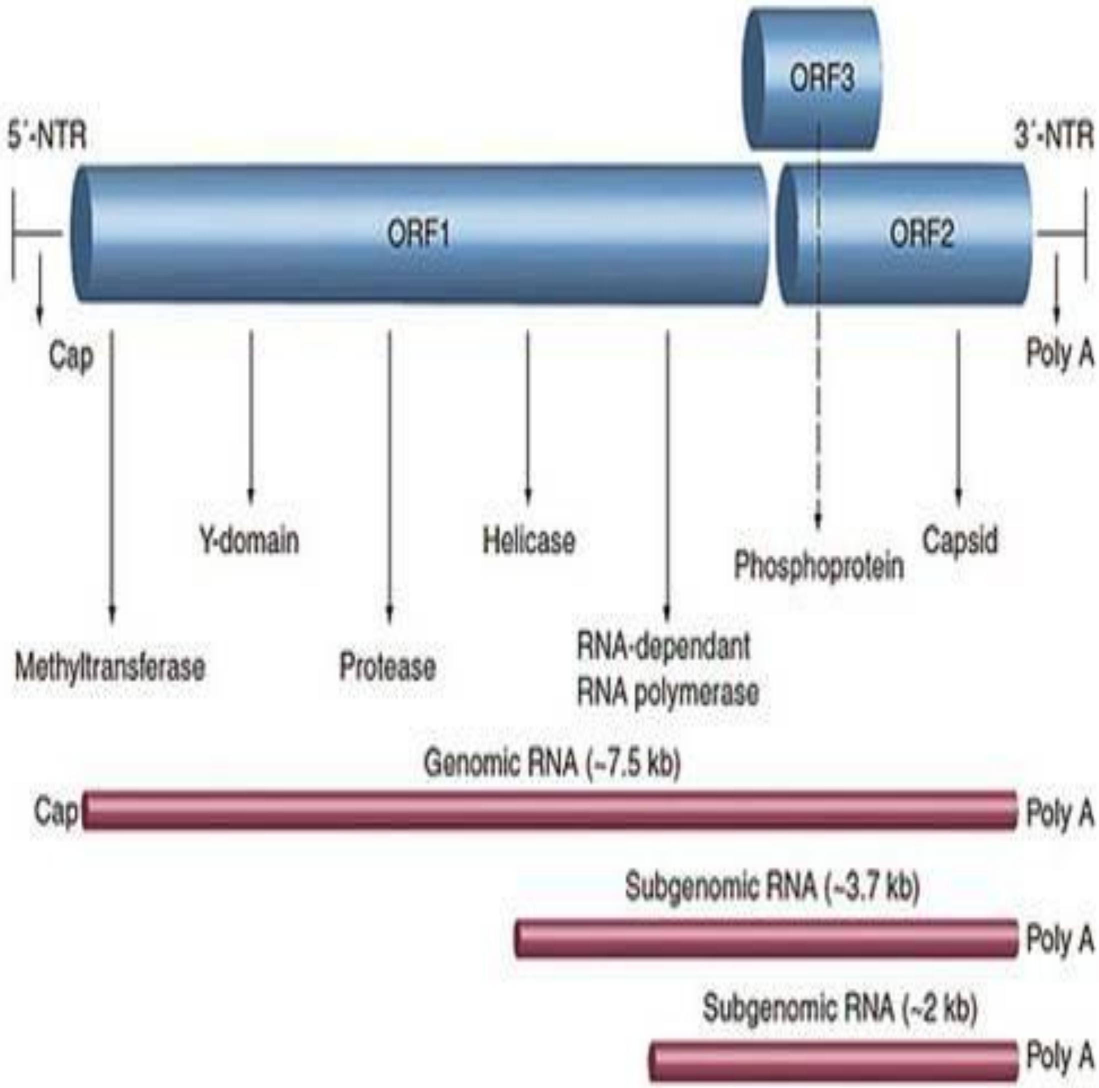


Figure 2.1: Crystal structure of Hepatitis E virus (rickjohnson303.wordpress.com)

2.2 HEV Viral Encoded Proteins

2.2.1 ORF1 Non-structural proteins

ORF1 encodes an approximately 1693 amino acid polyprotein encompassing multiple non-structural (NS) gene motifs: methyltransferase, papain-like cysteine protease, helicase and the RNA-dependent-RNA-polymerase (Tam *et al*., 1991). Total ORF1 polyprotein has successfully been expressed in a cell free translation system, in *E. coli* and in HepD2 cells as a protein of Mr 186 000. Transfection of *in vitro* synthesized RNA of the complete HEV genome into HepG2 cells resulted in detection of processed ORF1 active components.

2.2.1.1 Methyltransferase

The putative functions of HEV ORFs were assigned via a computer-assisted study. It was suggested that the amino-terminal domain from 60 to 240 amino acids is a methyltransferase (Koonin *et al*., 1992;Tam *et al.*, 1991). The m7G cap at the 5´ end of the HEV genomic RNA confirms a functional role for the viral methyltransferase. The methyltransferase domain is the hallmark of alphavirus-like superfamily (Rozanov *et al*., 1992) which contains sequences similar to cellular S-adenosylmethionine dependent methyltransferases and also contains guanylyltransferase activity. Expression of cDNA (encoding for aa 1 to 979 of non-structural ORF1 of HEV) in insect cell lines culminated in the synthesis of a protein of Mr 110 000 (P110), an immunopurified fraction which catalyzed the transfer of a methyl group from S-adenosylmethionine to GTP and GDP leading to m7GTP and m7GDP. HEV P110 capping enzyme has similar properties to methyltransferase and guanylyltransferase of alphavirus nsP1, tobacco mosaic virus P126, brome mosaic virus replicase protein 1a and bamboo mosaic virus non-structural protein, thus demonstrating a common evolutionary origin of these remotely related animal and plant viral families.

2.2.1.2 γ- domain

The γ-domain is significantly similar among HEV, rubella virus and beet necrotic yellow vein virus and consists of about 200 amino acid residues downstream of the methyltransferase domain (Panda *et al*., 2007). Within the polyprotein of HEV and rubella virus, the amino terminus of the γ-domain overlaps the conserved motif at the carboxy terminus of the methyltransferase (Koonin *et al*., 1992). Up until now, no specific function has been assigned to this domain.

2.2.1.3 Papain-like cysteine protease

Downstream the γ-domain is the putative papain-like protease encompassing 440 to 610 amino acid residues. This protein is flanked by the carboxy terminus of the γ-domain upstream and the amino terminus of the proline-hinge region downstream and spans about 300 amino acid residues (Koonin *et al*., 1992). The unique conservation of the X-domain which is an exclusive event in association with viral papain-like proteases indicates that HEV encoded protease harbors some similarity to proteases seen in other positive strand RNA viruses, eg, alphaviruses and the rubella virus. This protease is said to be involved in co- or post-translational processing of viral polyprotein to deliver discrete non-structural gene fragments. Viral protease domains have also been detected and defined in other hepatitis causing viruses (Panda *et al*., 2007). In hepatitis C virus, the NS3 serine protease exists and two papain-like proteases have been predicted in mouse hepatitis virus (Bonilla *et al.*, 1995) . The only evidence which supports polyprotein processing by the protease domain of HEV is preliminary. The exact cleavage sites of the ORF1 polyprotein are still unknown; however, partial cleavage of the full-length ORF1 polyprotein into processed products was achieved.

2.2.1.4 Helicase

The HEV helicase domain covers amino acid residues 960-1204 of the entire polypeptide and possesses all seven conserved segments of a typical Helicase Superfamily I. Comparison with other positive-strand RNA viral helicases which belong to the same Superfamily indicated the highest total similarity with the helicase of bee necrotic yellow vein virus and to a lesser extent, the helicase of rubella virus. Within this domain, two conserved amino acid sequence motifs exist and are associated with purine nucleotide triphosphate (NTP)-binding activity. They are GVPGSGKS located at amino acid position 975-982 and DEAP at amino acid positions 1029-1032 (Tam *et al*., 1991). The latter motif is said to interact with the Mg^{++} cation of the Mg-NTP complex for RNA- or DNA-dependent NTPase activity. Hence, these motifs generally involved in purine NTPase activity are located in viral helicases (Geider and Hoffmann-Berling, 1981). These viral helicases facilitate the unwinding of DNA, RNA or DNA-RNA duplexes necessary for genome replication, recombination, repair and transcription.

2.2.1.5 RNA-dependent RNA polymerase

The carboxy terminal region of HEV ORF1 polypeptide includes the RdRp homology domain encompassing amino acid residues 1200-1700 (Tam *et al*., 1991). A highly conserved amino acid motif recognized in all positive-strand RNA viruses as the canonical Glycine-Aspartate-Aspartate (GDD) tripeptide is indicated by amino acid residues 1550-1552 in the ORF1. The polymerase proteins of rubella virus, beet necrotic yellow vein virus and HEV together constitute a discrete group within the supergroup III of viral RNA polymerases with HEV polymerase demonstrating a difference from the supergroup consensus, having an alanine in place of a cysteine in motif VII (Koonin *et al*., 1992). By means of homology modeling, a crystal structure has been predicted for HEV RdRp and this comprises a 'palm' (five motifs), 'fingers' and 'thumb' domains.

2.2.2 ORF2 structural proteins

Amino acid translation of the second major ORF which is about 1980 nucleotides in length, results in the HEV structural polypeptide (pORF2). It is separated from the ORF1 with only a few nucleotides. It is a glycoprotein of about 71-88 kDa, 660 amino acids and with a potential signal region (high arginine and lysine concentrations and about 7 proline residues) (Zafrullah *et al.*, 1999). Amino acid sequence analysis of pORF2 reveals an extensive hydrophobic domain towards the amino terminus and a region rich in basic amino acids. This domain is said to be involved in virion maturation in conjunction with the endoplasmic reticulum post pORF2 translation. The pORF2 protein is synthesized as a precursor and then subsequently changed into a mature protein and glycosylated. The expression of ORF2 has been studied during viral infection through seroconversion in human subjects against epitopes found in pORF2.

2.2.3 ORF3

The third ORF is ORF3 and it is the smallest. It is situated between ORF1 and ORF2 and it is approximately 369bp (Kaba *et al.*, 2013). This molecule overlaps ORF1 at its 5´ end by a single nucleotide and overlaps ORF2 at its 3´ end by 328 nucleotides (Ahmad *et al.*, 2011;Tam *et al*., 1991). HEV genotype IV translates this ORF into a protein of 114 amino acids while other genotypes deliver a protein of 123 amino acids; thereby lacking the first 9 of 32 amino acids (Vasickova *et al*., 2007). ORF3 is said to code for an immunogenic phosphoprotein with several functions, but the main functions remain unclear (Emerson and Purcell, 2003;Zafrullah *et al*., 1997).

2.3 HEV in Animals

The gastro-intestinal tract of several animal species including pigs, deer, warthogs, wild boar, rodents, birds, etc carry HEV and shed the virus. However, the heaviest viral load is said to be found in the liver and gall bladder (bile). HEV strains isolated from reservoir animals are genotype 3 and 4 zoonotic strains. The only genotype 1HEV said to be detected in pigs occurred in Cambodia (Caron *et al.*, 2006). This result was not verified and till date neither genotype1 nor 2 have been detected in pigs.

The first experimental infection of domestic pigs with HEV was conducted in India. An Asian strain of human origin was said to have been used (Balayan, 1997). A later retrospective study revealed that the virus used in that study was not a human strain but rather it was HEV strain of swine origin. Further evidence of the inability of human strains to infect swine was provided by two separate and independent studies in the United States of America where laboratories failed to amplify HEV infections in pigs with well characterized genotype 1 Asian strain (Sar-55) and a genotype 2 Mexican strain (Mex-14) of human HEV (Meng, 2003). Shortly after, anti-HEV antibodies and RNA were detected in pigs in Nepal in 1995 but the molecular identity of the infecting virus was unknown (Clayson *et al.*, 1995a). By 1997, the index animal strain of HEV was identified and characterized from domestic pig in the United States (Meng *et al*., 1997). The scientists discovered by serendipity, that a large number of pigs in the U.S were IgG seropositive, suggesting that pigs in US were exposed to a HEV-related agent. A prospective study was then conducted to identify the agent. From that time, HEV has since crossed the species barrier and found to be present and circulating in other mammals.

Apart from pigs and members of the family *Suidae* being the most important reservoir, HEV is being increasingly found in other animal species. HEV has been reported in deer in Europe and Asia (Choi *et al.*, 2013;Kukielka *et al.*, 2015) but no such reports have been documented in Africa. The import of discovery of HEV in deer is the transmission to humans, especially through hunting activities or direct consumption. At the moment, there is no exhaustive range for the available reservoirs of HEV.

The frequency of occurrence of HEV in pig farms world-wide with at least one pig positive for anti-HEV antibodies and or HEV RNA may rise up to 100% (Pavio *et al.*, 2010). Although the first documented evidence of HEV was in 1997, retrospective analysis indicates that HEV had been circulating since about 1985 in Spain and India (Casas *et al.*, 2009). In Nepal, anti-HEV antibodies were reportedly detected from domestic pigs in 1995; the specific identity of that virus is not known because the virus was not sequenced (Clayson *et al.*, 1995b). Presently, swine HEV infection is widespread in farms worldwide in both developing and industrialized countries regardless whether HEV is endemic in the human populations or not (Meng, 2010). In general, swine become infected between 2-4 months of age, display a viraemia for about 1-2 weeks and shed infectious virus for about 3-7 weeks (Takahashi *et al.*, 2003;Yazaki *et al.*, 2003). It is known that the main source of virus transmission in pigs is the virus-containing faeces with large amounts of infectious virus. By this cycle, naïve pigs can become infected via direct contact with other infected pigs or through ingestion of faeces-contaminated feed or water (Bouwknegt *et al.*, 2008b).

HEV infection rates vary globally; for instance sero-prevalence in the United Kingdom is 85.5%, in China 78.8% and in Indonesia 72% (Banks *et al.*, 2004;Wang *et al.*, 2002, Wibawa *et al.*, 2004). This variation is most probably due to difference in farming practices, age at disposal and other obscure factors. In other animals, HEV has been widely detected by both serology and molecular methods. The geographical spread of HEV globally is wide; the virus has also been detected in small and large islands. For instance, HEV was detected in domestic pigs in New Caledonia in a province far from the capital (Kaba *et al.*, 2011a). This study is the first of its kind in the Islands of Oceania but it indicates that the virus may be present on other Islands in the region. In the larger Island of Australasia, domestic pigs have been found to harbour HEV and it is widespread in Australian pig farms.

2.3.1 HEV in avian species

Avian hepatitis generally is a relatively new disease in poultry. The virus was first detected in chickens with clinical signs of big liver and spleen disease in Australia (Payne *et al.*, 1999). Shortly after, a variant avian virus was identified from chickens with hepatitis-splenomegaly syndrome in the US (Haqshenas *et al.*, 2001). Both pathogens were later concluded to be variants of the same virus and known to be avian hepatitis E virus (aHEV). Since then, the virus has been identified in several countries, eg., Spain (Peralta *et al.*, 2009), Russia (Sprygin *et al.*, 2012) and Hungary (Banyai *et al.*, 2012). In a particular serological study of aHEV in Taiwan, a prevalence rate of 40.57% was obtained. On the other hand, 72-100% of the chicken liver tested in a another study in the US yielded aHEV RNA (Gerber *et al.*, 2014) indicating that aHEV maybe more widespread in poultry populations than previously speculated. aHEVs are known to possess high levels of genetic diversity within their genome; and this is partly responsible for detection limitation in some samples along the helicase gene region from different geographic locations (Sprygin *et al*., 2012). Globally, a full-scale outbreak of aHEV has not been experienced in any flock but the seroprevalence data suggests that aHEV is endemic in many countries. The public health potential of the virus and its cross-species infection ability has not been fully understood.

2.3.2 HEV in fish and shell fish

Apart from mammalian hosts, a few aquatic species harbouring HEV have been identified. HEV has been identified in oysters in Korea (Song *et al.*, 2010). In summary, 8.7% of the samples tested were positive for HEV RNA and specimens were not confined to a single geographic location. All oyster HEVs were classified as genotype 3 similar to human and swine HEV from Japan and USA, although one oyster cluster was formed with Korean swine HEV strains. Only recently, HEV was identified in fish (cutthroat trout) in the US (Batts *et al.*, 2011). This represents the first fish reservoir of HEV to be discovered. The cutthroat virus (CTV) sequences deferred significantly from other mammalian or avian HEV and thus a new genus, *Cutrovirus* was proposed. The study also revealed that CTV appeared to be widespread in western United States and therefore, may also be present in aquatic animals elsewhere globally. Unlike mammalian HEV, it is not completely clear how HEV is maintained in the cutthroat trout but plausible suggestions are the presence of HEV in other reservoir species or trout life stages and spread to broodstocks at spawning stage when they become more susceptible. The second possibility is that cutthroat trout maintains a chronic infection throughout life similar to what is obtained for *infectious pancreatic necrosis virus* (IPNV) and then the virus is transmitted horizontally to naïve animals and also vertically (Reno, 1999). This virus is now known to have been discovered over 20 years ago but molecular tools were only recently used to determine its true identity.

2.4 HEV in Foods

The presence of HEV in foods was first reported in Asia. It was observed in a group of Japanese hospital patients with the history of consumption of raw and undercooked meats from wild boar and Sika deer (Tei *et al.*, 2003). HEV sequences recovered from these patients were identical to sequences detected in some leftovers of meat. In India, studies revealed that HEV sequences were detected in pig livers from retail markets, which were meant for human consumption. Sequencing and phylogenetic analysis revealed HEV genotype 4 sequences and were >90% similar to HEV identified in pigs previously (Kulkarni and Arankalle, 2008). Elsewhere, pig livers bought from retail grocery shops were also found to be contaminated with HEV (Berto *et al.*, 2013a;Feagins *et al.*, 2007). This indicates that pork and pork product contamination is widespread and could be a ready means for transmission of HEV. In a separate study in Europe, investigation of autochthonous acute Hepatitis E traced the infection to the consumption of raw figatelli (a dried, smoked sausage containing about 30% pig liver) (Colson *et al*., 2010). Sea foods like bivalves are known to harbour and transmit enteric viruses to humans when eaten in raw or undercooked forms. The identification of HEV in oysters in The Republic of Korea (Song *et al*., 2010) is further evidence of the existence of enteric viruses in sea foods. Apart from meats, HEV has been detected in plant products recently. In a European study designed to trace viruses in the fruit supply process, HEV was identified in vegetables and fruits (Maunula *et al.*, 2013).

2.5 HEV in the Environment

Due to the zoonotic existence of HEV, the general environment is involved in the maintenance and transmission of HEV. The relationship between human activities and the environment cannot be separated. While animals remain reservoirs, environmental sources now play significant roles in the epidemiology of HEV. The environment often gets contaminated through shedding of enteric viruses. Water bodies also hold viruses and can be found in detectable limits. HEV has been found in flowing rivers and drinking water plants (Albinana-Gimenez *et al.*, 2006;Li *et al*., 2014). The exact routes of entry are not known, but human and animal pollution cannot be ruled out completely especially as sequences recovered from these sources are similar to those from animals and humans. Slaughter house effluents in developing countries may be responsible for environmental and surface water contaminations. In Brazil, waste water samples from a pig slaughter house were reported to be positive for HEV RNA (dos Santos *et al.*, 2011), these wastes were destined for the environment and viable viruses may leach into drinking water bodies. Manure from compost sites are also a source of HEV in the environment. In a single study in Spain, samples from manure composting plants were found to be positive for HEV RNA, and 80% of the sites studied were positive (Garcia *et al.*, 2014). However, it is noteworthy to mention that all positive cases were detected at the first phase of manure composting (slurry reception ponds) but could not be detected beyond this stage. This may mean that composting is a reliable means of inactivating HEV and other viruses from manure wastes. Sewage effluents from human excreta are also an important portal of environmental contamination. Molecular studies on environmental samples have revealed the presence of HEV in sewage (La Rosa *et al.*, 2010). These findings may also suggest that HEV contamination of water environment through sewage seepage contributes to the epidemiology of the virus and the disease. Although the detection of virus fragments by molecular methods does not ultimately translate to infectious agents, infectious HEV particles have been documented in raw sewage (Pina *et al.*, 1998). This heightens the public health hazards arising from contamination of surface water with HEV. Although HEV has not been observed in sewage in Nigeria, a relatively high percentage has been reported elsewhere. In Italy, 16% prevalence was obtained in a study spanning the whole country (La Rosa *et al*., 2010), whereas in Spain, only 1 out of 37 samples returned positive for HEV RNA and further analysis revealed that the particular virus was still infectious although the disease was not endemic in the geographical area (Pina *et al*., 1998). The study suggests that HEV in sewage may be more common in developing countries than industrialized countries. HEV fragments have also been identified in sewage of animal origin and the viral sequences were 92-94% similar to viral sequences of human origin (Pina *et al.,* 2000), providing further evidence that pig HEV sequences are closely related to human sequences.

2.6HEV in Humans

HEV infections are common in both developing and industrialized countries. HEV is described as an important pathogen among the causes of acute viral hepatitis in tropical and subtropical nations. However, in developed or industrialized countries, it was registered as a sporadic, imported disease often affecting individuals returning from endemic developing countries (Balayan, 1997). Currently, it has been established that HEV is also endemic in developed countries and that autochthonous HEV actually occurs in these countries (Duque *et al*., 2012;Miyamura, 2011). These reports are accompanied by high anti-HEV immunoglobulin G (IgG) prevalence in apparently healthy individuals and also there is an increase in the occurrence of acute hepatitis E with non-A, non-B and non-C hepatitis with no previous history of visit to hyperendemic countries. Besides, there is an increase in reports of HEV detection and transmission from animal reservoirs (pig, wild boar and deer) to humans in industrialized countries (Berto *et al*., 2013a;Di Bartolo *et al;* 2015;Suwannakarn *et al.*, 2010). Variants of HEV have so far been identified in human populations and animal reservoirs like pig, wild boar, deer, rabbit and rats. HEV variants which infect humans are grouped into four genotypes; genotypes 1 and 2 circulate faeco-orally among humans, whereas genotypes 3 and 4 can be transmitted to humans from animals and vice versa (Smith *et al.*, 2014). All existing mammalian genotypes are further grouped into subtypes based on molecular characterization (Lu *et al.*, 2006); however, the criterion for this further subtyping is ambiguous. In Africa, genotype 1 HEV appears to be most prevalent in North Africa around Egypt, Sudan, Chad and Central African Republic (CAR) and Namibia (Kim *et al*., 2014). This is followed by genotype 2 which is present in CAR, Chad, Namibia and Nigeria (Kim *et al*., 2014). Human strains of genotype 3 are rare in Africa as only one case has so far been detected.

Human HEV infection is a global disease. Previously, the natural history of the disease was largely unclear, but within the last decade, there has been improved understanding of the disease. The disease remains under-diagnosed, in part due to serological assays with sub-optimal sensitivity and also due to neglect in some developing countries (Kamar *et al.*, 2014). More recently, diagnostic capabilities have improved. The general epidemiology of HEV infection is not straight-forward, so also its clinical features. It differs from developing countries to developed countries. In general, the incubation period of HEV in humans is put at 2-10 weeks in outbreaks related to water contamination, but 4-5 weeks with post oral ingestion in volunteers (Kumar *et al*., 2013). Outbreaks have been observed to occur more frequently in the rainy season due to activity of flowing surface water from drainages and/sewage tanks and consumption of contaminated drinking water. Human overcrowding and overstretching of health facilities in refugee camps may also amplify the risk of infection (Boccia *et al.*, 2006).

2.6.1 Epidemiological patterns of HEV infection

The spatial distribution of HEV genotypes is complex and dynamic (Kaba *et al*., 2013). HEV strains specific to humans belong to genotypes 1 and 2 and cause sporadic and epidemic hepatitis E mostly in tropical and sub-tropical nations (Lu *et al*., 2006;Purcell and Emerson, 2008;Teshale *et al.*, 2010). HEV genotypes 3 and 4 are responsible for human autochthonous sporadic hepatitis infections in developed countries (Dalton *et al.*, 2013;Mansuy *et al.*, 2009a). Genotype 4 HEV infections were earlier limited to Asia, but recently genotype 4 sequences were identified in Italy, Belgium and untraveled persons in Germany, (Hakze-van der Honing *et al.*, 2011;Monne *et al.*, 2015) and may be spreading to other continents.The different epidemiological patterns present different disease characteristics in areas where the disease is endemic and non-endemic. Generally, in endemic areas epidemics of Hepatitis E occur more frequently and often appear after a few years interval; this has been observed in Africa, Middle East, China and the Indian subcontinent (Kumar *et al*., 2013). Differences in epidemiological patterns between HEV in developing and developed countries are presented in Table 2.1

2.6.2 HEV infection in developing countries

HEV infections are mostly self-limiting and last only a few weeks. After incubation period of about 2-6 weeks, clinical signs of hepatitis emerge accompanied by fever, nausea, abdominal pain, vomiting, anorexia, general malaise and hepatomegaly (Kamar *et al*., 2014). Infrequently, jaundice occurs, 40% of HEV infected patients develop jaundice (Labrique *et al.*, 2010). High mortality is experienced in pregnant women and other individuals harbouring chronic liver disease. Death in pregnant women is common in the third trimester and it is attributable to fulminant hepatic failure and obstetric complications like haemorrhages or eclampsia (Kamar *etal*., 2012).

Table 2.1: Epidemiological differences between developed country and developing country

Developing Countries	Developed Countries
• Genotypes: 1 and 2	Genotypes: 3 and 4
• Source: Human	Zoonotic: pigs, wild boar, deer, mink
• Route: Faeco-oral (contaminated water)	Faeco-oral: Infected meats
• Seroprevalence: Children less than 15 years, Individuals >15 to 30 years old	Steady increase in all age groups
• Outbreaks: Yes	Outbreaks: No
• Age Range: 15 to 30 years	> 50 years
• Death: 20 – 25%	None
• Burden: 3-4 million cases/year 70, 000deaths, 3, 000 still births	Data unknown

Mortality rates in epidemics range from 0.2% to 4.0% (in general population) and is higher in infants under 2 years old (Kamar *et al*., 2012). Genotypes 1 and 2 do not present chronic infections; this is corroborated by a study of renal transplant recipients in India where HEV genotype 1 is hyperendemic (Naik *et al.*, 2013). Person-to-person transmission of HEV in these areas is uncommon but household factors are important in outbreaks (Howard *et al.*, 2010).

Water-borne epidemics of HEV are common mainly in young adults and highest among men 15-35 years old (Purcell and Emerson, 2008). Clinical infection in men is two to five times higher than in women but no sex difference exists in exposure to HEV (Aggarwal *et al.*, 2002). Asyptomatic HEV infections are estimated to exceed symptomatic infections by up to four fold in water-borne outbreaks (Guthmann *et al*., 2006).

2.6.3 HEV infection in developed countries

The emergence of autochthonous HEV in developed countries has altered the otherwise incomplete understanding of hepatitis E. Such autochthonous infections are being reported in

Europe, Australasia and North America (Kamar *et al.*, 2014). HEV was reported to have been identified in every industrialized country where surveillance was executed except Finland (Kantala *et al.*, 2009). However, HEV was only recently identified and characterized in pigs in Finland where HEV genotype 3 was isolated (Kantala *et al.*, 2015). This zoonotic strain may also cross species barrier to humans; moreover, most strains identified in Europe belong to genotypes 3 and 4.

The overall manifestations of acute autochthonous HEV caused by HEV genotypes 3 and 4 cannot be distinguished from developing country hepatitis E, except that most of the patients are middle aged to elderly males. Most of the cases are sporadic acute cases, but pockets of cases from point source outbreaks exist (Tei *et al*., 2003) and sources of infection remain unknown. Results from a French study showed that clinical manifestations in a cluster of individuals with HEV genotype 4 were more severe than in those with HEV3. Similarly Japanese HEV patients presented with higher serum alaninetransferase levels (Mizuo *et al.*, 2005). In developed countries like France and UK, acute hepatitis E is more common than hepatitis A and in Southwest France, HEV is hyper-endemic (Kamar *et al*., 2014). In the USA, only a few cases of HEV have been reported so far despite seroprevalence rates of 21% and annual incidence of 0.7% (Kuniholm *et al.*, 2009). This scenario may mirror the fact that there are no licensed HEV diagnostic tests in the USA.

The exact burden of HEV infections in developed countries is difficult to determine. Although many studies report prevalence rates of <5% (Bouwknegt *et al.*, 2008a), the true figures may be much higher since the tests were carried out with assays with relatively poor sensitivity.

Chronic infection is commonly found in Hepatitis E caused by genotypes 3 and 4. It is observed as recurring HEV RNA in stools and serum for 6 months or greater in immunosuppressed patients mainly (Gerolami *et al*., 2008) and most cases have occurred in solid-organ transplant individuals. More to this group, a few cases have been reported in patients with HIV (Dalton et al., 2013;Kaba *et al.*, 2011b). Till date chronic infection is yet to be reported with HEV genotypes 1 and 2.

2.6.4 HEV infection and pregnancy

Apart from the devastating character of gradual progression to chronicity in immunocompromised persons, HEV also has immense deleterious effects in pregnant women. The incidence of HEV in pregnancy is rather high and a substantial number of pregnant women may progress to fulminant hepatitis with mortality rate rising above 30% (Navaneethan *et al*., 2008). The specific mechanisms of advanced hepatic damage in pregnant women are yet to be fully understood. Interestingly, in Northern India, HEV incidence and course is very severe while in endemic countries like Egypt, its course is benign with low levels of morbidity (Stoszek *et al*., 2006a). In one study in Egypt, where 2,428 pregnant women were recruited, anti-HEV antibody prevalence was 84.3%; however, no single case of acute viral hepatitis was reported. In another study from India, about 60% of viral hepatitis in pregnant women was attributed to HEV and fulminant hepatic failure (FHF) was more common among HEV-infected women (55%) than non-HEV-infected women. Also secondary maternal mortality due to FHF was higher in HEV-infected group (41%) than in the non-HEV group (Patra *et al.*, 2007). The actual reasons for the differences in response to HEV infection in different geographic areas are unclear but may be attributable to early exposure to HEV resulting in long lasting immunity and/or modification of subsequent reactions to exposure to the HEV (Navaneethan *et al*., 2008). On the other hand, the genotypes of HEV in Egypt may be of a lesser virulence than those in India and Asia (Stoszek *et al.*, 2006b).

During pregnancy, the general maternal immune system is greatly altered to accommodate the genetically different foetus (Marzi *et al.*, 1996). Certain factors immensely contribute to immunological tolerance during pregnancy; and the existence of trophoblasts between the foetal and maternal blood may be one factor (Navaneethan *et al*., 2008). Cytokines also modulate tolerance and both the trophoblasts and placenta are involved in the secretion of different classes of cytokines. Some researchers measured serum cytokine levels at different stages of pregnancy in mouse models and found out that the levels of different cytokines were low at the initial stage of pregnancy and then significantly increase at late pregnancy (Orsi *et al.*, 2006). It is also known that steroid hormones can greatly influence viral replication as it is observed in the replication of cytomegalovirus (CMV) when hormonal enhancement is performed. Pregnancy is directly related to high levels of steroid hormones and these are likely to enhance viral replication. Steroid hormones may also antagonize the liver cells and may eventually lead to hepatic failure in the presence of infectious agents. Steroid hormones are also immunosuppressive and mediate lymphocyte apoptosis through NF-κB. The p65 component of NF-κB has been shown to promote liver development and regeneration. In a study in mice, those lacking the p65 showed high evidence of apoptosis. This scenario was replicated in another study involving pregnant and non-pregnant women with FHF due to hepatitis B, C and E; p65 activity was nearly depleted in liver biopsy and peripheral blood mononuclear cells and this results in hepatic damage (Prusty *et al.*, 2007). This school of thought has further been supported by observations in other viral infections. The low levels of p65 expression in-vivo leads to liver fibrosis and liver damage in persons with Hepatitis C virus induced liver disease. It was observed that pregnant women infected with HEV had lower levels of CD4 and higher CD8 counts (Jilani *et al.*, 2007). Workers also observed that serum levels of oestrogen, progesterone and Ɓ–HCG were higher in HEV-infected pregnant women than in HEV-negative pregnant women. Furthermore, studies have revealed that cellular immune response in HEV-infected pregnant women was generally suppressed and indicated by decreased lymphocyte response to phytohaemagglutinin (PHA) and a T-helper type 2 (Th2) bias when compared with non-HEV infected pregnant women (Pal *et al.*, 2005).

2.6.5 Extrahepatic Manifestations of HEV

Apart from the classical signs of liver injury exhibited by HEV, it is also responsible for other associated non-liver disorders. These include renal dysfunction, pancreatic anomalies, hematological imbalance and neurological damages.

2.6.5.1 Neurological disorders

Signs of neurological impairment have been observed for HEV1 and acute and chronic HEV3 infections. The manifestations observed include Guillain-Barré syndrome, Bell's palsy, neuralgia amyotrophy, acute transverse myelitis and acute meningoencephalitis (Kamar *et al*., 2014). HEV neurological signs were studied in 126 patients and nervous signs were observed in about 5.5% of the enrolled patients including three immunocompetent individuals with acute HEV genotype 3 infection, three patients who had undergone solid organ transplants and one HIV-patient with chronic infection (Kamar *et al.*, 2011a). RNA was detected in the cerebrospinal fluid (CSF) of all patients with chronic HEV infection who had neurological symptoms, confirming that neurological disorders are associated with chronic HEV infections. Sequences of HEV were also identified in the CSF and serum of a kidney transplant recipient with chronic HEV case demonstrating neurological signs. In Europe, anti-ganglioside GM1- and GM2-positive Guillain-Barré syndrome in association with HEV infections were reported (Cronin *et al.*, 2011, Maurissen *et al.*, 2012).

2.6.5.2 Pancreatitis

Only in the developed world has pancreatitis been observed in HEV infection. Acute pancreatitis was seen in HEV genotype 1 infection (Deniel *et al.*, 2011). However, pancreatitis has not been associated with any other genotype of HEV anywhere in the world.

2.6.5.3 Haematological disorders

Haematological disorders are not very common manifestations of HEV infections. The only signs which were associated with acute HEV infections are thrombocytopenia and aplastic anaemia (Fourquet *et al.*, 2010).

2.7 Molecular Epidemiology of HEV

2.7.1 Genetic Variability of HEV

The molecular classification of variants of HEV is a dynamic and continuous process. Following its identification, the virus was characterized as a single-stranded positive-sense RNA genome encoding for three open reading frames (Koonin *et al*., 1992;Tsarev *et al*., 1992). The earliest classification schemes were based on only partial genome sequences. More HEV sequences were obtained from variants globally and a scheme of relatedness among viral isolates became evident and these variants were further grouped into genotypes and subtypes based on sequence similarity. It was hypothesized that variants with >20% nucleotide sequence divergence along the ORF2 be grouped into different HEV genotypes as was suggested for Noroviruses (Lu *et al*., 2006). However, there is no globally accepted criterion for subtype designation. In many publications where complete and partial nucleotide sequences were assembled, HEV subtyping was not done due to lack of agreed criteria. Subsequently, a detailed analysis of nodal support for phylogenetic groupings and distances between these groups in comparison of complete genomes or a combination of ORFs brought about the emergence of four genotypes and 24 subtypes. Later studies have failed to acknowledge the merit of subtypes as taxonomic units or differed in assignment of isolates to subtypes (Okamoto, 2007). In a more recent debate, rabbit and wild boar HEV are still pending as new genotypes or existing HEV subtypes (Smith *et al.*, 2013).

These taxonomic bottlenecks were borne out of the use of varying genomic regions (partial or complete genomes) and phylogenetic methods pairwise distances or bootstrap values of the neighbor-joining or maximum likelihood trees (Smith *et al.*, 2013). Against this backdrop, the International Committee on Taxonomy of Viruses (ICTV) *Hepeviridae* study group agreed that *Hepeviridae* consists of genotypes one to four along with unclassified strains from chickens and rats (Meng *et al.*, 2012). Further confusion emanated from the recent identification of HEV from bats, cutthroat trout and ferrets; and HEV "genotype 5" was designated to avian HEV, to variants of rabbit HEV, to rat HEV and also to variants of wild boar HEV (Smith *et al*., 2013). The actual number of published HEV sequences has astoundingly increased in the last few years and this has also increased the number of genomic groupings of the virus. The number of suggested new genera and genotypes has also increased (Smith *et al*., 2013).

2.7.2 Evaluation of the subtyping scheme

Although this scheme is a controversial one, there appears to be no other applicable scheme in the assignment of viral isolates into subtypes. The most accepted scheme was described by Lu *et al*. (2006) and designated all known HEV variants into 24 subtypes; genotype 1 (a-e), genotype 2 (a, b), genotype 3 (a-j) and genotype 4 (a-g). Sequence information limitation caused the subtyping to be based on both complete and partial genomic sequences from five genomic regions. Complete genomic sequences were available for only genotype 3a, 3b, 3g and 3j and genotype 4c, 4d and 4g. The subtyping system was recently re-evaluated (Oliveira-Filho *et al.*, 2013) and three genomic regions: ORF1 (1-287 nt of 5' end), ORF2 (301 nt of 5' end and 148 nt: 6390-6537) analyzed. The employment of different genomic regions gave varied genetic groupings of HEV variants without a distinct separation of subtypes (Oliveira-Filho *et al*., 2013). The results of the above analysis could not differentiate subtypes 3i, 3h and 3c, but placed them all on same branch using ORF1. Also when ORF2 (148nt) was used, it was impossible to differentiate subtypes 3i, 3b, 3h and also 3e, 3f and 3g. All the isolates which represented these subtypes were re-aligned and put together in two branches and a potential number of "new" subtypes could be deduced. It also revealed that subtypes 4a and 4f belong to one subtype.

2.7.3 Diversity pattern in the HEV genome

Coding sequences comprise greater than 95% of the HEV genome (Smith *et al*., 2013). Mean nucleotide distances are relatively homogenous over the complete genome except for two peaks of genetic variability in ORF1. The first variable peak is between nucleotides 1500 and 2000 which includes the papain-like cysteine protease domain and a downstream area of unknown function. The second region of high variability is around position 2200 and it is consistent with previously determined hypervariable region of ORF1; this region is proline- and serine-rich and predicted to be disordered intrinsically (Purdy *et al.*, 2012) and fails to deliver any meaningful sequence alignment among various genotypes. For a virus like HEV with varying degrees of genetic variability, molecular characterization to subspecies level with a percentage of reliability is achievable with complete genomic sequences. This greatly improves the robustness of the analyses (Oliveira-Filho *et al*., 2013). Only recently, Oliveira-Filho *et al*. (2013) re-analyzed global complete genomes of HEV and arrived at the conclusion that viral sequences grouped under five genotypes: the previously known genotypes 1-4 and two wild boar sequences from Japan which diverged considerably and were referred to as genotype 5. Within the genotypes, the maximum nucleotide distances observed were 13.16% for genotype 1, 27.10% for genotype 3 and 19.96% for genotype 4. Genotypes 2 and 5 have only a few complete sequences available so they could not be evaluated. Based on genetic tree topology, genotype 3 differentiates into three major subgroups; subgroup 3.1 includes animal and human viruses from Asia (Mongolia, Korea, China, and Japan), Germany and North America. Subgroup 3.2 comprises viruses from Japan, Thailand, Europe and a single sequence from Kyrgyzstan. Finally, subgroup 3.3 contains HEV viruses from rabbits from China. This resulting sub-division, downgraded nucleotide divergence within the subgroups. Maximum nucleotide divergence within subgroup 3.1 is 19.22%, 20.11% within subgroup 3.2 and 18.01% within subgroup 3.3. There are similar distances within genotypes 1 and 4 (Oliveira-Filho *et al*., 2013). The nucleotide distances between genotype 4 sequences were narrower compared to the other genotypes.

2.8 HEV-related viruses and the cutthroat trout virus

More HEV-related viruses are being increasingly discovered. HEV-like viruses have been reported in bats, ferrets, mink, rabbits, rats, etc (Drexler *et al.*, 2012;Han *et al.*, 2014;Krog *et al.*, 2013;Li *et al.*, 2015). The HEV sequences obtainable from animal species displayed high levels of divergence along their genomes, about 66.49-82.99% when compared to sequences from the traditional HEV genotypes (Oliveira-Filho *et al*., 2013). In the year 2011, Batts *et al*. (2011), identified an HEV-like virus in the cutthroat trout and the virus was accommodated in the family *Hepeviridae*. However, Oliveira-Filho *et al*. (2013) did not find sufficient genomic data and relatedness to HEV or other HEV-like viruses using complete genomic sequences. Due to this difference, alignment and phylogenetic analysis of CTV with HEV and HEV-like sequences is difficult. Amino acid sequence p-distances were higher than 0.6 over the entire genome except between residues 1480 to 1770 of ORF1 where the distance were generally between 0.4 to 0.6. Phylogenetic analysis of this particular genome region revealed a tree with the cutthroat virus branching separately and the human-related and rat/ferret sequences more closely associated with each other than to bat or avian viruses. This phylogeny opposes the suggestion that avian HEV be placed in a unique phylogenetic group from mammalian strains. A newly identified HEV strain, camel HEV (DcHEV) (Woo *et al.*, 2014) further compounds the problem of HEV diversity. The DcHEV strain had conserved sequences TLYTRTWS and RRLLXTYPDG in the HVR of HEV 1-4 and the two recently identified wild boar strains. Immediately preceding the HVR, the conserved motif (T/V)SGFSS(D/C)F(S/A)P in HEV3 and HEV4 is also present in the DcHEV strain as VSGFSSDFAP (Woo *et al.*, 2014). One of the DcHEV strains possesses a single nucleotide (U) insertion at nt 5146 similar to HEV4 and wildboar HEV thereby beginning its ORF2 at nt 5172 and ending at nt 7154. Another DcHEV strain lacks the U insertion similar to HEV1, HEV2, and HEV3; therefore ORF2 begins at nt 5171. Phylogenetic trees inferred using ORF1, ORF2, ORF3 and the concatenated ORF1/ORF2 excluding the HVR places DcHEV with different HEV strains in the different phylogenetic trees. In the ORF1 and concatenated ORF1/ORF2 excluding the HVR, DcHEVclusters with HEV3 but in the ORF2 and ORF3, DcHEC clusters with HEV1 and HEV2 (Woo *et al*., 2014).

Based on the differences and difficulties elucidated by various workers, it is evident that the evolutionary changes taking place in HEV and HEV-related viruses make it difficult to arrive at a universally acceptable classification model. Members of the ICTV and other workers arrived at a convenient classification method based on analysis of existing sequence information and proposed a taxonomic scheme dividing the genera into *Orthohepevirus* (mammalian and avian HEV isolates) and *Piscihepevirus* (cutthroat trout virus) (Smith *et al*., 2014). The second level classification is the species level, and the species within the genus *Orthohepevirus* are *Orthohepevirus* A (isolates from pigs, human, deer, wildboar, camel, rabbit, mongoose), *Orthohepevirus* B (avian HEV isolates), *Orthohepevirus* C (viruses from mink, ferrets, Asian musk shrew, rat, and bandicoot) and *Orthohepevirus* D (bat HEV isolates) (Smith *et al*., 2014). Proposals are also under way for genotype designation within rat and human HEVs. The proposed hierarchical system and the three classification levels (genotype, species and genotype) reflect discontinuities in the bracket of pairwise distances between amino acid sequences. The scheme also avoids the use of host names in taxonomic identifiers and ensures a logical platform for designation of novel variants.

2.9 Transmission of HEV

In most instances, the exact mode of HEV transmission remains unknown or controversial and sources of infection are more often than not ill defined. This is often the case in sporadic acute

HEV infections. In such scenarios, the infection may be related to level of population immunity, living standards, sanitary levels and other intrinsic factors. Generally, consumption of faecally contaminated water in hyper-endemic regions with vast poor sanitation and the ingestion of undercooked or raw meats from HEV-infected animals in industrialized nations accounts for majority of HEV transmissions (Mirazo *et al.*, 2014). In infected individuals, HEV is excreted via faeces over several days. In a different twist, HEV genomic material has been identified in urine of infected pigs (Kasorndorkbua *et al.*, 2004), which ultimately may play a major role in the transmission of HEV in pig populations. Apart from previously established transmission routes, three more important routes have been identified: parenteral route (blood borne transmission) (Gallian *et al.*, 2014), vertical transmission from mother to child (Krain *et al.*, 2014) and human to human transmission (Mansuy *et al.*, 2009b). Though these novel routes appear to be infrequent, there is a growing list of evidence in this regard and it promotes preventive measure to assist in control of HEV globally.

2.9.1 Waterborne Transmission

The first and earliest documented occurrence of Hepatitis E was a widespread epidemic of water origin in India (Khuroo, 2011). HEV has been reported to occur as large waterborne epidemics and small outbreaks. In developing endemic countries, HEV infections may occur in association with Hepatitis A virus thereby complicating the infection and its epidemiology (Mirazo *et al.*, 2014). All waterborne HEV outbreaks are strictly caused by HEV genotypes 1 and 2 strains (Guthmann *et al.*, 2006;Maila *et al.*, 2004). HEV particles have been frequently reported in environmental sources in both developing and developed countries, raw seawage, slaughter house effluents and also seawater have been demonstrated to contain infectious viral fragments which are closely related to strains circulating in humans (dos Santos *et al*., 2011;Vivek *et al.*, 2013).

2.9.2 Food-borne transmission of HEV

Swine HEV is now an important variant of HEV and the virus has been discovered in other animal species that also have capacity of acting as reservoirs. HEV sequences have been identified in swine, wild boar and deer (Pavio *et al*., 2010). Aquatic species like oysters, cockles and mussels are also documented to harbour zoonotic HEV (Donia *et al.*, 2012;Namsai *et al.*, 2011). The identification of infectious HEV particles in raw pig liver and other by-products of pig origin (Berto *et al*., 2013a) poses a major route of transmission to humans. In certain parts of the globe, the ingestion of blood, raw or semi-cooked pig liver and offals are important food sources; these uncommon feeding habits are important risk factors of HEV infection. Food-borne transmission by ingestion of raw or undercooked meats of wild game (deer and wild boar) and domestic pig have been reported in Asia and Europe (Choi *et al*., 2013;Li *et al*., 2005b); there are no such reports from Africa. In Japan, infections due to ingestion of undercooked pork products are known to occur. Out of about ten HEV cases, nine admitted to the consumption of pig livers 19 days to two months before the commencement of symptoms (Vasickova *et al*., 2007). Following this discovery, packages of raw liver from the affected area were tested and HEV RNA was identified in about 1.9% of samples tested.

Food products made from venison have also been reported to be important risk factors. In a group of people who had history of consumption of venison, anti-HEV antibodies were detected in 17.7% of those tested. On the other hand, in a control group of individuals who did not eat venison, only one person was anti-HEV IgG positive indicating that venison and its associated by-products cannot be jettisoned as a route of transmission. Reports are also available on possible natural interspecies transmission of HEV in the wild and tendency to cause infection in humans (Takahashi *et al*., 2011).

2.9.3 Person-to-person transmission of HEV

This route of transmission is still thought not to be an important factor in the epidemiology of global HEV. In developing countries, poor levels of sanitation and hygiene, hand washing and drinking water act as agents of food and water contamination thereby exacerbating person-to-person transmission during active outbreaks (Aggarwal and Naik, 1992). Many workers report that person-to-person transmission is uncommon but in endemic areas most HEV outbreaks have been associated with common drinking water source. Prolonged outbreaks could possibly be as a result of extensive human-to-human transmission. This mode of transmission was evident in an outbreak in northern Uganda (Teshale *et al*., 2010).

2.9.4 Parenteral transmission

Parenteral transmission is not believed to be a frequent and important route of HEV spread. In recent time, HEV has emerged as a transfusion transmittable pathogen. Though the consequences of this route have not been systematically evaluated, the dangers which abound by transmission through blood transfusions have been described by several workers (Gallian *et al*., 2014;Nelson *et al.*, 2011). Furthermore, genetic analyses of HEV sequences from blood donors and recipients have been performed, and very identical sequences were recovered from these two groups of individuals in which genotypes 1 and 3 were involved (Colson *et al.*, 2007). In Germany, IgG seroprevalence was estimated at 6.8% and HEV RNA was identified in 0.08% of donated blood

(Juhl *et al.*, 2014), and between 1,600 and 5,900 HEV RNA-positive blood donations could take place every year. In The Netherlands, about one HEV-positive donation per day is documented. These reports indicate that this route of transmission is significant and that blood transfusion which is a common event is a likely route in both developed and developing countries. However, an approach for screening of HEV in blood donors (Nucleic acid amplification techniques) has been developed in Germany.

2.9.5 Vertical transmission of HEV

HEV is the major cause of hepatitis during pregnancy. However, the real causes for the increase in severity of the viral infection is not known. The excessively high mortality in pregnant women has always been observed in HEV infections with genotype 1 viruses only and there is no single report of mortality associated with genotypes 3 or 4 viruses (Mirazo *et al*., 2014). In Africa, though reports are rare, a genotype 2 infection which resulted into fulminant hepatic failure in a pregnant woman was documented in Namibia (Maila *et al*., 2004).

From an infected mother, HEV is transmitted to the foetus through intrauterine and perinatal routes; in these neonates it results in severe liver damage and high mortality (Khuroo and Kamili, 2009). In survivor babies, the clinical course of HEV infection is self-limiting with only a very short period of viraemia (Khuroo and Kamili, 2009).

HEV is likely to be responsible for 2,400-3,000 stillbirths per year and antenatal deaths in developing countries; although it is not clear whether the increase in stillbirths is as a result of vertically transmitted HEV or due to maternal complications of hepatitis E. Till date, there is no report of HEV transmission through breast milk. More investigations are required to understand the pathogenesis of HEV in pregnant women and the role of trans-placental transmission in foetal loss and stillbirth.

2.10 Diagnosis of HEV Infection

HEV infections are usually diagnosed by the detection of serum anti-HEV antibodies or directly by the detection of HEV genome segments in blood, other body fluids and faeces. Liver biopsies are also possible specimens for HEV RNA analysis. The sensitivity and specificity of commercial immune assays are variable and not always optimal in performance; these depend on laboratories and assay manufacturers. In recent years, new diagnostic flows for HEV are being proposed due to a better understanding of its pathogenesis and epidemiology, there is still need for standardization of diagnostic criteria (Wu *et al.*, 2014). The incubation period is about 2-6 weeks, and a short lived IgM response is elicited, followed by the long-lasting IgG antibodies. Commercial rapid immunochromatographic and enzyme assays are developed based on ORF2/ORF3 peptides or recombinant technology of HEV genotype 1. This detects HEV IgM and IgG from all four genotypes since they all belong to a single serotype (Emerson *et al.*, 2006). No genotype-specific serological test exists for HEV and no cross-reactivity of HEV antigen with any other pathogen.

2.10.1 Immunologic assays

Very little information was available about the immunological aspects of HEV until in the last few years after which a trend of antibody response was observed and followed. In the acute stage when IgM is elicited, strongly positive response are rare after three months of infection. In 90% of patients with acute HEV infections, anti-HEV IgM is detectable within the first two weeks of onset of illness (Favorov *et al.*, 1992) and IgG is elicited immediately after IgM is secreted. Since both classes of antibodies are present simultaneously in the acute phase, it is confusing and difficult to distinguish an acute serological diagnosis of HEV infection. However, IgM quickly wanes in a few weeks to months, IgG remains in circulation for highly variable period of time between 1 to 14 years (Dawson *et al.*, 1992). The commercially available immunoassays for identification of specific anti-HEV antibodies in acute phase infection yield different specificities and sensitivities, resulting in variation in seroprevalence rates seen in different studies.

In general, the variability in strains of HEV causes significant modifications of antigenic sites and these have very important effects in the development of reliable assays. In spite of this observed variability, the four established HEV genotypes share cross-reactive neutralizing domain along the capsid protein ORF2 comprising amino acids 458-607 (Mushahwar, 2008). In the light of this, available commercial assays have a sensitivity of between 90-97% and false positives of up to 2.5% (Drobeniuc *et al.*, 2010); several of these assays produce false negative results in patients infected with genotype 1 HEV. Newer attempts at development of diagnostic assays dwell on improvement of recombinant ORF2-derived antigens expressed in *Baculovirus* or *Escherichia coli*; those assays which use VLP technologies in eukaryotic-expressing systems appear to be more appropriate. This more appropriate condition has resulted in the high improvement in the sensitivity of ORF2- based assays; the specificity still requires improvement in low endemic countries.

2.10.1.1 Enzyme immunoassay

The ELISA is very user friendly, sensitive and affordable tool for the determination of anti-HEV antibodies in humans and reservoir animals. Antigenic domains which are required for detection are located in all three ORFs of HEV: in ORF1, about 12 antigenic domains exist especially in the RNA-dependent RNA polymerase domain; in ORF2, six antigenic domains have been identified so far; and in the ORF3, three antigenic domains exist (Khudyakov *et al.*, 1999). Recombinant proteins which appear to produce better results and originate mainly from ORF2 and ORF3 C-end or from a larger ORF2 fragment and the complete ORF3 are used for detection of IgM and IgG of HEV. Synthetic peptides have also been developed and used recently with encouraging results in serum antibody detection of HEV but antibodies could not be detected in the convalescent stage due to low sensitivity.

Commercially, three main ELISA kits for HEV serum antibody detection are available. Two are available from Genelabs® and they employ four short recombinant proteins sourced from ORF2 (42 amino acids) and ORF3 (33 amino acids) for the genotype 1 (Burmese) and genotype 2 (Mexican) strains for general detection of IgM and IgG. The third ELISA kit is Abbott®, with its two peptide antigens derived from complete ORF3 (123 amino acids) of the genotype 1 strain.

The evaluation of the sensitivity, specificity, positive predictive and negative predictive values of serological tests is difficult (Vasickova *et al*., 2007). Over and above the observable differences in sensitivity and specificity among commercially available assays, most anti-IgM detection assays are better than anti-IgG assays (Mirazo *et al*., 2014). In practice, it is possible to raise sensitivity up to 100% and also increase specificity by combining IgM and IgG from same manufacturer or two different IgM detection assays. This approach is only feasible in nonendemic areas and for use as screening tools in the identification of acute HEV infections in immunocompetent patients.

Apart from the inconsistencies reported in commercial immunoassays, false reactivity in anti HEV IgM with other liver viruses like *Epstein-Barr virus* (EBV) and *Cytomegalovirus* (CMV) is documented. This is a major clinical problem particularly because these viruses are part of the most important differential diagnosis of acute hepatitis in the absence of hepatitis A virus. In retrospective study of serological cross reactivity (Hyams *et al.*, 2014), only 1.9% and 9.3% were clearly IgM and IgG positive. Cross-reactivity to anti-HEV IgM with EBV and CMV were relatively high at 33.3% and 24.2% respectively; only 13.3% of tested samples which were IgM positive correlated to HEV PCR positivity.

Acute HEV infection diagnosis in patients with illnesses linked to immunocompromise or immunosuppression like HIV, lymphoma or leukemia and solid organ transplant is very pivotal and has been investigated. In these patients, serological response to HEV infection is either delayed or totally absent. Human sera from organ transplant recipients, haematological patients and panels of sera from HEV-infected immunocompetent patients and HEV-negative blood donors were compared by two leading HEV diagnostic companies in Europe (Abravanel *et al.*, 2013). Both IgM assays demonstrated good specificity and sensitivity in immunocompromised patients and remarkably lower in immunocompetent HEV-infected group. The assays failed to detect IgG antibodies in acute phase indicating that the commercialy available kits in those settings were reliable as first line tools for diagnosis of acute HEV infection, in both healthy and immunocompromised patients.

2.10.1.2 Fluorescent microbead-based immunoassay (FMIA)

FMIA employs microspheres which are detected by a specific spectrum. Results point to an improvement in sensitivity of HEV detection than conventional ELISA assays. The assay also possesses the capacity to identify more than one pathogen in a single reaction, thus saving labor and time and costs. A highly sensitive and specific HEV FMIA was recently developed for the detection of swine anti-HEV IgG (Owolodun *et al.*, 2013). This assay demonstrated 100% specificity and 100% sensitivity.

2.10.1.3 Immune fluorescence microscopy (IFE)

Some highly specialized laboratories in industrialized countries use this method for identification of anti-HEV antibodies. The technique detects antibodies semiquantitatively. Anti-HEV antibodies successfully block the binding of fluorescein-conjugated anti- HEV IgG to HEV antigen in frozen liver tissues and the concentration of anti-HEV antibodies are estimated semiquantitatively (Krawczynski and Bradley, 1989).

2.10.1.4 Immune electron microscopy (IEM)

This technique is not a common detection tool. It has the capacity to detect virus like particles in diagnostic samples (Balayan *et al.*, 1983). Using native antibodies from acute or convalescent-phase serum, HEV particles are precipitated and anti-HEV antibodies are then semi-quantitatively determined by estimating the coating of the antibodies. IEM possesses a high level of specificity but it is deficient in sensitivity and therefore it is not recommended for routine diagnostic functions. The technique is laborious and time consuming and above these, most clinical specimens do not contain sufficient amounts of detectable virus like particles.

2.10.2 Virus Isolation

The major problem with isolation of HEV is the identification and establishment of a practical and sustainable cell culture system which will allow the growth of HEV. Several in-vitro cell culture systems have been developed, eg., macaque hepatocytes and human lung, kidney or liver. Most of these culture systems do not offer high titres of virus like particles or ideal HEV particles; they also lack reproducibility (Worm *et al.*, 2002) and until recently there was no reliable cell culture system for HEV propagation. The first cell culture systems to be developed were capable of releasing infectious HEV progenies in high titres in culture media using PLC/PRF/5 and A549 cells from human lung cancer cells (Okamoto, 2013). Recently, Berto *et al*. (2013b) developed a 3D cell culture system with the capacity to replicate HEV. Not only did this culture system replicate HEV in human hepatoblastoma PLC/PRF/5 cells for up to 5 months, it gave evidence that HEV progeny were viable and was able to infect fresh 3D cultures. This culture system is able to produce infectious hepatitis E virions. Other functional cell culture systems are swine cell (LLC-PK1) and human cell (HepG2/C3A) (Okamoto, 2013), though the former was more efficient than the latter. The cell culture systems along with reverse genetics system have the capacity to solve several problems posed by the HEV syndrome and provide answers to questions surrounding the epidemiology of HEV infection.

2.10.3 Molecular diagnosis

RT-PCR is one the conventional methods for the detection of viral RNA, not only in sera and faeces of humans during acute stages of the infection but also in clinical samples of animals like wild boar, Sika deer, mongoose, chickens and also contaminated water (Drexler *et al*., 2012;Haqshenas *et al*., 2001;Krog *et al*., 2013;Li *et al*., 2014;Takahashi *et al*., 2011). This technique consists of two or three steps (i.e nested RT-PCR): reverse transcription in the first step, utilizes specific primers, Oligo DT or random hexamers to rewrite viral RNA to cDNA. The second or next step is PCR or nested PCR using specific primers to amplify specific segments of viral RNA. Nested PCR may however be prone to contamination.

The increasing evidence of diverging performance abilities of commercially available and in-house diagnostic serological assays especially in endemic but also nonendemic regions, together with false negative results and cross-reaction with some hepatotrophic viruses gives room for debate on whether molecular based techniques should be performed on all HEV suspected cases or not. In recent times, nucleic-acid based technologies like nested real-time RT-PCR and RT-PCR have been extensively used as first choice tests for sensitive and specific tools for detection of RNA genome viruses. These methods are very important in the molecular characterization of diverse strains of HEV whose serological status were not detectable by some tests, most of which are in HEV-nonendemic areas (Worm *et al*., 2002). The World Health Organization (WHO) Expert Committee on Biological Standardization approved the establishment of an international standard for the detection and quantification of HEV RNA through the Paul-Ehrlich-Institute, Germany (Baylis *et al.*, 2013). It also provides a control material (genotype 3a) for comparing analytical sensitivities of various nucleic acid based techniques. The variability observed in sensitivity of nucleic acid based assays highlights the need for standardization of genotype-specific nucleic acid techniques.

Despite the heavy reliance on molecular methods, their overall sensitivities depend much on timely reporting and collection of serum, stool or both samples. It also relies on appropriate transportation and storage systems. HEV RNA is detectable at the onset of infection and remain for up to 4 to 6 weeks in serum and stool respectively, but viral RNA may also be low thereby reducing chances of detection.

Real-time RT-PCR is a reliable, fast and dependable technique for initial screening of large samples in surveillance situations; it is applicable in both biological and environmental samples. One such widely used quantitative PCR was developed by Jothikumar *et al.* (2006). This assay targets a short fragment within the HEV ORF3 and it is broadly reactive, detecting all four HEV genotypes. Other quantitative PCR approaches exist but with various sensitivity and specificity ratios. However, for HEV detection, identification of viral RNA remains the gold standard for confirmation of acute hepatitis of HEV origin since they detect active infection and also confirm immune diagnosis (Mirazo *et al*., 2014). The drawback of these molecular methods is that they are extremely expensive, may not be readily available in diagnostic laboratories especially those in resource limited nations and they require specialized equipments and trained personnel.

2.11 Treatment and Clinical Management of HEV infection

In general, acute HEV infections are self limiting in immunocompetent patients, although some of these patients may require symptomatic treatment. In patients with debilitating disease conditions such as underlying liver disease or immune suppressing conditions, HEV infection may progress to FHF or chronic liver disease. In these situations, administration of Ribavirin (antiviral agent) is reported to effect complete recovery and skip the need for liver transplant (Gerolami *et al.*, 2011). A major problem with HEV (most especially genotype 1) is its propensity to progress to fulminant hepatitis in pregnant women with concomitant high mortality. There are no records of treatment of HEV in pregnant women with acute Hepatitis E, in non-pregnant patients with acute hepatic failure or during neonatal infection. The cases described above most commonly occur in highly endemic regions, differ significantly from chronic HEV infection because the range of time available for effective treatment is often shorter (Arends *et al.*, 2014).

Chronic HEV infections are mostly observed in highly immunosuppressed patients. A marked reduction of immunosuppressive therapy in (solid organ transplant) SOT patients, most especially agents which target T cells results in obvious viral clearance in about one-third of patients (Kamar *et al.*, 2011b); in the remaining SOT patients antiviral therapy is often administered. It is noteworthy to point out that all data enumerated are based on case reports and series as no randomized treatment trials have been carried out so far (Kamar *et al*., 2014). From experience, a three-month therapy of pegylated interferon therapy (135µg/week) was administered to three liver transplant patients and a haemodialysis patient who previously received a kidney allograft; a sustained virological response was observed in all four patients (Kamar *et al.*, 2010a;Kamar *et al.*, 2010b). Interferon cannot be utilized post kidney, heart and lung transplantation due to increased risk in tissue rejection. Therefore, short courses of ribavirin monotherapy are recommended with excellent results. The mechanism of viral clearance by ribavirin is still unclear but maybe related to inhibition of viral replication or immunomodulatory effect (Feld and Hoofnagle, 2005).

A few studies have been performed on HIV-HEV infected patients. In one study, five patients were administered antiviral therapy: one patient received pegylated interferon for six months, another patient ribavirin for three months, two patients received ribavirin for six months and a fifth patient received pegylated interferon for six months followed by a combination therapy with pegylated interferon and ribavirin for three months. There was a sustained virological decline in all the patients except the patient with a six-month ribavirin monotherapy (Kamar *et al*., 2014).

Pegylated interferon alone and ribavirin alone for three months have been used experimentally with sustained virological response in three haematological patients with chronic HEV infections (Mallet *et al.*, 2010).

A few patients with acute HEV genotypes 1 and 3 infections were successfully treated with antiviral therapy. They were considered to be high risk patients with either preexisting chronic liver disease or severe fulminant hepatitis; they were treated with ribavirin monotherapy resulting in rapid viral clearance (Gerolami *et al*., 2011;Goyal *et al.*, 2012).

Suitable prophylaxis must be deployed to prevent HEV infection in especially high risk groups such as pregnant women, immunosuppressed patients and solid organ transplant recipients.

2.12 HEV Risk Groups in the Population

Specific high risk individuals or groups have been defined by the WHO.

i. Individuals living in areas or quarters where community outbreaks occur
ii. Individuals travelling to regions with endemic occurrence of Hep E
iii. Individuals living in overcrowded refugee camps like Sudan, Somalia, Kenya and Ethiopia. Recently refugee camps have increased globally due insurgent activities; countries like Syria, Iraq, Congo DRC, South Sudan and Nigeria are at high risk.
iv. People with chronic liver disease
v. Individuals working with non-human primates, pigs, cows, sheep and goats.

2.13 HEV Vaccines

The development of safe, effective and cheap hepatitis vaccine is instrumental to global public health especially in developing countries because antiviral therapy is very unlikely to change the general epidemiology of HEV and burden of infection. The exact populations that need to be vaccinated is not yet agreed upon once an acceptable vaccine is derived. Those populations that are at high risk may be the earliest and prime targets.

All HEV strains belong to one serotype (Emerson *et al*., 2006), therefore any Hep E vaccine that elicits antibodies or demonstrates efficacy to one HEV genotype may also offer immunological protection against other genotypes (Li *et al.*, 2005a).

Several attempts have been made at the development of efficacious and immunological responsive HEV vaccines (Arankalle *et al.*, 2009;Cheng *et al.*, 2012;Kamili *et al.*, 2002). Early experiments with HEV vaccines were carried out in Cynomolgus macaque; results indicated that immunization with fragments of truncated ORF2 protein evokes a protective immune reaction against homologous wild-type HEV challenge. Subsequent challenge studies proved that macaques which were vaccinated with a baculovirus-expressed 55-kDa protein from ORF2 (genotype 1) were protected against heterologous challenge with the HEV Mexican strain (genotype 2) (Purdy *et al.*, 1993). A HEV ORF2 DNA vaccine was evaluated in a group of Cynomolgous monkeys challenged with a heterologous Mexican strain HEV, all macaques seroconverted and were protected against the challenge virus (Kamili *et al*., 2002).

Rabbits have also been used as models for HEV vaccine trials (Cheng *et al*., 2012). In this trial, groups of rabbits were intravenously inoculated with different strains of genotypes 1, 3, 4 and rabbit HEV. Following three doses of the candidate vaccine (HEV p179), the animals were challenged with field strains of HEV. The HEV genotype 1 was found not to be infectious to the rabbits challenged, but were infected by the rabbit strains and the genotypes 3 and 4 strains. Infection status was confirmed by viral shedding in faeces, histological changes and elevation of serum enzyme values. Two vaccine doses of 10μg and 20μg were effective in preventing alanine transferase (ALT) elevation in the serum post HEV challenge. All the animals in the 20μg group did not show viral excretion in faeces but 40% of those in the 10μg group showed evidence of viral shedding and was consistent with previous reports. This set of experiments showed that rabbits could serve as a suitable non-primate model for HEV infection and subsequent vaccine trials.

In all, two candidate recombinant HEV vaccines have been developed and tried on human subjects and were both found to be safe and immunogenic (Kaba *et al*., 2013;Kamar *et al*., 2014). The first vaccine rHEV, is a 56-kDa protein encoded by the ORF2 of a HEV genotype 1 strain was expressed in insect cells (*Spodoptera frugiperda*). Its overall immunogenicity and safety were evaluated in a cohort of male army volunteers in Nepal who were HEV-free (Shrestha *et al.*, 2007). The efficacy of the three-dose intramuscular vaccination at 20μg/dose at month 0, 1 and 6 was 95% (CI: 85.6-98.6) and were followed up for 804 days, by which time the efficacy had waned to 56.3%. The second vaccine (HEV 239), is a 26-kDa protein encoded by ORF2 of HEV genotype 1, expressed in *Escherichia coli*(Zhu *et al.*, 2010). The phase three trial took place in 11 townships in China, where volunteers received three intramuscular doses at month 0, 1 and 6 or hepatitis B vaccine as placebo and were monitored till month 19. The vaccine was well tolerated and protective against Hepatitis E with efficacy rate of 100% (95% CI: 72.1-100.0). The vaccine has been approved for use in the Peoples' Republic of China.

2.14 Prevention and Control of HEV Infection

Almost all HEV infections are distributed by the faeco-oral route. Thus good personal hygiene, high quality standards for public water supply and proper disposal of sanitary waste in developing countries are strongly recommended. Travelers to highly endemic regions are advised to adhere to basic local food hygiene practices such as avoidance of drinking water or ice of unknown origin, eating of uncooked shellfish and pork products or unpeeled vegetables.

Hepatitis E of genotype 1 origin may be prevented in developing countries by availability of potable drinking water and observation of sanitation and hygiene. HEV genotype 3 infection is preventable by avoiding consumption of raw and undercooked meats, most especially pork and pork products. Research shows that HEV can be totally destroyed when subjected to temperatures greater than 70°C (Emerson *et al.*, 2005b). Infection can also be prevented by proper handling of wildlife meat and byproducts, thorough cooking of venison and education of those at risk.

2.15 HEV in Nigeria

Nigeria is a HEV-endemic country and HEV RNA has been detected in the major HEV reservoir, the pig (Owolodun *et al*., 2014). HEV was also previously identified in humans in south-south Nigeria where HEV genotype 2 was confirmed (Buisson *et al*., 2000). There is a growing body of evidence implicating swine as the major reservoir globally. However, the two studies which demonstrated the circulation of HEV in Nigeria did not show that the same viral genotypes were involved. Invariably, it suggests that the HEV circulating in swine are completely different and are independent of the virus circulating and causing disease in humans in Nigeria. Besides these two reported studies, a few other reports are available to demonstrate HEV circulation in humans, although they only detected anti-HEV antibodies.

Adesina *et al*., 2009, conducted a serological study to determine the occurrence of HEV in Ekiti state. The study population recruited for the study was 360 and the age ranges included were from 1-10 years to greater than 70 years old. In general, the study population was categorized into healthy pregnant women, healthy individuals and sick individuals visiting a University

Teaching Hospital. Overall, about 25 (13.4%) of all individuals tested were positive for HEV antibodies. Males were found to have a higher prevalence than women; also seroprevalence increased with age for both sexes, ie., 7.8% among 11-20 year olds to 20% in 61-70 year olds. The limitation of that study was that all samples were collected from a hospital setting and so could not give the true population prevalence and the study did not indicate whether it was IgM or IgG that was assayed for.

In a similar study in Ibadan, Odaibo and Olaleye (2013) investigated the circulation of anti-HEV antibodies in HIV positive patients. The study determined both IgM and IgG in anti-retroviral therapy (ART) naïve and experienced patients visiting the ARV treatment clinic of the University College Hospital, Ibadan. The combined rate of positivity was 12.2%; 11.1% were positive for IgG while 1.7% positive for IgM. The IgG prevalence was higher among ART drug naïve patients. Only two individuals were positive for anti-HEV IgM and were also ART drug naïve. The study was also limited by study subjects who were drawn from those visiting the hospital. However, it provides a baseline mark for further studies on HEV prevalence in Ibadan and its environs.

In a separate study in Ibadan, health care workers (HCW) were screened for anti-HEV antibodies (Ola *et al.*, 2012) in association with nosocomial infection. Sera from 88 HCWs and 44 non-HCWs were obtained and tested for antibodies using commercially available kits. Anti-HEV antibodies were estimated at 43.0% among HCWs and 94.0% among controls. The prevalence was highest among surgeons (55.0%) and least among paediatricians (18.0%).

In north-central Nigeria, a seroprevalence study was conducted in domestic animals in Plateau state (Junaid *et al.*, 2014). The animal species included in the study were pigs (67), goats (43), sheep (19) and cattle (37). The overall prevalence of anti-HEV antibodies was 24.1%. The HEV

IgG seroprevalence was 16.3% while IgM was 7.8%. Results from the study revealed that goats had a higher seroprevalence (37.2%) than pigs (32.8%). The number of study subjects was too small to extrapolate for the entire Plateau state especially given the fact that pigs are regarded as the main reservoir for HEV globally.

Most of the HEV studies carried out in Nigeria were serological based; therefore there is a dearth of information on the molecular identification of HEV in both humans and animals. There is a great need for increased surveillance activities in general human and animal populations to be able to establish true prevalence and identify all current strains of HEV and determine whether there are common strains in humans and the main animal reservoir of HEV.

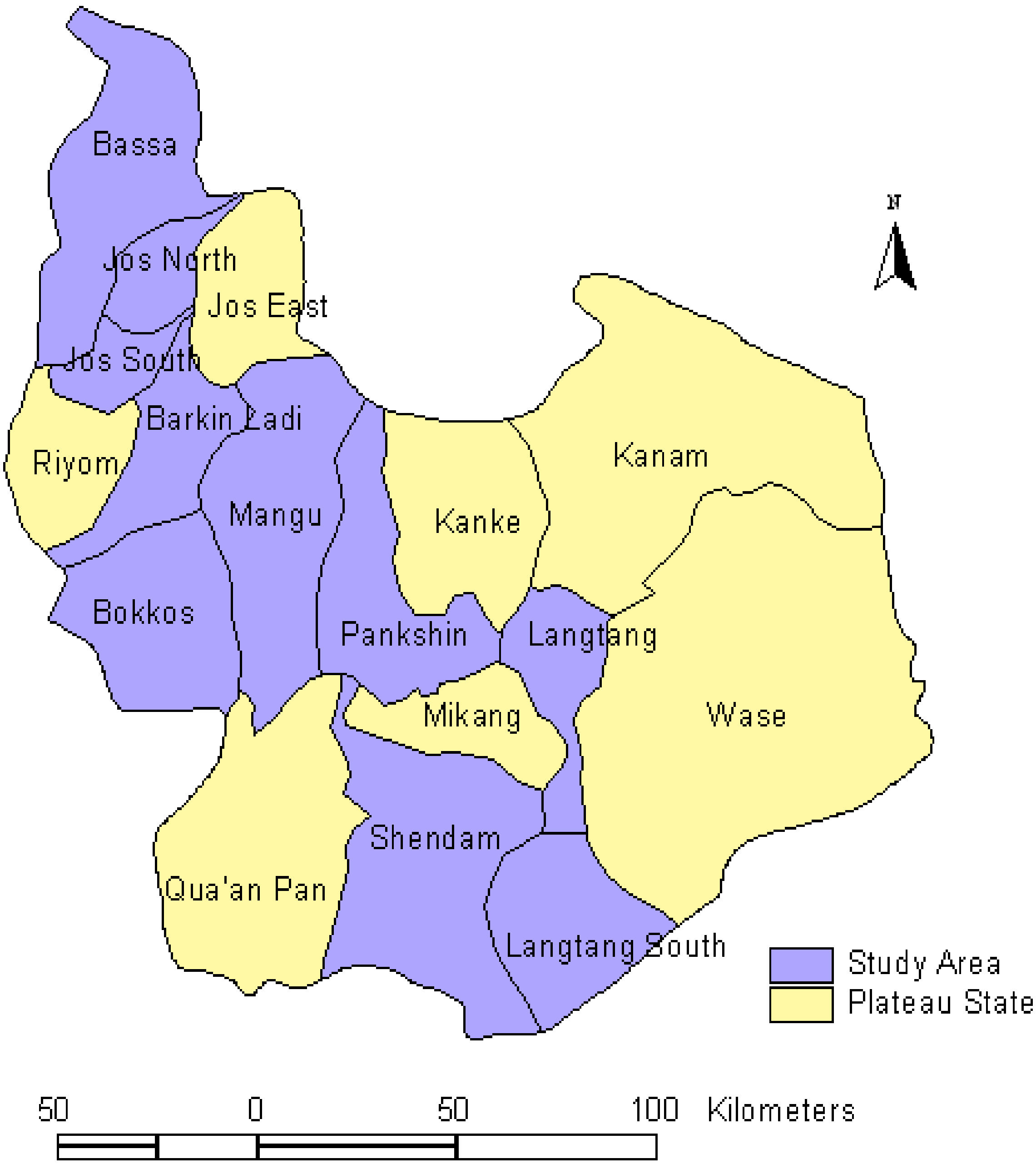
CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Areas- Plateau State

Plateau state is is located in the north-central part of the country, which corresponds to the Guinea savannah zone. Plateau state shares geographical borders with four other Nigerian states namely Bauchi state to the north-east, Taraba state to the south-east, Nasarawa state to the southwest and Kaduna state to the north-west. The state has a land mass of 26, 899 square kilometers and a population of about three million people. The altitude of the state ranges from 1,200meters to a high peak of 1,829meters above sea level. It comprises of deep gorges and lakes as a result of extensive tin mining activities.Plateau state is situated in a tropical zone but exhibits temperate-like climate; it has average temperature of 18°C and 22°C. The weather is coldest between December and February due to north-east harmattan winds, and the warmest temperatures occur between the months of March to April. Mean annual rainfall varies, between 131.75cm in the south to 146 cm on the Plateau. The state consists of granite rock dating 160 million years old. Various historical volcanic activities make Plateau state a mineral rich region.

Plateau state is an agricultural zone with high plant and animal husbandry activities. The state is a pig producing state responsible for large proportion of pigs consumed in southern part of Nigeria. Incursion of African swine fever (ASF) in 2001 decimated millions of pigs and reduced pig production to backyard pig farming in recent years. The study area (for faecal sample collection) comprised of 10 selected local government areas (LGA) namely Bassa, Jos-north, Jos-south, Barkin-Ladi, Bokkos, Mangu, Shendam, Pankshin, Langtang-north and Langtang-south (Fig 3.1). These LGAs were purposively selected. Blood/serum was collected from three sites in Plateau state: The Jos main abattoir, Mangu slaughter slabs and Langtang slaughter slabs.



Source: Map created by Dr. Pius S. Ekong, 2013

Figure 3.1: Geographical map of Plateau state indicating the study areas in blue.

3.1.1 Study Design

The study design was a cross-sectional study. Sampling started in 2011 and ended in 2012. Pig population for the entire state could not be determined and could not be accessed, due to the fact that pig population census has not been carried out in Plateau state in recent time and most pig farms are scattered and backyard in nature. The criteria for selection of the LGAs were availability of pigs as indicated by the Plateau State Ministry of Agriculture, Department of Veterinary Services. All sick animals and animals whose owners were not favorably disposed to sample collection were excluded. Target animals were apparently healthy pigs of all ages, both sexes and breeds available for sampling.

3.1.2 Sampling Technique

A non-probability convenience sampling method was employed, (Noordhuizen *et al.*, 2001) and animals were sampled from backyard farms (household farms) who were willing to cooperate in sampling, as quite a number of pig farmers declined participation for fear of introduction of ASF into their farms.

3.1.3 Sample size

Sample size for faecal collection was calculated using the formula, n = Z^2pq/d^2(Noordhuizen *etal*., 2001), where:

n= Sample size
p= 50%
q= 1-p
Z= appropriate value for the standard normal deviation for the desired confidence = 1.96
d= 5% allowable error

50% was chosen because at the time of sampling there was no literature available for prevalence rate, therefore a maximum of 50% was taken into effect.

Total sample size calculated from the formula above was 384.However, since this study was first of its kind in Plateau state and Nigeria as a whole, sample size was increased to 500so as to increase chances of detection of the virus.

3.1.4 Faecal sample Collection

Sample collection was carried out with pigs under standard restraint; young pigs were held by the forelimbs by an assistant with heads pointing down. About 10-20 grams of fresh faeces was collected per rectum from pigs on site by a gentle digital manipulation, 50 samples were collected from each LGA (a total of 184 herds were sampled and maximum number of pigs sampled per herd was 9). A fresh latex glove was used each time for a different animal. Evacuated faeces were transferred into well labeled 20 ml Falcon tubes (Sterilin UK®). Every tube was labeled serially according to LGA of origin, sex and age. All samples were then placed on wet ice and transported to the National Veterinary Research Institute (NVRI), Vom. All samples were transferred into -80°C freezers for long term storage. Faecal samples were next shipped to Emerging Pathogens Laboratory, Department of Virology, Central Veterinary Institute, Edelhertweg, Lelystad, The Netherlands on dry ice.

3.1.5 Blood/serum Sample Collection

Blood/serum was collected from three sites in Plateau state. The Jos main abattoir, Mangu slaughter slabs and Langtang slaughter slabs were selected for blood collection. These points were selected based on availability of slaughter pigs on designated slaughter days. About 10 ml of blood was collected from the jugular vein of pigs at slaughter and retained in slanting position to promote blood clotting and separation of serum. All pigs presented for slaughter were sampled each time. Three aliquots of each serum sample were made and properly labeled. Aliquots of sera were transferred in clearly labeled 1.8 ml micronic cryovials (Sterilin®) and immediately placed on wet ice (Ice packs) and taken to NVRI, Vom. Samples were transferred to -80°C freezers till use. A total of 500 sera samples were collected: Jos main abattoir (n=300), Mangu area (n=100) and Langtang area (n=100).

3.2 Shipment of Samples to Specialized Laboratories in the USA and The Netherlands

Both faecal and sera samples were split into three aliquots depending on sample quantity. The complete listing of field samples was provided and export permits were sought through the Federal Department of Veterinary & Pest Control Services, Federal Ministry of Agriculture and Rural Development, Abuja. Import permits were also sought from the Veterinary Diagnostic Laboratory, College of Veterinary Medicine, Iowa State University Ames and the Department of Virology (Animal Sciences Group), Central Veterinary Institute, Lelystad, The Netherlands. The samples were packaged in dry ice resistant polythene bags containing 100 samples each. A triple packing was done for the samples and placed in dry ice-filled boxes and shipped to the specialized laboratories mentioned above via World Courier®.

3.2.1 Sample Sorting and Archiving

On arrival in The Netherlands and USA, all samples were moved into a specialized P3 laboratory and sorted according to LGA of origin and given specific laboratory numbers. Samples were stored in reserve freezers (-80°C). All shipping containers were disinfected and destroyed by incineration.

3.3Detection of anti-HEV IgG by in-house ELISA

A commercially available *Escherichia coli*-derived recombinant protein about 44kDa spanning the 452-617 aa of the HEV ORF2 (Genway®, California, USA) was used as antigen. This protein is known to contain immunodorminant regions and has a glutathione S-transferase (GST) tag. Briefly, the ELISA was performed according to protocols described (Meng *et al.*, 1997), with the following minor modifications. One hundred microliters of the antigen was used to coat each well of a microtitre plate (Nunc, Thermo Fischer Scientific Inc) at a concentration of 0.6 µg per ml in phosphate buffered saline (PBS) with a pH of 7.4. The plates were incubated at room temperature overnight. Antigen concentration was optimized for sensitivity and specificity purposes. The microtitre plate was washed four times with PBS containing 0.05% Tween20 (wash buffer) and subsequently blocked with 1.0% foetal bovine serum (FBS) (Gibco®, New York, USA) at room temperature for 2 hrs. Serum samples at a dilution of 1:100 with serum dilution buffer (PBS containing FBS) were added into each well and incubated for 30 min at 37º C. The plates were washed four times and 100 µl of a 1:25,000 dilution of horse radish peroxidase goat anti-swine IgG conjugate (Jackson ImmunoResearch, Inc, USA) was added into each well and incubated at 37ºC for 30 min. Plates were again washed four times and the reaction was visualized by using tetramethylbenzidine-hydrogen peroxidase (TMB) as substrate (KPL, Gaithersburg, MD, USA). The reaction was terminated after 15 min by adding 50µl of 2.5M sulphuric acid to each well and the plates read at 450nm using a spectrophotometer.

3.3.1 Evaluation of IgG Results

Raw data were transferred unto spreadsheets and index ratios were calculated by applying the formula, Index = sample OD/Cut-off control.

i. Grey range lower limit- cut-off value.

ii. Grey range upper limit- cut-off value x 1.2.

iii. All samples above grey range were considered positive.

iv. All samples with values below the grey range were considered negative.

v. All samples with values which fall within the grey range area were considered suspect.

Preparation of appropriate buffers are presented in Appendix I.

3.4 Detection of anti-HEV IgM by CVI ELISA format

A recombinant baculo-protein in coating buffer was used to coat the polystyrene plates and incubated over-night. Into each well, 80µl of serum dilution buffer was dispensed with a multi-channel pipette; and 20µl of positive reference serum into wells A1 and B1, negative reference serum into wells C1 and D1. 20µl of diluted test serum was dispensed into each well according to test panel schedule. The Polysterene plate (Costar 9018) was sealed and left to incubate for 1 hr at room temperature. The ELISA plate was washed three times using an ELISA plate washer. The MCA goat anti-swine IgM Horse Radish Peroxidase conjugate was diluted 1:2000 and the MCA anti-swine L-chain conjugate 1:1000 in conjugate buffer. Then 100µl of dilute conjugate was dispensed into each well. The ELISA plate was covered with a plate sealer and incubated for 1 hr at room temperature. The plates were again washed three times with wash buffer containing 0.05% Tween80. To visualize the antibody-antigen reaction, 100µl of TMB substrate was dispensed into each well and incubated for 10 min at room temperature in the dark. The ELISA reaction was terminated by the addition of 100µl of 0.5M sulphuric acid into each well. The OD_{450} of the substrate was read in a Multiscan ELISA reader within 15 min after stopping the reaction(van der Poel *et al.*, 2014).

3.4.1 Interpretation of IgM Test Results

The mean OD450 values of the positive reference sera = OD_{Max}, and the negative reference sera =OD_{Neg}, were calculated. The corrected OD of the mean positive sample = Corrected OD Max was calculated by subtracting the mean OD_{Neg} value from OD_{Max}.

The Percentage Positivity, PP for each test sample was calculated according to the formula:

PP of sample = (OD450/corrected OD_{Max})X 100

*Samples with PP values <25PP were considered negative and samples with PP values 25PP and above were positive.

Appendices I, II and III provide details on preparation of buffers, storage of buffers and preservation of coated ELISA plates respectively.

Controls Samples *(in duplicate)*

	1	2	3	4	5	6	7	8	9	10	11	12
A	Cc	Cc	1	5	9	13	17	21	25	29	33	37
B	C++	C++	1	5	9	13	17	21	25	29	33	37
C	C++	C++	2									
D	C+	C+	2									
E	C+	C+	3									
F	Cm	Cm	3									
G	Cm	Cm	4									40
H	C-	C-	4									40

Cc = conjugate control (no serum/no monoclonal antibody)
C++ = strong positive serum
C+ = moderate positive serum
Cm = monoclonal antibody control (no serum)
C- = negative serum control

Figure 3.2: Schematic representation of the ELISA template used in this study.

3.5 Detection of HEV using One-Step qRT-PCR

3.5.1 Faecal Sample Preparation

Sample preparation for PCR was carried out from faeces. A 10% faecal suspension was prepared with PBS (Sigma-Aldrich Inc., Saint Louis, MO, USA). Briefly, 0.1 gm of frozen faeces was weighed out and deposited into a 1.5 ml Eppendorf tube containing 900µl of PBS. Each tube was vorted at maximum capacity (2500 rpm) and centrifuged for 10 min at 8, 000 x g. The supernatants contained viral particles and the sediments only faecal material and intestinal debris.

3.5.2 Genomic RNA Isolation

Viral genomic RNA was extracted from 10% faecal suspensions using the QIAamp® MinElute® Virus Spin kit (QIAGEN, Germany). Viral genomic RNA extraction was carried out according to manufacturer's instructions from 200µl of faecal supernatants. Briefly, 25µl of protease was aliquoted into sterile 1.5 ml eppendorf tubes and 200µl of faecal suspensions were added. Next, 200µl of buffer AL was added and vortexed for 15 sec. Mixture was incubated on heating block at 56ºC for 15 min. This stage was followed by addition of 250µl of absolute ethanol and vortexing for 15 sec. 500µl of buffer AW1 was added and centrifuged at 8, 000 rpm for 1 min, followed by addition of 500µl of buffer AW2 and centrifugation at 8, 000 rpm for 1 min. To the column, 500µl of absolute ethanol was added and centrifuged. The QIAamp MinElute column was placed in a sterile 1.5 ml microcentrifuge tube and 100µl of buffer AVE was added to the middle of the silica membrane and centrifuged at 14, 000 rpm for 1 min.HEV control standards and nuclease-free water (negative control) were subjected to extraction procedures and were also re-suspended in 100µl of AVE elution buffer. Aliquots of extracted RNA were stored at -80°C till utilized.QIAamp MinElute Virus Spin Kit Components are listed in Appendix IVa and reagent preparation IVb.

3.5.3 HEV Real-Time RT-PCR

Quantitative real-time PCR was carried out according to protocols described by Jothikumar *et al*. (2006). The master mix for qPCR (Table 3.1) was carried out in a SterilGARD Biosafety cabinet class II (The Baker Company®, Sanford, Maine, USA) after it was disinfected and exposed to UV light for 30 min. All necessary pipettes, Eppendorf and PCR tubes were specific for this cabinet and were also sterilized by UV light. Master mix was performed and aliquots of all reagents were mixed in a 1.5 ml eppendorf tube and subsequently split into the corresponding number of test samples. A qPCR kit RNA UltraSense™ One-Step qRT-PCR System (Invitrogen™, Carlsbad, CA, USA) was used for master mix. The primers JVHEVF and JVHEVR target the ORF3 of the HEV genome (Table 3.2). Amplifications were carried out in a 20µl reaction volume consisting of 10µl of extracted RNA and 1µl enzyme mix; forward primer, reverse primer and probe concentrations were 10µM. One-Step qRT-PCR was performed with a LightCycler® 480 thermal cycler apparatus (Roche Diagnostics, GmbH, Germany), a 96-well platform. Thermal cycling profile used was: reverse transcription at 50°C for 15 min, followed by 95°C for 2 min. DNA amplification for 45 cycles at 95°C for 10 sec, 55°C for 20 sec and 72°C for 15 sec. Positive and negative controls were included in each run.

Table 3.1: Real-Time RT-PCR Master Mix: Invitrogen®

Reagent Components	Conc.	1X
Nuclease-Free H_2O		3.8µl
5X RT-PCR buffer		4.0µl
JVHEVF	10µM	0.5µl
JVHEVR	10µM	0.5µl
Probe	10µM	0.2µl
Enzyme mix		1.0µl
		10.0µl
RNA template		**10.0µl**
Total Reaction Volume		**20.0µl**

Table 3.2: Oligonucleotide primers used in qPCR

Primer	Orientation	Target	Nucleotide sequence 5'-3'
JVHEVF:	Sense	ORF3	5'-GGT GGT TTC TGG GGT GAC-3'
JVHEVR:	Antisense		5'-AGG GGT TGG TTG GAT GAA-3'
JVHEVP:	Probe		5'-(FAM) TGA TTC TCA GCC CTT CGC(BHQ)-3'

3.6 cDNA Synthesis

All qPCR positive faecal samples were selected for conventional PCR. RNA was converted to cDNA prior to PCR. cDNA synthesis was carried out according to protocols developed by National Institute of Public Health (RIVM), Bilthoven, The Netherlands (Table 3.3). Briefly, five microlitres of RNA was deployed for cDNA synthesis in a reaction volume of 10µl comprising of 200U/µl of Superscript II RT (Invitrogen, CA), 40U RNase Inhibitor, 0.1M DTT, 10mM dNTPs (Takara, Japan), 5x RT Buffer (Invitrogen™, Carlsbad, CA, USA) and 100µM external forward primer HEV222alt (Eurogentec, San Diego, CA, USA). cDNA profile was 22°C for 10 min, 42°C for 60 min, inactivation at 95°C for 5 min and immediately used for PCR.

Table 3.3: cDNA Master Mix

Reagent Components	Conc.	1X
5X RT Buffer		2.0µl
dNTPs	10mM	1.0µl
DTT	0.1M	0.5µl
HEV222alt (Ext. Forw. Primer)	100µM	0.5µl
RNasin	40U	0.5µl
Superscript II RT	200U/µl	0.5µl
		5.0µl
	RNA template	**5.0µl**
	Total Reaction Volume	**10.0µl**

3.7 HEV Conventional PCR

Only qPCR positive samples were subjected to nested PCR, therefore, qPCR was only used as a screening tool. Real-time PCR was used to quantitatively detect HEV only and so lacked the capacity to distinguish among circulating HEV genotypes. This conventional end-point PCR was embarked upon to provide DNA material which will be useful for sequencing and further characterization of available pig strains in Nigeria.

All faecal samples with Ct values 38 or less than 38 in the real-time RT-PCR were identified and subjected to a conventional nested RT-PCR. Nested PCR targeting partial regions of ORF2 was carried out on all qPCR positive stool samples according to protocols described previously by National Institute of Public Health (RIVM), Bilthoven, The Netherlands on a GeneAmp PCR System 9700 (Applied Biosystems, USA). The external PCR was carried out in a 50µl reaction volume consisting of 10X PCR buffer (Invitrogen™, Carlsbad, CA, USA), 50mM $MgCl_2$(Roche, Germany), 10mM dNTPs mix, 100µM external reverse primer HEV224alt, 2.5U of Taq DNA polymerase (Invitrogen™, Carlsbad, CA, USA) and 10µl of cDNA (Table 3.4). A positive HEV control, Netherlands strain and a non-tempelate control were included in each run. Thermal cycling conditions consisted of denaturation at 95°C for 2 min, 35 cycles of 95°C for 30 sec, 42°C for 30 sec and 60°C for 45 sec; amplicons were held at 4°C.

Table 3.4: External PCR Master Mix

Reagents Components	Conc.	1X
MilliQ H_2O		31.5µl
10X PCR Buffer	1X	5.0µl
$MgCl_2$	50mM	1.5µl
dNTPs	10mM	1.0µl
HEV224alt	100µM	0.5µl
Taq polymerase	2.5U	0.5µl
		40.0µl
	cDNA	**10.0µl**
	Total Reaction Volume	**50.0µl**

The internal (nested PCR) was similarly carried out in a total volume of 50µl, consisting of 10X FastStart Taq buffer (Roche Diagnostics GmbH, Mannheim, Germany), 10mM dNTPs, 100µM forward and reverse primers HEV-AN88alt and HEV-AN89alt respectively (Eurogentec, San Diego, CA, USA), 2.5U of FS Taq Polymerase (Roche Diagnostics GmbH, Mannheim, Germany) and 1µl of outer PCR product (Table 3.5). This preparation was submitted to the following PCR conditions: initial denaturation at 95°C for 6 min, followed by 40 cycles of 95°C for 30 sec, 60°C for 20 sec and an extension at 72°C for 15 sec. Products were held at 4°C. The external and internal amplicon sizes are 900bp and 580 bp respectively. Details on primers used for both outer and internal PCRs and sequencing are available in Table 3.6.

Table 3.5: Master Mix for Internal PCR

Reagents	Conc.	1X
MilliQ H_2O		42.1µl
10X FS PCR Buffer	1X	5.0µl
dNTPs	10mM	1.0µl
HEV-AN89alt	100µM	0.2µl
HEV-AN88alt	100µM	0.2µl
FS Taq Polymerase	2.5U	0.5µl
		49.0µl
First round PCR product		**1.0µl**
Total Reaction Volume		**50.0µl**

- Expected band size = 580bp

Table 3.6: Oligonucleotide primers used in nested PCR (RIVM, Bilthoven, Netherlands)

Primer	Orientation	Target	Nucleotide sequence 5'-3'	Size
HEV222alt:	Sense	ORF2	5'-CCI GCY CCI TCI CGY CCI TTY TC-3'	
HEV224alt:	Antisense		5'-AAY CAR GGI TGG CGY TCI GTI GAR AC-3'	
				900bp
HEV-AN89Falt:	Sense	ORF2	5'-GAG GAG GAA GCT ACC TCY GGY YTI GTI ATG CTY TGY AT-3'	
HEV-AN88Ralt:	Antisense		5'-AAY CAR CAY GAR CAR GAY CGA CCA ACT CCT TCT CC-3'	
				580bp
HEV-AN89seq:	Sense	ORF2	5'-GAG GAG GAA GCT ACC TC-3'	Sequencing
HEV-AN88seq:	Antisense		5'-CGA CCA ACT CCT TCT CC-3'	primers

3.8 Agarose Gel Electrophoresis and Image Capture

A 1.5% agarose gel was prepared by weighing out 1.5 gm of Agarose MP powder (Roche Diagnostics GmbH, Mannheim Germany). This was dissolved in 100 ml of 1X Tris-Boric acid-EDTA (TBE) under rotation in a microwave oven set to maximum heat capacity. About 5µl of Molecular Biology Grade Ethidium bromide (5µg/ml) (Promega®, Madison, USA) was added to the molten liquid to stain. This was performed in a PCR Work station and allowed to cool. The gel was cast in a BioRad® electrophoresis assembly containing a tray and 'comb' for well formation.

About 5 µl of all test samples were pippetted out and thoroughly mixed with 6X Orange DNA gel loading dye to prevent floating of amplicons. Eight µl of each amplicon was added to 2µl of loading dye on a parafilm (Pechiney® Chicago, Illinois, USA). The firm gel was placed in the electrophoresis tank containing 1X TBE. Altogether, 10µl of amplicons + loading dye was transferred into each well in the stained gel. Positive and negative reference controls were also loaded simultaneously. A 200bp ready –to-use DNA ladder (Promega®, Madison, WI, USA) was included in each electrophoresis run for discrimination of bands. Electrophoresis was performed at 120 volts for 55 min.

The gel was visualized in an improved gel documentation system, Molecular Imager® Gel Doc™ XR^{+} (BioRad Laboratories www.bio-rad.com). The gel was visually observed and analyzed with the help of the DNA ladder for band size estimation. Gel images were corrected and adjusted accordingly and were captured and saved in appropriate format (jpeg). A 580 bp fragment is expected as confirmatory for all four mammalian HEV genotypes and strains (RIVM, Bilthoven, The Netherlands).

3.8.1 Amplicon excision and purification

Following 55 min of amplicon separation in a 1.5% agarose gel, discreet amplicon bands of interest were identified/extrapolated against the molecular weight marker. UV protective goggles and gown were worn to prevent irradiation. Specific bands of interest were neatly cut out from the gel under UV light each with a sterile new scapel blade, care was taken to avoid excess gel surrounding bands of interest. These were deposited in pre-weighed Eppendorf tubes for easy calculation of reagent ratios during purification.

The amplified DNA was purified using the Zymoclean™ Gel DNA Recovery Kit (www.zymoresearch.com). It provides for quick recovery of ultra-pure DNA through the use of column and filtration design. This kit utilizes the advantage of avoidance of glass beads and organic denuratants and it elutes DNA suitable most for downstream operations including DNA sequencing. Briefly, three volumes of ADB buffer was added to each known gel weight and incubated at 55°C for 10 min. The melted solution was transferred into a Zymo-spin column placed in a 2 ml collection tube and centrifuged at 8, 000 rpm for 10 sec; the eluent was discarded. Then 200µl of wash buffer was added to the column and centrifuged at 8, 000 rpm for 10 sec, immediately after which 200µl of wash buffer was added again and centrifuged for 10 sec. The eluent was discarded. This step was repeated for the third time. The Zymo-Spin column was placed in to a sterile, labeled 1.5 ml Eppendorf tube and 10µl of DNAse-RNAse free water was added to the centre of the column matrix and allowed to stand at room temperature for 5 min. The labeled tubes were centrifuged at 13, 000 rpm for 30 sec. The pure sterile DNA was collected in the 1.5 ml tubes. The ultra pure DNA was quantified spectrophotometrically at wavelength 260 nm and stored at 4°C till use.

3.9 Nucleotide Sequencing

Following purification and quantification, 10µl of each amplicon was transferred into specially provided tubes and labeled accordingly. Both forward and reverse sequencing primers (Table 3.6) were prepared into 10µM each and dispensed into 20µl aliquots and included in the shipment. The amplicons were packaged and sent for commercial sequencing at BaseClear BV, Leiden, The Netherlands at room temperature.

3.9.1 Sequence assembly and similarity search

Successful nucleotide sequences were received online as *.seq*, *.scf* and *.seq* formats. Nucleotide sequences were analyzed and assembled using online programs: Chromas® (http://technelysium.com.au/?page_id=13), BioEdit (Hall, 1999) and MEGA6 (Tamura *et al.*, 2013). The sequences were checked for base calling and edited accordingly. Nucleotide BLAST homology search was performed with the 580 bp partial HEV sequences on the BLASTn programe of the PubMed database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for identification of reference strains which were included in the multiple sequence alignments. Highly similar viral sequences were identified by percentage identity status and were subsequently used in the final analysis. Using the BioEdit programe, HEV nucleotide sequences from this study were aligned to determine their similarity or diversity with each other. Reference HEV sequences from the Gen Bank data base were also aligned with sequences from this study. All study and reference sequences were aligned using ClustalW in the MEGA6 programe and trimmed for phylogenetic analysis.

3.10 Phylogenetic Analysis

Phylogenetic analysis was performed using the MEGA6 software programe (Tamura et al., 2013). The phylogeny reconstruction was based on the statistical method of Neighbor-joining and bootstrap method was the test of phylogeny for robustness utilized 1, 000 replications. The method of inference was p-distance approach. All the 19 successful sequence taxa were included in the analysis.Different phylogenetic trees were constructed; initially all recovered HEV sequences from this study were aligned and submitted to Neighbor-joining tree construction. The tree was observed for geographical clustering.HEV sequences from this study were subsequently aligned with global prototype HEV genotypes and a neighbor-joining tree was constructed with a robustness of 1, 000 replications; this phylogenic analysis groups HEV sequences of the same genotype together. For all trees constructed, avian hepatitis virus USA (AY043166) was used as outgroup.

3.11 Subtyping of HEV Sequences

In order to determine the subtype of the 19 HEV ORF2 sequences from Plateau state, a subtyping scheme evolved by Lu *et al*. (2006) was employed. Published sequences from all 10 subtypes of G3 HEV were mined from the Gen Bank and added to the alignment and sequence analysis of HEV sequences from this study. Two tree statistical methods were created in order to establish Nigerian HEV subtypes and to check whether the tree type could change identity of the subtypes; both Neighbor-joining and UPMGA methods were deployed using 1, 000 replications each time to test tree robustness.

3.12 Nucleotide sequence analysis andPairwise distances computation

A comparison of nucleotide sequences of the 19 taxa was done to observe for polymorphisms. Sequence alignment in ClustalW of MEGA6 was used. Since HEVs especially genotype 3 are known to be highly diverse, the evolutionary divergence (genetic distances) between isolates and among all HEV sequences in this study was computed using the MEGA6 software programe. The resulting distances are presented in a diagonal form.

3.13 Amino Acid Sequence Analysis

The deduced amino acids of the HEV sequences recovered from this study were subject to analysis. Also included in this analysis were sequences from all four major global mammalian prototype strains of HEV for comparison. All 19 Plateau state strains and seven prototype strains were aligned with the ClustalW alignment program of MEGA6 and trimmed. All sequences were also analysed based on plot to first sequence for easy comparison. A Nigerian sequence from this study, BK20-HEV-swNig was used as reference strain (first sequence). Prototype strains used are:M73218 Bur-82 (Burma 1a)-Burmese strain, M80581 Sar-55 (Macaca Pakistan 1b), M74506 (M1 Mexico 2a)-Mexican strain, AB097812 (HE-JA1 Japan 4c)- Japan Asian strain, AY594199 (swCH25 Pig 4d)-China Asian strain, AF060668 (HEV-US1 3a) and AB189070 (JBOAR1-Hyo04 3b)-Japanese strain.

3.13.1 Conserved sites

The deduced amino acid sequences were analyzed for conserved domains within the partial ORF2 segment that was amplified. All sequences were plotted to a consensus sequence (BK20-

HEV-swNig) in the MEGA6 software. Though HEV ORF2 is not known to be a highly conserved region, short stretches of the sequence are conserved and therefore these regions in Nigerian swine viruses were compared to such regions in the prototype strains. The resulting comparison is presented as plots.

3.13.2 Variable sites

The HEV strains from Plateau state were analyzed based on variable sites compared to prototype strains. Strains of HEV from different geographical regions showed different levels of genetic variation, therefore, variation is unpredictable.

3.13.3 Parsimony informative sites

Analysis of the inferred amino acids of the partial ORF2 sequence was performed. This was done and compared to published HEV mammalian prototype strains in the GenBank. Analysis was performed on the MEGA6 software following ClustalW alignment.

3.14 Data Presentation, Analyses and Archiving

All data from the current study were segregated into serological data and data on molecular analysis. Data on serological studies were coded and presented in contingency tables and further analyzed by chi-square and odds ratio and 95% confidence level on the odds ratio were calculated to assess associations between HEV positivity and sex of pigs, location of sampling and breed of pig sampled. Positivity or negativity of both seum and faecal samples were presented as prevalence rates in percentages. Sera were analyzed based on age group of pigs, sex and breed. For age, pigs were categorized into pigs < 9 months of age and those > 9 months of age. For breed, pigs were grouped into indigenous and mixed breed populations; while sex was stratified into male and female. Molecular analysis (Real-time RT-PCR) was presented as percentages (prevalence rates) with respect to location of sampling. Overall data on molecular analysis were summarized as statistical cladograms (phylogenetic trees) and further comparisons of gene sequences are presented as figures and sequences to first plots.

CHAPTER 4

4.0 RESULTS

4.1 Seroprevalence of HEV Plateau State

The occurrence of both IgM and IgG anti-HEV antibodies were investigated in pigs in three major slaughter areas in Plateau state; Jos main abattoir, Mangu slaughter slabs and Langtan g slaughter slabs. Anti-HEV antibodies were detected in sera from all three slaughter areas but with different levels of seroprevalence. The overall seroprevalence of HEV in Plateau state was 71.1%.

4.2 Seroprevalence of HEV in JosArea

The overall seroprevalence of HEV in Jos is 67.5% out of 301 serum samples tested. Of the 102 from <9 month-old pigs, 85.3% were IgM positive while 47.0% were IgG positive and of the pigs >9 months, 91.3% were IgM positive and 44.3% IgG positive. There was no statistically significant association between age and seropositivity (OR=1.8, 95% CI= 0.9-3.8). About 90.5% of 116 of female pigs were IgM positive and 45.7% IgG positive and 163 of the 185 males were positive. Female pigs in Jos area were 0.8 times less likely to have acute HEV infection than males though this was not statistically significant (OR=0.8, 95% CI on OR=0.4-1.7).

Indigenous breeds were found to be less likely to be IgM positive (O.R= 0.8, CI: 0.4-1.7) and also less likely to be IgG positive (O.R= 0.8, CI: 0.5-1.3) than exotic breeds (Table 4.1)

Table 4.1: Seroprevalence of HEV in pigs slaughtered in Jos main abattoir

		IgM				IgG			
		No. Tested	No. Pos(%)	O.R(95%CI)	P-Value	No. Tested	No. Pos(%)	O.R(95%CI)	P-Value
***Age**	**<9M**	102	87(85.3)	Ref		102	48(47)	Ref	
	>9M	183	167(91.3)	1.8(0.9-3.8)	0.2	183	81(44.3)	0.9(0.6-1.5)	0.7
Sex	**F**	116	105(90.5)	Ref		116	53(45.7)	Ref	
	M	185	163(88.1)	0.8(0.4-1.7)		185	86(46.5)	1.0(0.6-1.6)	0.9
Breed	**Ind**	124	112(90.3)	Ref		124	61(49.0)	Ref	
	Mix	177	156(88.1)	0.8(0.4-1.7)		177	78(44.0)	0.8(0.5-1.3)	0.5

Pos.= positive, O.R= Odd's ratio, CI= Confidence interval, Ref= Reference, Ind= Indigeneous, *Age in months

4.3 Seroprevalence of HEVin Mangu Area

A total of 99 sera samples were collected from slaughter slabs in Mangu area. The overall seroprevalence of HEV is 72.7%. IgM prevalence is 93.9% and IgG is 51.5%.Based on IgM, 90.0% of the 30 pigs younger than 9 months were positive while 56.7% were IgG positive. Younger pigs were more likely to have IgM antibody than older ones (O.R= 2.4, 95% CI: 0.5-12.9).The IgM ELISA result revealed that 41out of 44 female serum samples were positive representing 91.2%, while 52 of the 55 male samples were IgM positive (94.6%) (Table 4.2). There was no statistically significant association between sex and seropositivity (O.R= 1.3, 95%CI: 0.2-6.6). The seroprevalence of IgM in indigenous breeds of pigs was 95.5%, and that of mixed breed pigs was 92.7%. Breed association was not statistically significant (O.R= 0.6; 95%CI: 0.1-3.5). The IgG seroprevalence was 47.7% in indigenous pigs and 54.6% in mixed breed pigs indicating that indigenous pigs were also less likely to be IgG positive (O.R= 1.3; CI: 0.6-2.9).

Table 4.2: Seroprevalence of HEV in slaughtered pigsin Mangu area

		IgM				**IgG**			
		No. Tested	No. Pos(%)	O.R(95%CI)	P-Value	No. Tested	No. Pos(%)	O.R(95%CI)	P-Value
***Age**	**<9M**	30	27(90.0)	Ref		30	17(56.7)		Ref
	>9M	69	66(95.7)	2.4(0.5-12.9)	0.5	69	34(44.3)	0.7(0.3-1.8)	0.7
Sex	**F**	44	41(91.2)	Ref		44	24(54.6)	Ref	
	M	55	52(94.6)	1.3(0.2-6.6)		55	27(49.1)	0.8(0.4-1.8)	0.7
Breed	**Ind**	44	42(95.5)	Ref		44	21(47.7)	Ref	
	Mix	55	51(92.7)	0.6(0.1-3.5)		55	30(54.6)	1.3(0.6-2.9)	0.6

Pos.= positive, O.R= Odd's ratio, CI= Confidence interval, Ref= Reference, Ind= Indigeneous, *Age in months

4.4 Seroprevalence of HEV in Langtang Area

The overall seroprevalence of HEV in Langtang slaughter area was 73.0%.In Langtang area, 100 serum samples were collected and analyzed by both IgM and IgG ELISA techniques. Of the 100 samples, 33 belonged to young pigs <9 months old, while 67 were from older pigs. Stratified by age, 93.9% of the young pigs were IgM seropositive and were 0.6 times less likely to be IgM positive compared to older pigs (O.R= 0.6; C.I: 0.1-2.8). On the other hand, 63.6% of the younger pigs were IgG positive while only 50.8% of the older pigs were IgG positive. About 86.1% of the 43 female pigs were IgM positive and 46.5% IgG positive. About 94.7% of the 57 male pigs were IgM positive while 61.4% IgG positive. (Table 4.3).

Table 4.3: Seroprevalence of HEV in slaughterrd pigsin Langtang area

		IgM				IgG			
		No. Tested	No. Pos(%)	O.R(95%CI)	P-Value	No. Tested	No. Pos(%)	O.R(95%CI)	P-Value
***Age**	**<9M**	33	31(93.9)	Ref		33	21(63.6)	Ref	
	>9M	67	60(89.6)	0.6(0.1-2.8)	0.7	67	34(50.8)	0.6(0.3-1.4)	0.3
Sex	**F**	43	37(86.1)	Ref		43	20(46.5)	Ref	
	M	57	54(94.7)	2.9(0.7-12.4)	0.3	57	35(61.4)	1.8(0.8-4.1)	0.2
Breed	**Ind**	84	75(89.3)	Ref		84	43(51.2)	Ref	
	Mix	16	16(100.0)	undefined	0.4	16	12(75.0)	2.9(0.9-9.6)	0.1

Pos.= positive, O.R= Odd's ratio, CI= Confidence interval, Ref= Reference, Ind= Indigenous, *Age in months

4.5 Co-detection of IgM and IgG in Serum

The co-circulation of both IgM and IgG revealed a significant presence of both classes of antibodies in serum of pigs in all regions sampled in Plateau state. Out of the 301 serum samples from Jos abattoir, a total of 135 had both immunoglobulin classes; representing 44.9%. In Mangu abattoir, 41 serum samples out of a total of 99 had both IgM and IgG, amounting to 41.4%. Langtang abattoir had the least with 34 samples (36.7%) having IgM and IgG. The total number of samples in Plateau state with co-existence of antibodies was 210 (Table 4.4). This figure represents 41.0% of the total samples collected. Results are summarized in a 2 x 2 contingency table (Table 4.5).

Table 4.4: Distribution of both IgM and IgG HEV antibodies in pigs in Plateau State

Location	No. of sera tested	IgM Positive (%)	IgG Positive (%)	IgM + IgG Positive (%)
Jos abattoir	301	268(89.0)	139(46.2)	135(44.9)
Mangu abattoir	99	93(93.9)	51(51.5)	41(41.4)
Langtang abattoir	101	91(91.0)	91(91.0)	34(36.7)
Totals	**500**	**362(72.4)**	**281(56.2)**	**210(41.0)**

Table 4.5: A 2x2 contingency table of frequency of occurrence of anti-HEV antibodies in pigs, Plateau state

	IgG	IgM	TOTALS
+	281 a	362 b	**643 a+b**
-	219 c	138 d	**357 c+d**
	500 a+c	**500 b+d**	**1000**

H_0: There is no significant difference between IgG and IgM detection in pigs in Plateau state.

H_a: There is a significant difference between IgG and IgM detection in pigs in Plateau state.

Alpha(α) = 0.05; Degree of freedom (df) = (r-1)(c-1)

= (2-1)(2-1)

= (2-1)(2-1)= 1x1= 1

Critical Value (cv) = $\chi^2\alpha$df; χ^2 0.05,1 =$\chi^2\alpha$df=**3.84**

Condition= Reject H_0 if $/\chi^2/ \geq \chi^2\alpha df$

$$/\chi 2/ = \frac{n\left(/ad - bc/ - \frac{n}{2}\right)2}{(a+d)(c+d)(a+c)(b+d)}$$

$$/\chi 2/ = \frac{500\,\left(/281x138 - 362x219/ - \frac{500}{2}\right)2}{(419)(357)(500)(500)}$$

$$/\chi 2/ = \frac{500(/38778-79278/-250)2}{37395750000}$$

$/\chi 2/$ = 500(- 40500)-250)2

37395750000

/χ2/ = <u>500(40250)2</u>
37395750000

/χ2/ = <u>40250000</u>
37395750000/**χ2/ = 0.0011.**

Conclusion: There is no significant difference between IgG and IgM detection in pigs in Plateau state.

4.6 HEV Prevalence in Plateau State by qPCR

Out of the 444 samples screened by qPCR, HEV RNA was identified in 114 samples. HEV RNA was identified in 67 of 184 households representing 36.4%. Individual pig prevalence is 25.7%. HEV Real-time amplification curves are seen in Plate I. The results are summarized in Table 4.4.

Table 4.6: Distribution of HEV RNA positive herds by LGA and proportion of HEV positive pigs

LGA	No. HH Sampled	No. HH Pos. (%)	No. Pigs Sampled	No. Pigs Pos.(%)
Bokkos	20	9(45.0)	47	10(21.3)
Jos-North	9	3(33.3)	45	4(8.9)
Jos-South	18	1(5.6)	41	1(2.4)
Barkin-Ladi	19	6(31.6)	42	10(23.8)
Shendam	20	3(15.0)	46	7(15.2)
Mangu	16	10(62.5)	44	21(47.7)
Pankshin	18	1(5.6)	46	5(10.9)
Langtang-North	27	14(51.9)	50	16(32.0)
Langtang-South	21	14(66.7)	43	27(62.8)
Bassa	16	6(37.5)	40	13(32.5)
Total	**184**	**67(36.4)**	**444**	**114(25.7)**

LGA= Local Government Area; HH= Households, Pos.= Positive

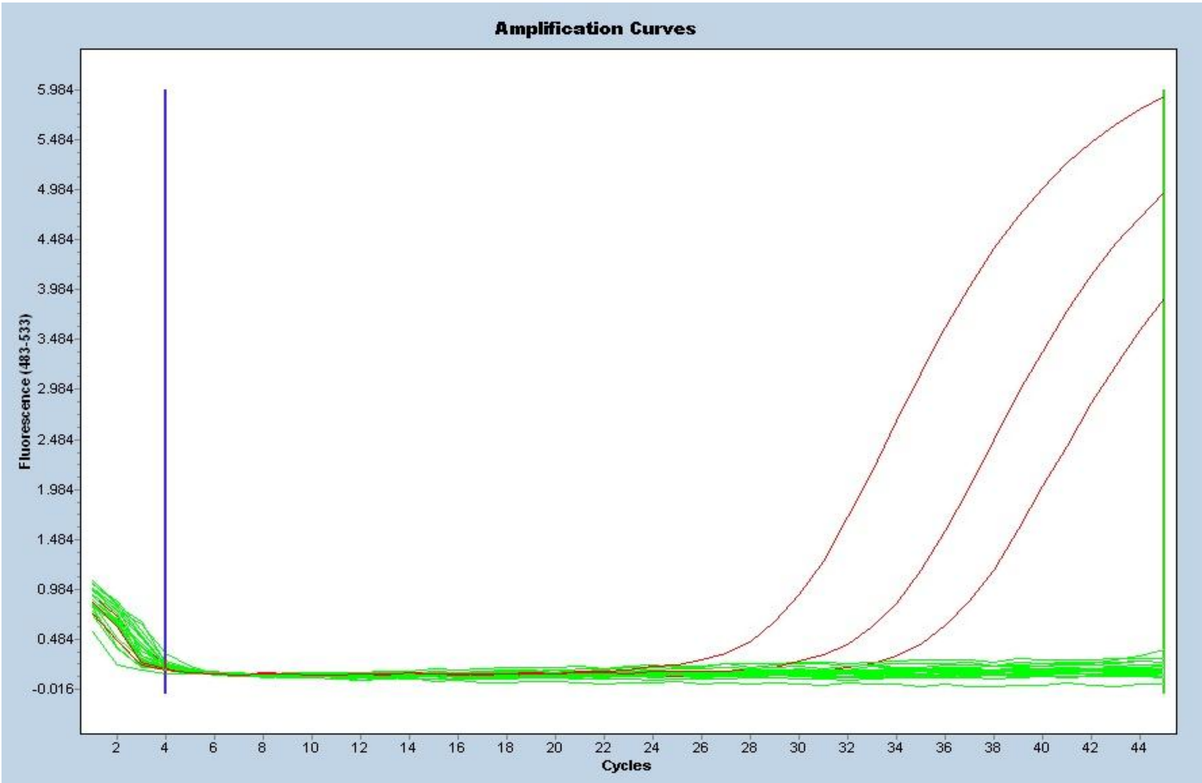


Plate Ia: qPCR amplification plots indicating three positive samples. Positive samples dislay a sinusoidal curve beginning from the X-axis (cycles). Strength of positivity is inversely proportional to Ct value, ie, the lower the Ct value, the higher the strength of positivity.

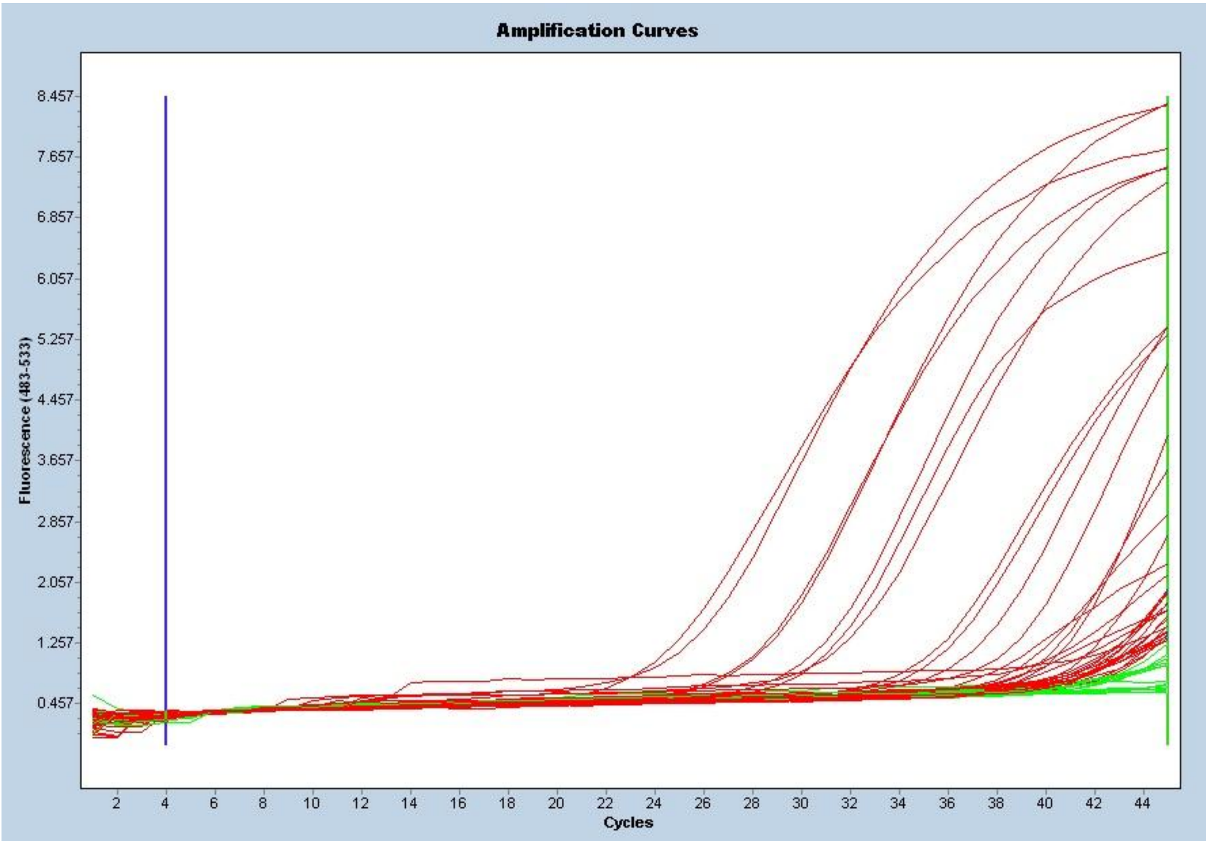


Plate Ib: qPCR amplification plot indicating multiple positive samples.

4.7 Prevalence of HEV RNA in Plateau State Stratified by LGA

When compared to Jos-South, Langtang-North (OR=10.18, 95% CI=1.23-84.27), Langtang-South (OR=30.53, 95% CI=3.58-260.21) and Mangu (OR= 15.47, 95% CI=1.76-135.83) had significantly higher association with HEV prevalence. The highest prevalence 62.8% was obtained in Langtang-South and the lowest (2.40%) in Jos-South (Table 4.5).

Table 4.7: Prevalence of HEV RNA in Plateau State stratified by LGA

LGA	# Samples Tested	# Samples Pos.	% Pos.	O.R	95% CI	p-value
Jos-South	41	1	2.4	1	Ref.	
Barkin-Ladi	42	10	23.8	6.34	0.68-59.17	0.11
Bassa	40	13	32.5	7.27	0.80-66.11	0.08
Bokkos	47	10	21.3	5.83	0.65-52.50	0.12
Jos-North	45	4	8.9	2.36	0.18-31.37	0.52
Langtang-North	50	16	32.0	10.18	1.23-84.27	0.03
Langtang-South	43	27	62.8	30.53	3.58-260.21	0.00
Mangu	44	21	47.7	15.47	1.76-135.83	0.01
Pankshin	46	5	10.9	1.61	0.09-28.09	0.74
Shendam	46	7	15.2	3.62	0.34-38.36	0.29
TOTALS	**444**	**114**	**25.7**			

Overall p = 000.9; $p < 0.05$
O.R= Odds Ratio, CI= Confidence interval, Pos.= Positive

4.8 Nested PCR

All faecal samples with Ct values less than 38 in the real-time RT-PCR were identified and subjected to conventional nested RT-PCR. The PCR product of the internal PCR was used to confirm positive samples, the expected band size was 580bp. Amplicons from this study were analyzed in comparison with a 200 bp molecular weight ladder and all samples from the 114 tested that displayed a discreet, clear amplicon at 580bp were regarded positive for HEV. Summarily, 22 samples were nested PCR positive (nPCR). Some of the nPCR positive samples are presented in Plate II.

4.9 DNA Sequencing, Phylogenetic Analysis and Genotype Assignment

The nucleotide sequences of the 22 nPCR positive faecal samples were determined from the cDNA amplified transcripts. However, 19 sequences were readable and complete, the remaining three were discarded. After checking sequences for base calling, manual editing and trimming, the resultant sequences were submitted to a BLASTn for highest similarity search. Nucleotide sequencing confirmed that all positive samples belonged to HEV genotype 3. Sequencing of three samples from Bokkos, one from Pankshin, five from Langtang-South, five from Mangu, two from Shendam, one from Jos-North and two from Barkin-Ladi LGAs demonstrated that the recovered strains from this study had 90.6% to 100.0% nucleotide identities to each other on the 580 bp partial sequence of the ORF2. The nucleotide sequences of the samples from Plateau state were assembled and subjected to a neighbor-joining phylogenetic analysis. From the inferred cladogram, 4 clusters emerged based on geographic origins. The first cluster contained three sequences from Bokkos LGA with two very closely related (bootstrap support 76.0%) and one of them less closely related. The largest cluster is the cluster containing sequences from Mangu LGA, which in turn contains two sub-clusters. Each of these two sub-clusters has bootstrap support of 98.0%. The third cluster also contains two sub-clusters; one contains 2 sequences from Shendam LGA while the other contains 2 sequences from Langtang South LGA. The fourth cluster is divided into three sub-clusters: 2 sequences from Langtang South LGA group together with 100% bootstrap support indicating that they are very identical to each other; the second contains 2 sequences from Barkin Ladi origin that are also identical and the third sub-cluster comprises of a single sequence from Jos North LGA. This sequence from Jos North is closely related to sequences from Barkin Ladi with nodal support of 88.0%.In close comparison, the most identical sequences are MG7 and MG8 which both share 100.0% nucleotide identity. The least identity is between BK20-HEV-swNig and BL49-HEV-swNig with 90.6% nucleotide identity. In the inter LGA sequence comparison, BK22-HEV-swNig shared 98.0% nucleotide identity, the highest with any other sample; BL37-HEV-swNig shared 96.9% with JN119-HEV-swNig, LS9-HEV-swNig.

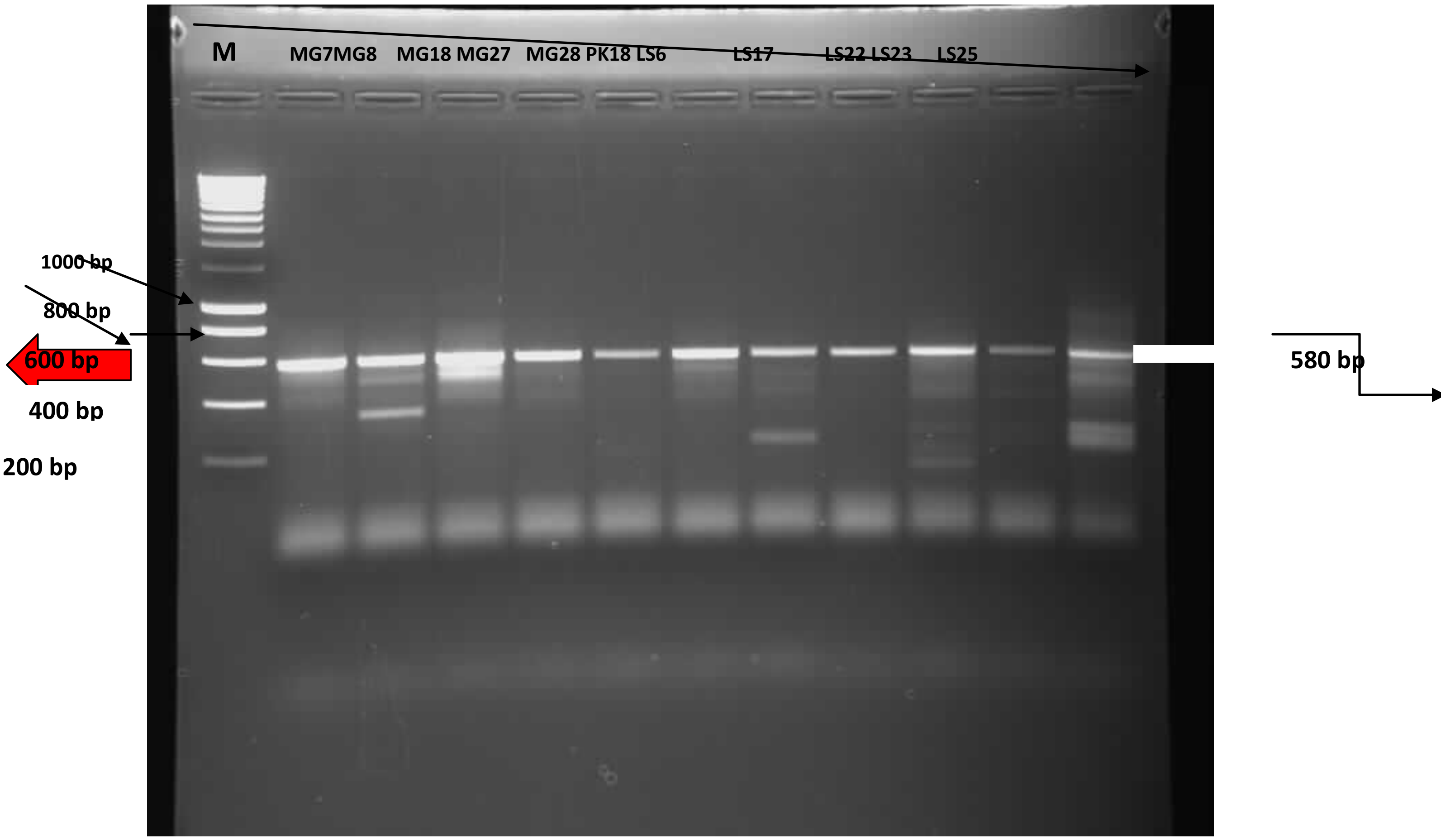


Plate IIa: Agarose gel electrophoresis of HEV ORF2 positive faecal samples. Arrow indicates positive bands at 580 bp. Lane M: 200 bp molecular weight marker, lanes 1-11: (with isolate numbers) are positive for the expected amplicon size.

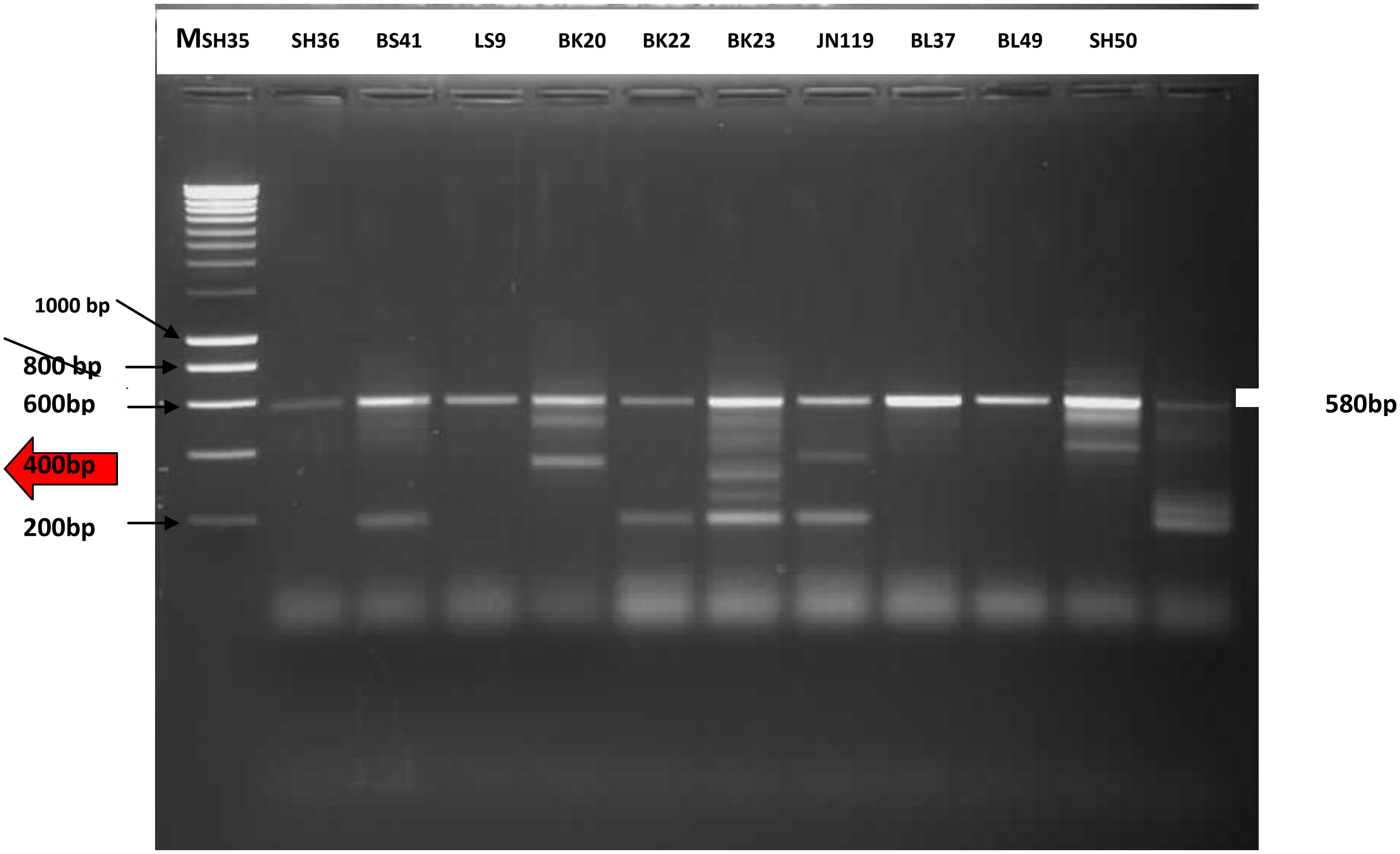


Plate IIb: Agarose gel electrophoresis of HEV ORF2 positive faecal samples. Red arrow indicates positive bands at 580 bp. Lane M: 200 bp molecular weight marker, lanes 1-11: (with isolate numbers) are positive for the expected amplicon size.

shared 96.7% with SH36-HEV-swNig and BK20-HEV-swNig shared 96.2% with PK18-HEV-swNig.

The Nigerian strains from Plateau state are very closely related to HEV strains from Japan (Gen Bank accession number AB248521) and European strains from Hungary (EF530669) and France (JQ953665) with nucleotide identities between 92% and 90%.

All 19 partial ORF2 sequences were included in the first phylogenetic analysis performed to determine the phylo-relationship between these strains and to evaluate for any geographical clustering. A phylogenetic tree of the nucleotide sequences was inferred by the Neighbor-Joining (NJ) method (Figure 4.1). From the tree, local diversity was observed with sequences from Mangu forming a distinct genetic cluster with 96% bootstrap support. The five sequences from Langtang-South showed the highest levels of genetic diversity by separating into three distinct clusters; one of these is LS25-HEV-swNig being more closely related to the isolate from Pankshin LGA, PK18-HEV-swNig than to any other Langtang-South strain. Strains LS22-HEV-swNig and LS23-HEV-swNig share 100% similarity and cluster closely together with a 100% bootstrap support. The Shendam LGA sequences also formed a cluster but were less related to a certain cluster of two from Langtang-South LGA. The only strain sequenced from Jos-North is more closely related to HEV strains from Barkin-Ladi, this genetic relatedness is supported by a nodal agreement of 87%. All three sequences from Bokkos LGA also form a distinct cluster in the tree.

Estimate of the evolutionary divergence among the sequences from this study was also determined; from the chart, the highest number of base difference per site between any two sequences was 0.07(Figure 4.2).

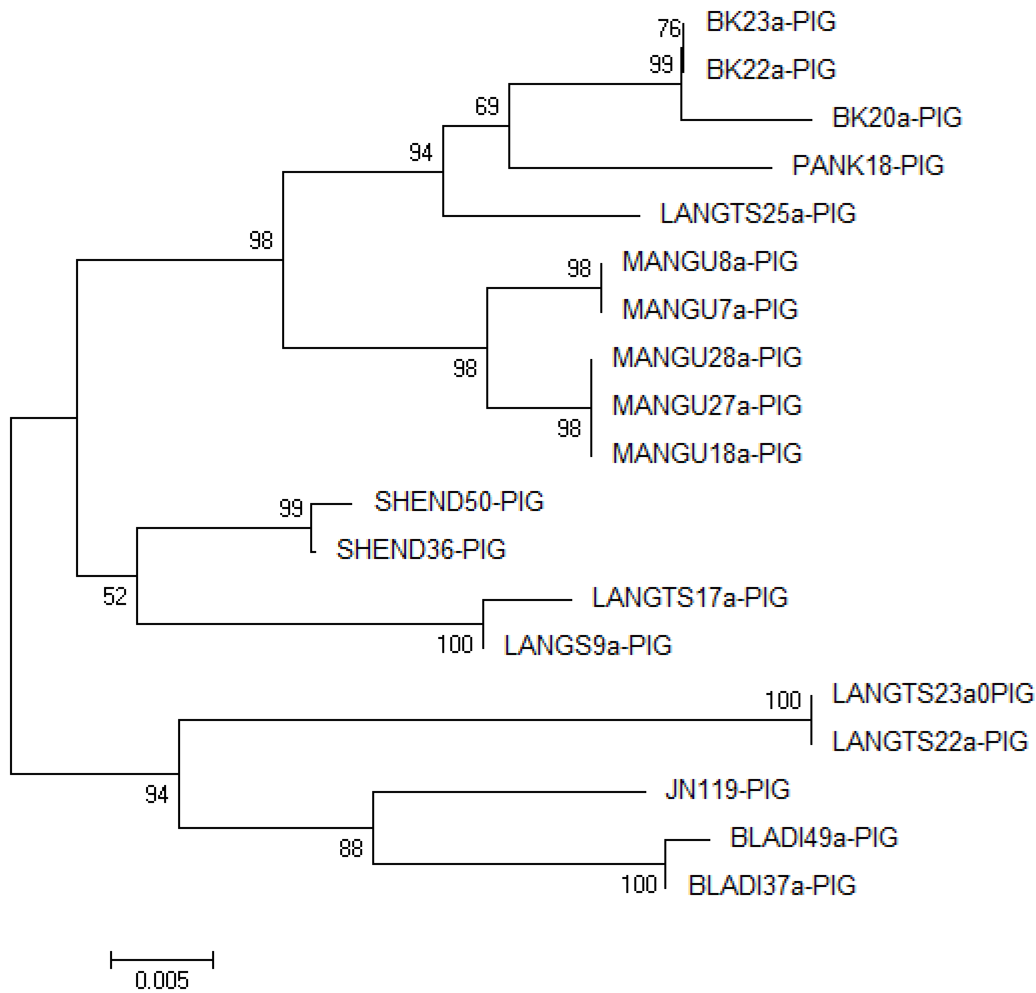


Figure 4.1: A phylogenetic tree constructed with the 19 HEV3 partial coding ORF2 sequences recovered from Plateau state. A NJ consensus tree depicts their genetic relationship. The cladogram which utilized 1,000 replications is shown. The scale bar shown below the tree represents substitutions per site.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1. SHEND50-PIG		0.00	0.04	0.04	0.04	0.04	0.04	0.04	0.03	0.06	0.06	0.03	0.03	0.05	0.05	0.05	0.04	0.04	0.05
2. SHEND36-PIG	0.00		0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.06	0.06	0.03	0.03	0.05	0.05	0.04	0.04	0.04	0.05
3. PANK18-PIG	0.01	0.01		0.04	0.04	0.04	0.04	0.04	0.03	0.07	0.07	0.06	0.05	0.07	0.07	0.07	0.02	0.02	0.03
4. MANGU28a-PIG	0.01	0.01	0.01		0.00	0.00	0.01	0.01	0.03	0.07	0.07	0.05	0.05	0.06	0.06	0.06	0.03	0.03	0.04
5. MANGU27a-PIG	0.01	0.01	0.01	0.00		0.00	0.01	0.01	0.03	0.07	0.07	0.05	0.05	0.06	0.06	0.06	0.03	0.03	0.04
6. MANGU18a-PIG	0.01	0.01	0.01	0.00	0.00		0.01	0.01	0.03	0.07	0.07	0.05	0.05	0.06	0.06	0.06	0.03	0.03	0.04
7. MANGU8a-PIG	0.01	0.01	0.01	0.00	0.00	0.00		0.00	0.03	0.06	0.06	0.05	0.04	0.06	0.07	0.06	0.04	0.04	0.04
8. MANGU7a-PIG	0.01	0.01	0.01	0.00	0.00	0.00	0.00		0.03	0.06	0.06	0.05	0.04	0.06	0.07	0.06	0.04	0.04	0.04
9. LANGTS25a-PIG	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01		0.07	0.07	0.05	0.05	0.06	0.07	0.06	0.02	0.02	0.03
10. LANGTS23a0PIG	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01		0.00	0.07	0.06	0.06	0.05	0.05	0.07	0.07	0.08
11. LANGTS22a-PIG	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00		0.07	0.06	0.06	0.05	0.05	0.07	0.07	0.08
12. LANGTS17a-PIG	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01		0.00	0.05	0.06	0.05	0.06	0.06	0.06
13. LANGS9a-PIG	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00		0.05	0.05	0.05	0.05	0.05	0.06
14. JN119-PIG	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01		0.03	0.03	0.07	0.07	0.07
15. BLADI49a-PIG	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01		0.00	0.07	0.07	0.07
16. BLADI37a-PIG	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00		0.07	0.07	0.07
17. BK23a-PIG	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01		0.00	0.01
18. BK22a-PIG	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00		0.01
19. BK20a-PIG	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.00	

Figure 4.2: Estimates of evolutionary divergence between sequences. The number of base difference per site from between sequences are shown. Standard error estimates are shown below the diagonal and were obtained by a bootstrap procedure of 1000 replicates. The analysis involved 19 nucleotide sequences. All positions containing gaps and missing data are eliminated. There were a total of 470 positions in the final data set.

To determine genotype identity of the Nigerian HEV strains, seven global prototype strains from the GenBank database were added to the analysis and these include two strains belonging to genotype 1, one prototype genotype 2 sequence, two genotype 3 sequences and also two sequences belonging to genotype 4. A NJ tree was constructed and all 19 Plateau state sequences belonged to genotype 3 HEV (Figure 4.3).

The Plateau state swine HEV sequences from this study were compared to a human HEV sequence (AF173231) previously identified in Nigeria (Buisson *et al*., 2000) and swine HEV sequences that originated from other parts of Africa. The human isolate from Nigeria, clustered together with the prototype genotype 2 Mexican isolate (GenBank accession no.: (M74506) confirming its identity as genotype 2. The two African strains from Cameroon (KC012634) and Democratic Republic of Congo (DRC) (FJ600536) were only distantly related to the Nigerian strains but more closely related to each other. The strain from the DRC clustered closely with HEV strain from The Netherlands sharing 95% bootstrap support (Figure 4.4).

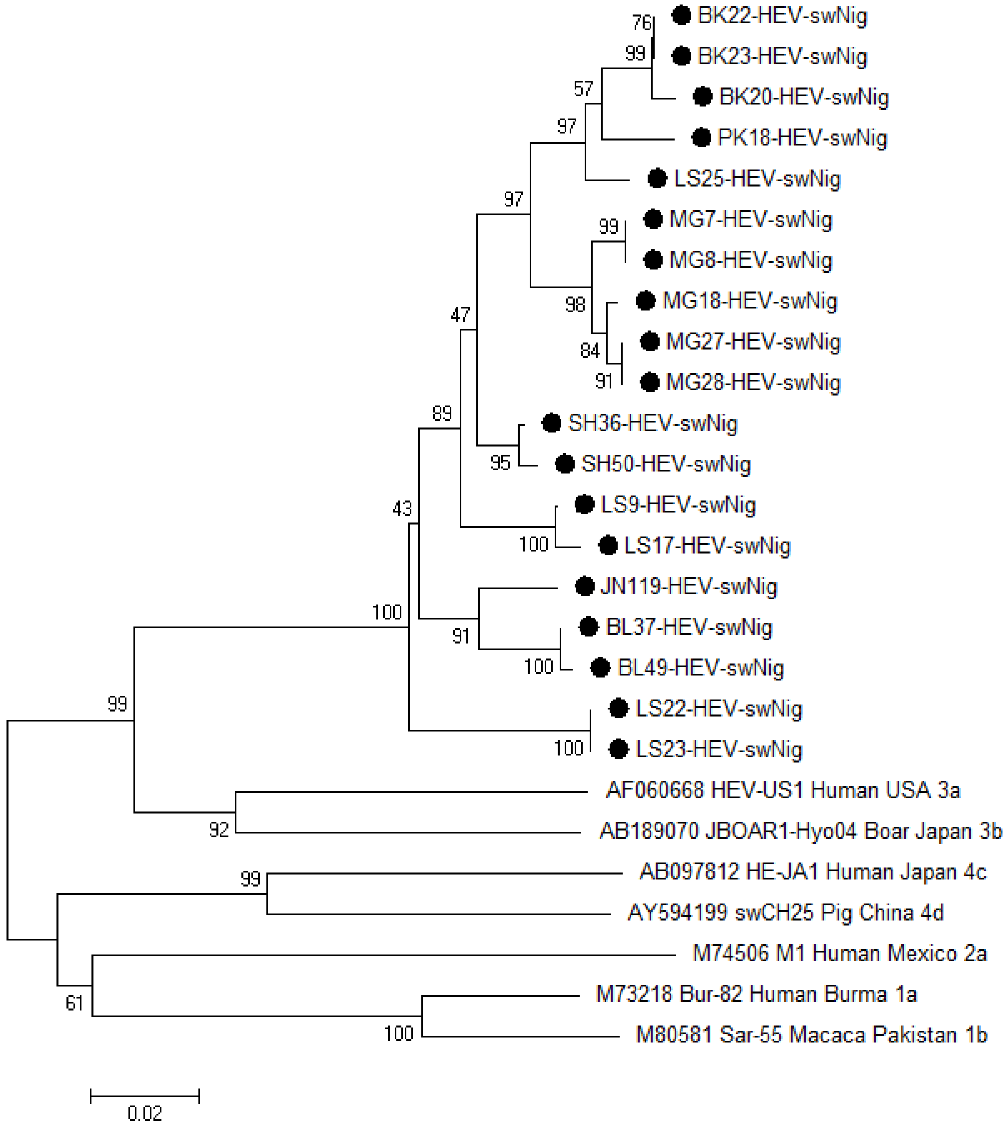


Figure 4.3: Phylogenetic analysis conducted on 580 bp fragment of 19 HEV sequences recovered from this study and seven prototype full genome sequences. A 1000 replicate bootstrap resampling was utilized. Figures at the nodes indicate bootstrap confidence values. All Nigerian sequences are represented by a black circle. The scale bar represents nucleotide substitutions per site.

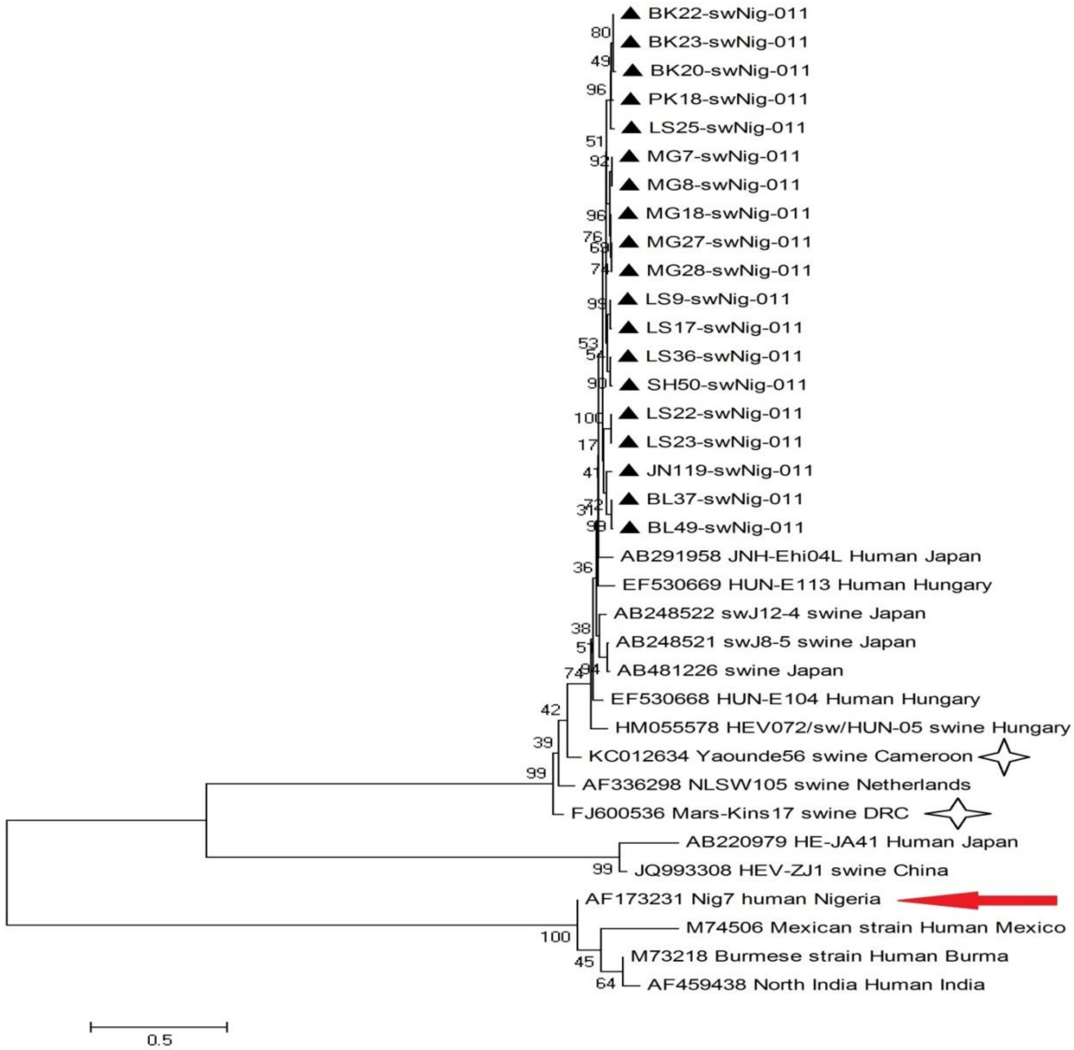


Figure 4.4: Phylogenetic analysis of partial ORF2 HEV sequences from this study with a previously characterized human isolate from Nigeria, swine HEV sequences from Cameroon and DRC. The NJ tree was constructed with 1000 replicates. Sequences from this study are indicated with a triangle, the Cameroon and DRC strains with a star and the human strain from Nigeria with a red arrow. The scale bar represents nucleotide substitutions per site.

4.10 Subtyping of Nigerian Genotype 3 Strains

Further genetic characterization was performed to determine the subtype of circulating swine HEV strains in Plateau state. Twenty four (24) previously published and characterized strains were used for subtyping and a NJ tree was inferred (Figure 4.5). In the subtyping tree, the subset of Plateau state strains grouped most closely to the swine HEV genotype 3e strain (FR-SHEV3e) from France; strains from The UK, Hungary and Japan also belonged to the same group, thus placing the Nigerian strains in subgroup 3e.

4.11 Amino Acid Sequence Analysis

The nucleotide sequence analysis did not reveal unique differences when compared to prototype mammalian genotypes. The region sequenced is a variable region, so genetic differences were spread across the fragment length. However, there were conserved sites.

Sequence analysis could only be meaningful when amino acid translation of the 580-nt region was performed. Following translation, 153 amino acids were deduced and these correspond to 459-nt after treating and trimming. The significance of the amino acid changes were not however evaluated in an in vitro system. Generally, most amino acid positions were conserved among strains recovered from this study.

The following predicted amino acid sites were conserved among all taxa including the prototype strains: A42 except in the Nigerian strains BL37-HEV-swNig, BL49-HEV-swNig, JN119-HEV-swNig and both Chinese genotypes 4c and 4d where it was replaced by a “V”; T46 was conserved in all except Chinese genotype 4d where it was replaced by “I”. The aa position G54 was replaced by “V” in both Chinese genotype 4c and 4d. Position S57 was substituted by “N” in the Chinese strain 4d. One uniquely conserved site was aa position 67 where there was a gap in

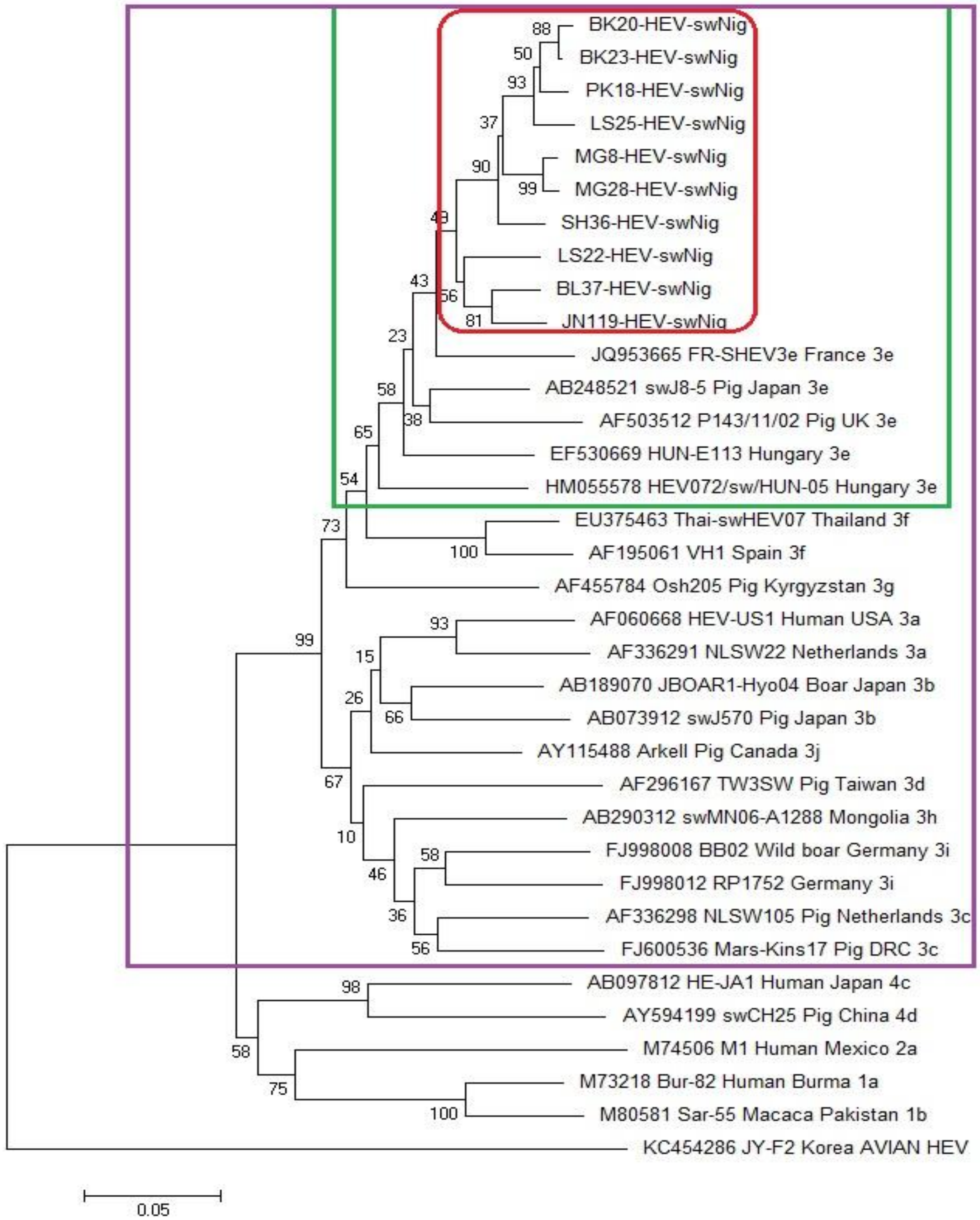


Figure 4.5: Neighbor-Joining tree of the 580-nt fragment of the ORF2 protein of strains from Plateau state and other known HEV subtypes. The strains from Plateau state are in the red box. Scale bar represents nucleotide substitutions per site.

all sequences including the prototype strains and this is represented by a “*” within the sequences. Other conserved sequences but with single substitutions are V79A in JN119-HEV-swNig and R80K in the Mexican prototype genotype 2 sequence; and R134 and R140 which were conserved across strains and genotypes. However, there were two conserved motifs; positions 101-107 were conserved and produced the sequence “RQN*FRR” (* position 104 is a common gap) and predicted aa positions 121-124 represented by “PMAS”. These positions do not represent aa positions on any consensus HEV genome, but positions as indicated by the translation of the 459-nt fragment (Appendix V).

Further analyses of the predicted aa sequences of strains from this study are summarized as follows: The aa sequences were examined for conserved sites across the entire length of the 26 isolates and in all, 19 conserved sites were identified (Appendix VI).

All sequences were also scrutinized for variable sites. Analysis revealed that the partial ORF2 sequences are more variable than conserved; aa variation was observed in vast parts of the sequences (Appendix VII).

The predicted aa sequences were analyzed for parsimony. The partial ORF2 sequence had multiple parsimony informative sites across its entire length confirming that it is a variable region as previously known (Appendix VIII).

CHAPTER FIVE

5.0DISCUSSION

Hepatitis E virus antibodies and RNA were detected by ELISA, RT-PCR followed by sequencing. The results of this study indicate that natural infection of HEV maybe widespread in domestic pig populations in Plateau state and possibly in Nigeria. HEV was detected in all the LGAs sampled, suggesting that there is a mechanism responsible for the spread of the virus. The ubiquitous nature of the virus implies that the pig population in Plateau state is at risk of HEV infection, especially pigs born naive into backyard farm settings. Experimental evidence suggests that trans-placental transmission of HEV has not been reported in gilts (Kasorndorkbua *et al.*, 2003). By extension, it also implies that humans, especially pig handlers and probably pork consumers are at high risk of HEV infection.

The pigs tested in this study were all backyard subsistence farming pigs; this system of rearing is common within rural communities where pigs are allowed to forage for food and are herded back to their pens at night. This farming system exposes pigs to other pigs, open water sources, open faeces of other pigs, human excreta and open farming fields where manure is dumped. Very few commercial pig farming systems remain standing in Plateau state. This is due to the continuous outbreaks of African swine fever (ASF) which depletes pig populations and makes it impossible to restock ASF-free piglets.

Viral hepatitis caused by other agents is common in Nigeria (Forbi *et al.*, 2013;Okusanya *et al.*, 2013). However, the occurrence and epidemiology of HEV is still widely unknown both in human populations and in the reservoir hosts. This situation is probably due to lack of knowledge of the disease and its causative agent in some areas and also due to negligence and underreporting. HEV is not totally unknown in Nigeria. It has been detected and documented in humans in the eastern part of the country where genotype 2 was characterized (Buisson *et al*., 2000). Serological evidence of HEV circulation in both ill and apparently healthy individuals and also health care givers have been documented (Adesina *et al*., 2009;Odaibo and Olaleye, 2013;Ola *et al*., 2012).It is not known when HEV was first introduced into Nigerian pig populations or how long it has been in circulation. However, this study and a recent report put its first identification as 2014 (Owolodun *et al*., 2014).

The seroprevalence obtained in this study indicates that HEV is abundant and the risk for human infection may be high. Samples for the serological study were collected from three distinct geographical areas, Jos area, Mangu area and Langtang area. In Jos area, the prevalence of anti-HEV IgM was 89.0%, which is in a range consistent with prevalence figures in the other two areas sampled. The result in pigs from Mangu area was 93.9% which is higher than that obtained in Jos area. Langtang area yielded a prevalence rate of 91.0%, slightly lower than Mangu area but higher than Jos area. These results appear to be quite high, but not totally unexpected of serological results from an HEV endemic developing country. The IgM prevalence rate recorded in this study is similar to studies carried out elsewhere in some developed countries. For instance, 85.5% prevalence rate was recorded in Great Britain (Banks *et al*., 2004) and 78.8% in China (Wang *et al*., 2002). Interestingly, Junaid *et al*. (2014), carried out a HEV survey in farm animals in Plateau state; IgM seroprevalence was 16.4%. This prevalence in this study was higher than in the previous study mentioned above.

The seroprevalence of HEV in pigs in Plateau state based on IgG was 50.8%. This prevalence rate indicates that HEV infection is widespread in pigs in Plateau state and that these antibodies (IgG) remain in circulation after clearance of virus presence or multiplication (virus shedding). HEV is known to be most commonly found in young pigs 2-4 months old and much less in older pigs. Here, pigs <9 months old had a higher overall IgG prevalence rate than older pigs. This situation has also been reported in Phillipines (Liu *et al.*, 2015). In Europe where pig farming is predominantly intensive system and hygiene of pens is much higher, serological findings present contrasting results; for instance in Scotland, seroprevalence of swine anti-HEV IgG in a recent study is 29.0% (Crossan *et al.*, 2014). This figure is far less than findings in Plateau state. In a bid to explain this marked difference prevalence, is attributable to the fact that samples from this study were collected from household farmed pigs and free range pigs that move from place to place to scavenge for food and wallow in common waters thereby contaminating the environment with faeces and urine, which may serve as sources of infection for all other susceptible pigs within the same vicinity. On the other hand reports from Belgium and Italy reveal that seroprevalence of anti-HEV IgG in both countries were 73.0% and 93.7% respectively (Ponterio *et al.*, 2014, Thiry *et al.*, 2014). The reasons for these high rates are not clear but it is probably due to the seeming evidence of HEV endemicity in swine even in developed and industrialized countries.

The simultaneous detection of both IgG and IgM in individual pigs highlights the re-infection of adult pigs by the HEV virus. The spike in IgG reveals this. Both IgG and IgM were detected in 210 pigs (41.0%). This scenario may have been caused by the poor housing conditions, free roaming nature of some pigs and poor faeces disposal practices of backyard pig farmers. This indicates that before previous infections are cleared, contact with sufficient doses of live virus can result in new infections in pigs.

A few swine HEV studies exist in Africa and involve detection of HEV RNA and subsequent molecular characterization. In Madagascar, where pig farming is very similar to the practice in Nigeria, a serosurvey of anti-HEV antibodies was carried out for the first time. An overall IgG seroprevalence of 71.2% was determined (Temmam *et al.*, 2013) across 18 districts. This percentage is higher than the 50.8% found in Plateau state. While farming practices may be comparable to Nigerian backyard system.

Pig HEV ELISA results differ from region to region and from country to country; this may be due to the farming system under which such pigs are housed prior to sampling. In backyard pig farming, the chances of contamination of feed and water are very high due to the poor hygienic conditions under which such pigs are kept. Another important factor in the seroepidemiology of HEV globally is the age at which pigs are eventually slaughtered for human consumption. In Nigeria, slaughter age of pigs as observed at the slaughter house generally exceeds 9 months, whereas, in developed countries slaughter age of pigs rarely exceeds 6 months. Generally, it is not well understood whether re-infection with HEV occurs in adult pigs following a prior infection at a younger age.

In this study, an attempt to detect HEV RNA was successful. To the best of my knowledge, this is the first report of molecular detection and characterization of HEV in pigs in Nigeria. HEV RNA was detected in all the 10LGAs sampled indicating the widespread presence of the virus in Plateau state pig populations. The rate of detection, however, diferred from LGA to LGA. The overall prevalence of HEV by qPCR was 25.7%. The highest prevalence was obtained in Langtang South LGA (62.8%), followed by Mangu LGA with 47.7%. Surprisingly, Jos South and Jos North LGAs had the lowest detection rates of 2.4% and 8.9% respectively. These two LGAs are believed to have the highest number of pigs in the state and also where commercial pig activities take place. Furthermore, the activity of pig middle men in pig establishments in these two areas is greater and is believed to have negative effects on pig health as was observed in the spread of ASF in South West Nigeria. However, it may be justifiable to conclude that the high detection rate in Langtang South LGA is probably due to the rampant extensive system/free range system under which pigs are kept in that area. Although overall 25.7% detection rate in a cross-sectional study appears high for a zoonotic agent with grave public health importance, this rate is consistent with faecal HEV shedding detection in other pig producing countries (Ning *et al.*, 2007;Ning *et al.*, 2008). In Lao Peoples's Democratic Republic also in Asia, a prevalence rate of 11.6% was deduced from swine faecal specimens (Conlan *et al.*, 2011); this is much lower than the prevalence detected from Plateau state. Interestingly, all pigs sampled in that study were also from smallholder/backyard farming setting, so the actual reason for a lower prevalence rate is not known, though the level of exposure to environmental factors by these pigs and the level of farm level hygiene practiced are unknown. In New Caledonia, Oceania, a surveillance program was instituted for HEV in growing pigs; HEV RNA was identified in 6.5% of pigs tested and subsequently characterized to be genotype 3 (Kaba *et al*., 2011a). The result from the present study in Nigeria is much higher than the results from New Caledonia.

On the African continent, the results of this study can only be compared to survey reports from Cameroon and Democratic Republic of Congo. HEV RNA prevalence in this study is higher than in Congo where 2.5% of the pigs tested were HEV positive (Kaba *et al*., 2010). Compared to results of a HEV study in Cameroonian pigs, the prevalence in Nigerian pigs is higher. About 1% of the pigs in Cameroon were HEV RNA positive.

Following qPCR detection, age distribution of HEV in Nigerian pigs was evaluated. The highest detection rate was in 4-month old pigs, 36.4%; which is in agreement with global findings of HEV in pigs (Kase *et al.*, 2008). The lowest rate of 15.1% was in 6-month old pigs, which also agrees with findings from other studies. This suggests that growing pigs 2-4 month old pigs may be the most susceptible age group. In this study however, 1-month old pigs had a higher detection rate (23.8%) than 2 and 3-month old pigs (15.4% and 20.8% respectively). This may mean that these piglets either did not acquire maternal antibodies or there was rapid disappearance of these antibodies.

Within the phylogenetic tree, the lone sequence from Jos-North clusters closely with HEV sequences of pigs from Barkin-Ladi. This occurrence may be due to introduction of young pigs from Jos-North as replacement stock. This may also explain why 2 sequences from Pankshin (Pk18) and Langtang South (LS25) LGAs cluster around sequences from Bokkos LGA. The phylogenetic tree comprising only sequences from Plateau state (Fig 4.1) strongly indicates that genetic diversity exists within HEV isolates from the state and that this virus is capable of constant genetic mutation. However, the genetic diversity observed among HEV strains from Plateau state may not be very significant when these strains are compared with other HEV sequences of swine origin from the rest of Africa and around the globe.

Results from BLAST homology search showed that strains from Plateau state were most closely related to swine HEV strains from Japan, Hungary and France. The sequence identity between HEV sequences from Plateau state and the HEV sequence from France (FR-SHEV3e accession number JQ953665) was about 90.0%. This identity was even higher when compared with swine HEV sequences from Hungary (HEV072/sw/HUN-05 accession number HM055578) and United Kingdom (P143/11/02 accession number AF503512) which was 91.0%. The highest sequence identity was with swine HEV sequence from Japan (swJ8-5 accession number AB248521), yielding 92.0% identity. The Nigerian sequences formed a large heterogenous group closely related to genotype 3. This close genetic relationship between the Nigerian strains with strains from Japan and Europe is akin to reports of the phylo-relationship between HEV sequences from Cameroon and Asian and European strains (S. de Paula *et al*., 2013). However, swine HEV sequences from Kinshasa, DR Congo also clustered closely with a European strain from Netherlands (Kaba *et al*., 2010). It is surprising that the Nigerian strains were not genetically close to HEV sequences in pigs from other African countries; it may be due to different sources on introduction into the continent. The close relationship to European strains maybe due to the fact that in a bid to improve pig production in Nigeria in the past, foreign breeds of pigs were imported into the country and may have introduced the virus into the country. This of course remains hypothetical, but HEV subtype 3e is frequently isolated in Europe especially United Kingdom, France and Hungary (Banks *et al*., 2004;Berto *etal*., 2013a;Reuter *et al*., 2009) and strengthens the argument for the introduction of the genotype into Nigeria. But the close nucleotide identity between sequences from this study and the Japanese strains remains unexplained; it may also be connected to importation of live pigs from Asia.

Hepatitis E is a globally important zoonosis and in most places where human HEV exists, there is a corresponding zoonotic presence of HEV in pigs. Very identical HEV sequences have been detected in both humans and pigs living in the same area (Suwannakarn *et al*., 2010;Wenzel *et al.*, 2011). This is currently not the same scenario in Nigeria, because whereas the sequences identified in this study belong to genotype 3; HEV recovered from humans previously belong to genotype 2 (Buisson *et al*., 2000). This situation has also been reported in two separate studies in different parts of India where human and swine HEV belong to different genotypes (Arankalle *et al*., 2002;Shukla *et al.*, 2007). It is noteworthy to mention that the human and swine studies were not carried out in the same geographical location; therefore further studies are required to establish the involvement of pigs in the transmission of HEV to humans in Nigeria.

Further typing (subgenotyping) of the strains from Plateau state placed them as genotype 3e viruses which is the same subgroup of swine HEV strains from France, Japan, United Kingdom and Hungary. But the swine HEV sequences identified previously in Cameroon and DR Congo did not belong to subgenotype 3e. (Kaba *et al*., 2010). The situation in Cameroon is quite different where swine HEV sequences did not cluster with any of the genotype 3 subtype reference strains in the phylogenetic tree; instead they clustered with a clade of undefined strains from Mongolia. This subtype classification is fast becoming obsolete in the taxonomy of HEV and many authors propose newer classification systems which will do away with confusions which arise from the present system in use. Currently, the most widely accepted proposed system is that emanating from Smith *et al*., 2014.

The nucleotide sequence analysis of the sequenced region did not yield much information because it was only a partial fragment of the ORF2 and secondly, HEV is known to have numerous mutations within this ORF, so the mutations detected were not unique.

The amino acid sequence analyses based on conserved sites, variable sites and parsimony informative sites yielded unique differences when the sequences from Plateau state were compared to genotypes 1, 2, 3 and 4 reference strains. The following deduced amino acid residues were conserved among all strains analyzed (strains from Plateau state and reference strains); V79, L93, R134 and R140. Also common to all was the conspicuous gap at aa position 67. Apart from these, there were 2 identified unique motifs within the 153 translated amino acid; the first was RQN*FRR from position 101-107 with a gap at position 104. The second conserved motif was identified between position 121-124 signaled by PMAS across all strains. There were also sites which were conserved in virtually all sequences except a few strains; for instance, A42 was conserved throughout except in strains BL37, BL49, JN119 and both genotype 4 strains from Asia. This difference especially within the three Nigerian strains placed them separately in one group when compared to other sequences from Plateau state; in all five sequences, ‘A’ was replaced by the amino acid ‘V’. Another minor alteration was observed at position T46 which was replaced by I46 in the genotype 4d Chinese isolate swCH25 accession number AY594199. Position G54 was replaced V54 in both Asian genotype 4 strains (AY594199 and AB097812). Similarly, S57 is replaced by N57 only in the Chinese isolate 4d swCH25. At position 68, R68 is replaced by K68 within the prototype Mexican genotype 2 isolate M1 (M74506) and both Asian genotype 4 strains. It is also noteworthy to state that the amino acid residue positions do not coincide with specific genomic or amino acid reference positions; and are simply assigned from nucleic acid translation. Analysis of conserved sites is depicted in Appendix VII. The implications of the associated changes in amino acid residues were not assessed. Therefore, it is difficult to state the difference in virulence or otherwise of the mutations. Additional studies will be required to investigate this.

On variable sites, amino acid variability was observed across the entire sequences. This is expected based on the known variability of the HEV genome (Oliveira-Filho *et al*., 2013;Smith *et al*., 2013;Smith *et al*., 2014). From this analysis, there were neither unique sites nor unique motifs, but within sequences from Plateau state, patterns could be observed and followed. This is because of the limited genetic variability between them. Even among reference sequences, genetic variability was observed and this is may be because the reference strains were from different geographical origins.The deduced amino acid residues were also analyzed for parsimony. Parsimonious sites were well distributed along the entire sequences of all HEV sequences included; this is also due to the high level of genetic variability present in HEV strains globally. Again, no particular pattern was established but details of parsimony informative sites are included in Appendix IX.

CHAPTER 6

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

This study has established the occurrence of zoonotic HEV in pigs from Plateau state, Nigeria and confirms that these pigs are potential reservoirs for HEV in the country.The current study has demonstrated that swine HEV antibodies are present and abundant in slaughter age pigs in Plateau state. Both IgM and IgG were detected in all the three geographical areas (Jos main abattoir, Mangu and Langtang slaughter slabs), therefore it is concluded that both acute and chronic HEV infections prevail in pigs in these areas.The results of this study indicate that HEV RNA was present in about 25.7% of the pigs tested. Young pigs constantly shed live HEV in faeces; this will have huge implications on the maintenance and transmission of the virus in naïve pigs within the same geographical areas.Genotype 3 HEV was the only genotype identified in this study and it may be the only genotype that circulates in domestic backyard pigs in Plateau state. Furthermore, molecular characterization revealed that the circulating viruses were HEV subtype 3e.The partial ORF2 sequences identified in pigs in Plateau state are hypervariable, this genetic variation gave rise to geographical clustering of isolates within the state.The amino acid sequence analysis also groups isolates based on geographical origin. However, a higher proportion of the translated amino acids was conserved in the isolates from Plate state indicating a common origin and may have been introduced from the same source.

This suggests that pig HEV strains in Nigeria are most likely to have emanated from pigs imported from Europe or Asia. The discovery of HEV in Nigerian pigs calls for greater public health attention (HEV is zoonotic) considering the close association of rural farmers and pig owners to their animals, and the attendant contamination of surface waters by pig excreta. This study demonstrates for the first time the circulation of HEV in Nigerian pigs and also characterizes the circulating strains to the subgenotype level.This research study has added to the existing knowledge gap on the presence of HEV in pigs in Plateau state, Nigeria and the potential role of pigs as reservoirs of HEV in the country.

6.2 Recommendations

Hepatitis E virus is a worldwide pathogen of public health importance and its presence has been well established in pig farms in developed and industrialized countries. In Africa, HEV is beginning to gain importance as a zoonosis that requires immediate intervention steps to control its spread and prevent transmission from animal reservoir to susceptible human hosts. Therefore, based on the findings of this study, the following recommendations are proffered:

i. Surveillance for HEV in pigs should be extended to other states. This extended surveillance should also aim to determine other reservoirs of HEV. HEV in humans should also be investigated to know its prevalence and to characterize the circulating genotypes.
ii. The impact of pig HEV in the environment needs to be assessed based on the indiscriminate disposal of pigsty waste by farmers and the random defecation by free roaming pigs into farm lands, natural and artificial water bodies.
iii. More isolates from pigs should be analyzed and characterized.
iv. The zoonotic potential of HEV isolated from pigs should be assessed.
v. A one-health group consisting of veterinary and medical personnel should be encouraged to develop modalities of educating the public, especially animal handlers on the dangers of HEV infection, mode of transmission and methods of prevention of infection.

vi. Pig farmers need to be educated on proper waste disposal methods in order to prevent further contamination of the environment and spread of HEV to other susceptible pig; and transmission of zoonotic strains of HEV to their human handlers. They should also be educated and trained on the need and how to wear protective gear during cleaning of pig pens and handling of pigs.

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APPENDICES

Appendix I

Ia Preparation of ELISA buffers for IgG detection

Carbonate coating buffer, pH 9.6

2.93 g sodium bicarbonate

1.59 g sodium carbonate

qs to 1 L with millipore H_2O

Store at 4° C

Elisa wash buffer, 20X

NaCl	160g
KCl	4g
KH2PO4	4g
Na2HPO4	9.2g
Tween 20	10ml

Millipore H2O up to 1L

Blocking Buffer

Sucrose	100g
Dextran	5g
Glycerol	50ml
Proclin300	1ml
BSA (Albumin)	10g

8.5mM PBS Buffer pH 7.2

NaCl	8g
KCl	0.2g
KH2PO4	0.35g
Na2HPO4	0.85g
Proclin 300	1ml

Millipore H2O up to 1L

Ib Preparation of ELISA buffers for IgM detection

Coating buffer

0.5M High salt buffer: 14 grm NaCl in 500 ml and add 2.5 ml of 10% Tween-80 solution

Low salt buffer: Add 2.5 ml of 10% Tween-80 solution to 500 ml PBS

Serum-dilution buffer

10% BSA + 10% skimmed milk powder (ELK) + 1% Tergitol (dissolved in PBS) to the low salt buffer

Conjugate buffer

Fetal calf serum (FCS) is added to high salt buffer to a concentration of 5% (2.5 ml FCS + 47.5 ml high salt buffer).

Appendix II

Storage of reagents and samples for ELISA

Storage Condition	Consumables
Room Temperature	High Salt buffer Low Salt buffer 0.5M Sulphuric acid
+4°C	Coated ELISA plates Coating buffer

	Stabilcoat Ready-to-use TMB
-20°C	Serum samples MCA anti-Swine IgM, IgG MCA anti-Swine L-chain conjugate Horse Radish Peroxidase conjugate

Positive and negative reference sera can be stored at -20C for at least 5 years without degradation

Appendix III

Preservation of coated ELISA plates

-Coating buffer was discarded from the wells of ELISA plates and dried.

-The plates were washed 3 times with water supplemented with 0.05% Tween80 using ELISA plate washer.

-The Stabilcoat was diluted 1:1 with PBS.

-100µl of diluted Stabilcoat was dispensed into all wells of the ELISA plates.

-The plates were incubated for 30 min at room temperature.

-The Stabilcoat was discarded from the ELISA plates.

-The ELISA plates were dried at 37C for 3 h.

-ELISA plates were stored at +4°C till use.

Appendix IV

IVa. QIAamp MinElute Virus Spin Kit Components

KIT COMPONENT	**QUANTITY**
QIAamp MinElute Columns	50
Collection Tubes (2 ml)	200
Buffer AL*	12 ml
Buffer AW1* (Concentrate)	19 ml

Buffer AW2 (Concentrate) Buffer AVE Protease Resuspension Buffer Carrier RNA QIAGEN Protease Handbook	13 ml 2 ml x 5 6 ml 310 µg 1 vial 1

- QIAamp MinElute columns were stored at +4°C on arrival.
- All buffers were stored at room temperature (15-25°C).
- Lyophilized RNA was stored at room temperature on arrival, carrier RNA was dissolved in buffer AVE and stored in aliquots at -20°C.
- QIAGEN Protease was dissolved in Resuspension buffer and kept at +4°C.

IVb. Reagent preparation for QIAamp MinElute Virus Spin

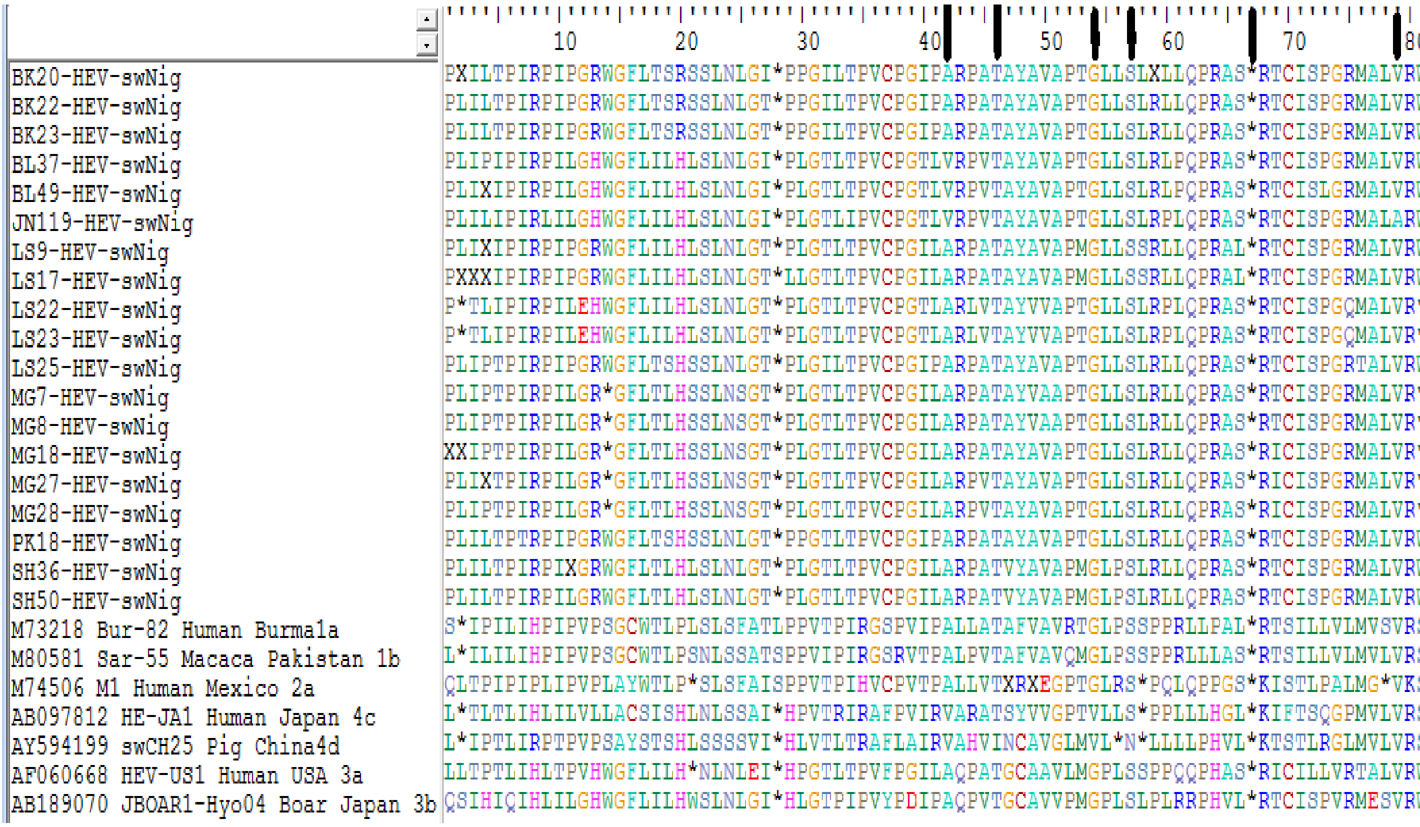
No. Samples	**Vol. Buffer AL (ml)**	**Vol. Carrier RNA-AVE (µl)**
1	0.22	6.2
2	0.44	12.3
3	0.66	18.5
4	0.88	24.6
5	1.10	30.8
6	1.32	37.0
7	1.54	43.1
8	1.76	49.3

9	1.98	55.4
10	2.20	61.6
11	2.42	67.8
12	2.64	73.9
13	2.86	80.1
14	3.08	86.3
15	3.30	92.4
16	3.52	98.6
17	3.74	104.7
18	3.96	110.9
19	4.18	117.0
20	4.40	123.2
21	4.62	129.4
22	4.84	135.5
23	5.06	141.7
24	5.28	147.8

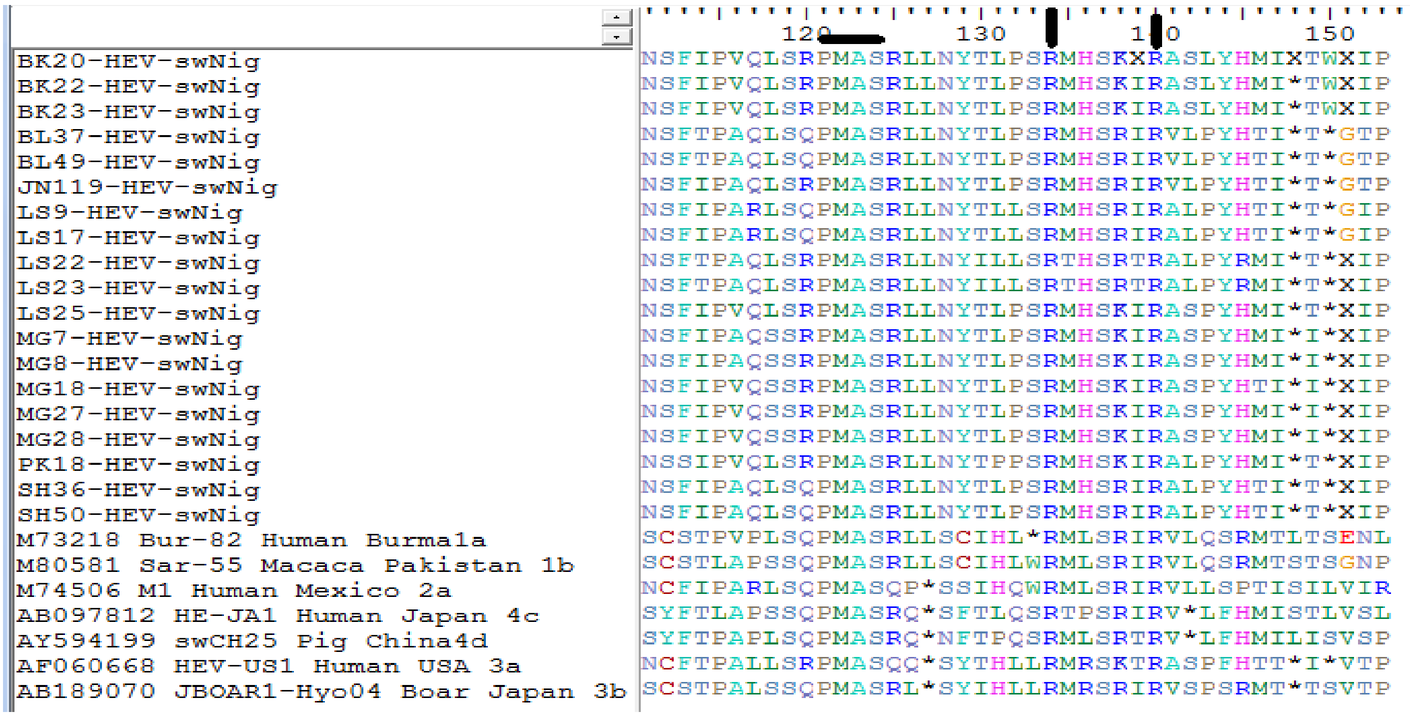
Appendix V

Amino acid sequence analyses

Va. Unique amino acid positions within the 459-nt translated sequences: aa 01-110 (Conserved sites are indicated by vertical black arrows, conserved motif indicated by horizontal black arrow)



Vb. Unique amino acid positions within the 459-nt translated sequences: aa 111-153 (Conserved sites are indicated by vertical black arrows, conserved motif indicated by horizontal black arrow)



Appendix VI

VIa: Amino acid analysis of the translated 459-nt partial sequence of ORF2; conserved sites are indicated in yellow

Name	Group	*	P	*	L	Y	S	T	S	L	T	H	F	S	A	V	C	R	Q	N	*	F	R	R	P	E	V	N	S	F	I	P	V	Q	L	S
☑1. BK20-HEV-swNig		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
☑2. BK22-HEV-swNig		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
☑3. BK23-HEV-swNig		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
☑4. BL37-HEV-swNig		.	.	.	P	.	.	.	L	.	I	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	A	.	.	.
☑5. BL49-HEV-swNig		.	.	.	P	.	.	.	L	.	I	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	A	.	.	.
☑6. JN119-HEV-swNig		.	.	.	.	.	.	.	.	.	I	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	A	.	.	.
☑7. LS9-HEV-swNig		.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	A	.	.	.	.	.	A	R	.	.
☑8. LS17-HEV-swNig		.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	A	.	.	.	.	.	A	R	.	.
☑9. LS22-HEV-swNig		.	.	.	P	.	.	I	L	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	A	.	.	.
☑10. LS23-HEV-swNig		.	.	.	P	.	.	I	L	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	A	.	.	.
☑11. LS25-HEV-swNig		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
☑12. MG7-HEV-swNig		.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	S	.
☑13. MG8-HEV-swNig		.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	S	.
☑14. MG18-HEV-swNig		.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.
☑15. MG27-HEV-swNig		.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.
☑16. MG28-HEV-swNig		.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.
☑17. PK18-HEV-swNig		.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.
☑18. SH36-HEV-swNig		.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
☑19. SH50-HEV-swNig		.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
☑20. M73218 Bur-82 Human Burma 1a		.	.	S	P	C	.	.	L	.	.	L	C	L	.	A	.	.	.	.	.	.	.	.	L	V	A	S	C	S	T	.	.	P	.	.
☑21. M80581 Sar-55 Macaca Pakistan 1b		.	R	L	P	C	L	.	L	.	.	P	C	L	.	.	Y	.	.	.	.	.	.	.	L	V	A	S	C	S	T	L	A	P	S	.
☑22. M74506 M1 Human Mexico 2a		.	L	.	H	.	L	.	L	.	.	R	S	.	.	G	S	.	.	.	.	.	.	.	L	A	G	.	C	.	.	.	A	R	.	.
☑23. AB097812 HE-JA1 Human Japan 4c		.	R	.	.	C	L	I	L	.	.	R	.	.	.	G	S	.	.	.	.	.	.	.	L	V	A	S	Y	.	T	L	A	P	S	.
☑24. AY594199 swCH25 Pig China 4d		.	R	.	P	C	L	I	L	.	I	R	C	.	.	G	F	.	.	.	.	.	.	.	L	.	A	S	Y	.	T	.	A	P	.	.
☑25. AF060668 HEV-US1 Human USA 3a		L	.	.	.	C	L	I	L	.	I	R	.	L	V	.	Y	.	.	.	.	.	.	.	L	G	.	.	C	.	T	.	A	L	.	.
☑26. AB189070 JBOAR1-Hyo04 Boar Japan 3		L	.	.	H	C	L	I	L	.	.	R	.	.	V	.	.	.	.	.	.	.	.	.	L	G	A	S	C	S	T	.	A	L	S	.

VIb: Amino acid analysis of the translated 459-nt partial sequence of ORF2; conserved sites are indicated in yellow

☑Name	Group	S	R	L	L	N	Y	T	L	P	S	R	M	H	S	K	?	R	A
☑1. BK20-HEV-swNig		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	?	.	.
☑2. BK22-HEV-swNig		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.
☑3. BK23-HEV-swNig		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.
☑4. BL37-HEV-swNig		.	.	.	.	.	.	.	.	.	.	.	.	.	.	R	I	.	V
☑5. BL49-HEV-swNig		.	.	.	.	.	.	.	.	.	.	.	.	.	.	R	I	.	V
☑6. JN119-HEV-swNig		.	.	.	.	.	.	.	.	.	.	.	.	.	.	R	I	.	V
☑7. LS9-HEV-swNig		.	.	.	.	.	.	.	.	L	.	.	.	.	.	R	I	.	.
☑8. LS17-HEV-swNig		.	.	.	.	.	.	.	.	L	.	.	.	.	.	R	I	.	.
☑9. LS22-HEV-swNig		.	.	.	.	.	.	I	.	L	.	.	T	.	.	R	T	.	.
☑10. LS23-HEV-swNig		.	.	.	.	.	.	I	.	L	.	.	T	.	.	R	T	.	.
☑11. LS25-HEV-swNig		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.
☑12. MG7-HEV-swNig		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.
☑13. MG8-HEV-swNig		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.
☑14. MG18-HEV-swNig		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.
☑15. MG27-HEV-swNig		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.
☑16. MG28-HEV-swNig		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.
☑17. PK18-HEV-swNig		.	.	.	.	.	.	.	P	.	.	.	.	.	.	.	I	.	.
☑18. SH36-HEV-swNig		.	.	.	.	.	.	.	.	.	.	.	.	.	.	R	I	.	.
☑19. SH50-HEV-swNig		.	.	.	.	.	.	.	.	.	.	.	.	.	.	R	I	.	.
☑20. M73218 Bur-82 Human Burma 1a		.	.	.	.	S	C	I	H	L	*	.	.	L	.	R	I	.	V
☑21. M80581 Sar-55 Macaca Pakistan 1b		.	.	.	.	S	C	I	H	L	W	.	.	L	.	R	I	.	V
☑22. M74506 M1 Human Mexico 2a		.	Q	P	*	S	S	I	H	Q	W	.	.	L	.	R	I	.	V
☑23. AB097812 HE-JA1 Human Japan 4c		.	.	Q	*	S	F	.	.	Q	.	.	T	P	.	R	I	.	V
☑24. AY594199 swCH25 Pig China 4d		.	.	Q	*	.	F	.	P	Q	.	.	.	L	.	R	T	.	V
☑25. AF060668 HEV-US1 Human USA 3a		.	Q	Q	*	S	.	.	H	L	L	.	.	R	.	.	T	.	.
☑26. AB189070 JBOAR1-Hyo04 Boar Japan 3		.	.	.	*	S	.	I	H	L	L	.	.	R	.	R	I	.	V

APPENDIX VII

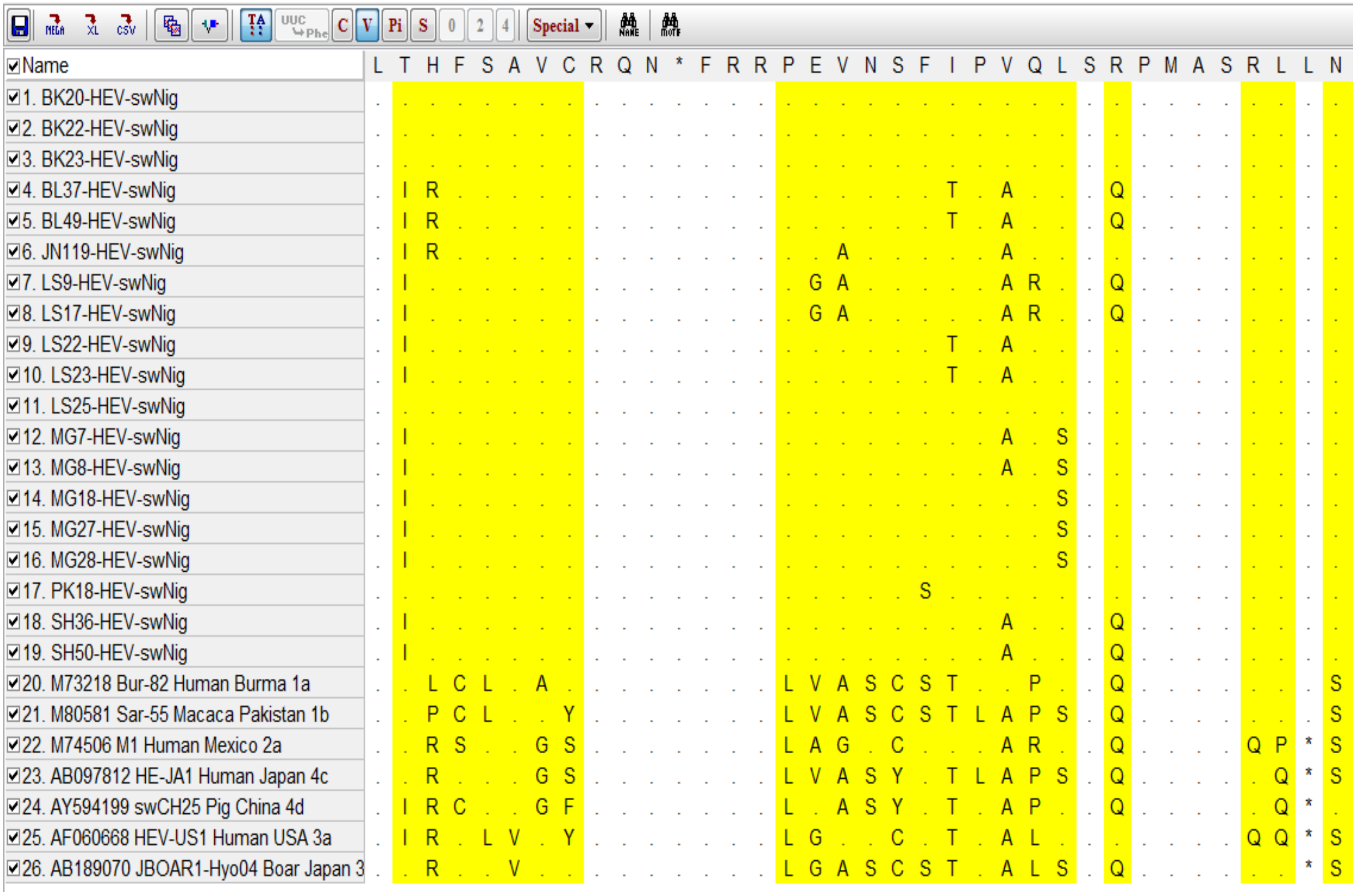
VIIa: Amino acid analysis of the translated 459-nt partial sequence of ORF2; variable sites are indicated in yellow

Name	P?ILTPIRPIPGRWGFLTSRSSLNLGI*PPGILTPV
1. BK20-HEV-swNig	.?..................................
2. BK22-HEV-swNig	.L........................T.........
3. BK23-HEV-swNig	.L........................T.........
4. BL37-HEV-swNig	.L.PI.....L.H....ILHL........L.T....
5. BL49-HEV-swNig	.L.?I.....L.H....ILHL........L.T....
6. JN119-HEV-swNig	.L..I...L.L.H....ILHL........L.T.I..
7. LS9-HEV-swNig	.L.?I............ILHL.....T..L.T....
8. LS17-HEV-swNig	.???I............ILHL.....T.LL.T....
9. LS22-HEV-swNig	.*T.I.....LEH....ILHL.....T..L.T....
10. LS23-HEV-swNig	.*T.I.....LEH....ILHL.....T..L.T....
11. LS25-HEV-swNig	.L.P...............H......T..L......
12. MG7-HEV-swNig	.L.P......L..*....LH....S.T..L.T....
13. MG8-HEV-swNig	.L.P......L..*....LH....S.T..L.T....
14. MG18-HEV-swNig	??.P......L..*....LH....S.T..L.T....
15. MG27-HEV-swNig	.L.?......L..*....LH....S.T..L.T....
16. MG28-HEV-swNig	.L.P......L..*....LH....S.T..L.T....
17. PK18-HEV-swNig	.L....T............H......T....T....
18. SH36-HEV-swNig	.L........?.......LHL.....T..L.T....
19. SH50-HEV-swNig	.L........L.......LHL.....T..L.T....
20. M73218 Bur-82 Human Burma 1a	S*.PIL.H...VPS.CW.LPL..SFATL..VTPIRG
21. M80581 Sar-55 Macaca Pakistan 1b	L*..IL.H...VPS.CW.LP.N.SSATS..V.PIRG
22. M74506 M1 Human Mexico 2a	QLTPI..PL..VPLAYW.LP*..SFA.S..VTPIH.
23. AB097812 HE-JA1 Human Japan 4c	L*T..L.HL.LVLLACSI.HLN.SSA..H.VTRIRA
24. AY594199 swCH25 Pig China 4d	L*.P.L...T.VPSAYS..HL.SSSV..HLVT..RA
25. AF060668 HEV-US1 Human USA 3a	LLTP.L.HLT.VH....ILH*N...E..H..T....
26. AB189070 JBOAR1-Hyo04 Boar Japan 3	QS.HIQ.HL.L.H....ILHW.......HL.TPI..

VIIb: Amino acid analysis of the translated 459-nt partial sequence of ORF2; variable sites are indicated in yellow

☑Name	A	Y	A	V	A	P	T	G	L	L	S	L	?	L	L	Q	P	R	A	S	*	R	T	C	I	S	P	G	R	M	A	L	V
☑1. BK20-HEV-swNig	.	.	.	.	.	.	.	.	.	.	.	.	?	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
☑2. BK22-HEV-swNig	.	.	.	.	.	.	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
☑3. BK23-HEV-swNig	.	.	.	.	.	.	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
☑4. BL37-HEV-swNig	.	.	.	.	.	.	.	.	.	.	.	.	R	.	P	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
☑5. BL49-HEV-swNig	.	.	.	.	.	.	.	.	.	.	.	.	R	.	P	.	.	.	.	.	.	.	.	.	.	.	L	.	.	.	.	.	.
☑6. JN119-HEV-swNig	.	.	.	.	.	.	.	.	.	.	.	.	R	P	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A
☑7. LS9-HEV-swNig	.	.	.	.	.	.	M	.	.	.	.	S	R	.	.	.	.	.	.	L	.	.	.	.	.	.	.	.	.	.	.	.	.
☑8. LS17-HEV-swNig	.	.	.	.	.	.	M	.	.	.	.	S	R	.	.	.	.	.	.	L	.	.	.	.	.	.	.	.	.	.	.	.	.
☑9. LS22-HEV-swNig	.	.	V	.	.	.	.	.	.	.	.	.	R	P	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Q	.	.	.	.
☑10. LS23-HEV-swNig	.	.	V	.	.	.	.	.	.	.	.	.	R	P	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Q	.	.	.	.
☑11. LS25-HEV-swNig	.	.	.	.	.	.	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.
☑12. MG7-HEV-swNig	.	.	V	A	.	.	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
☑13. MG8-HEV-swNig	.	.	V	A	.	.	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
☑14. MG18-HEV-swNig	.	.	.	.	.	.	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.
☑15. MG27-HEV-swNig	.	.	.	.	.	.	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.
☑16. MG28-HEV-swNig	.	.	.	.	.	.	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.
☑17. PK18-HEV-swNig	.	.	.	.	.	.	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
☑18. SH36-HEV-swNig	V	.	.	.	.	.	M	.	.	P	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
☑19. SH50-HEV-swNig	V	.	.	.	.	.	M	.	.	P	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
☑20. M73218 Bur-82 Human Burma 1a	.	F	V	A	V	R	.	.	.	P	.	S	P	P	R	L	L	P	.	L	.	.	.	S	.	L	L	V	L	.	V	S	.
☑21. M80581 Sar-55 Macaca Pakistan 1b	.	F	V	A	V	Q	M	.	.	P	.	S	P	P	R	L	L	L	.	.	.	.	.	S	.	L	L	V	L	.	V	.	.
☑22. M74506 M1 Human Mexico 2a	?	R	?	E	G	.	.	.	.	R	.	*	P	Q	.	.	.	P	G	.	.	K	I	S	T	L	.	A	L	.	G	*	.
☑23. AB097812 HE-JA1 Human Japan 4c	S	.	V	.	G	.	.	V	.	.	.	*	P	P	.	L	L	H	G	L	.	K	I	F	T	.	Q	.	P	.	V	.	.
☑24. AY594199 swCH25 Pig China 4d	N	C	.	.	G	L	M	V	.	*	N	*	L	.	.	L	.	H	V	L	.	K	.	S	T	L	R	.	L	.	V	.	.
☑25. AF060668 HEV-US1 Human USA 3a	G	C	.	A	V	L	M	.	P	.	.	S	P	P	Q	.	.	H	.	.	.	.	I	.	.	L	L	V	.	T	.	.	.
☑26. AB189070 JBOAR1-Hyo04 Boar Japan 3	G	C	.	.	V	.	M	.	P	.	.	.	P	.	R	R	.	H	V	L	.	.	.	.	.	.	.	V	.	.	E	S	.

VIIc: Amino acid analysis of the translated 459-nt partial sequence of ORF2; variable sites are indicated in yellow



Name	L	T	H	F	S	A	V	C	R	Q	N	*	F	R	R	P	E	V	N	S	F	I	P	V	Q	L	S	R	P	M	A	S	R	L	L	N
1. BK20-HEV-swNig	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
2. BK22-HEV-swNig	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
3. BK23-HEV-swNig	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
4. BL37-HEV-swNig	.	I	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	A	.	.	.	Q	.	.	.	.	.	.	.	.
5. BL49-HEV-swNig	.	I	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	A	.	.	.	Q	.	.	.	.	.	.	.	.
6. JN119-HEV-swNig	.	I	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.
7. LS9-HEV-swNig	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	A	.	.	.	.	.	A	R	.	.	Q	.	.	.	.	.	.	.	.
8. LS17-HEV-swNig	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	A	.	.	.	.	.	A	R	.	.	Q	.	.	.	.	.	.	.	.
9. LS22-HEV-swNig	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	A	.	.	.	.	.	.	.	.	.	.	.	.
10. LS23-HEV-swNig	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	A	.	.	.	.	.	.	.	.	.	.	.	.
11. LS25-HEV-swNig	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
12. MG7-HEV-swNig	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	S	.	.	.	.	.	.	.	.	.	.
13. MG8-HEV-swNig	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	S	.	.	.	.	.	.	.	.	.	.
14. MG18-HEV-swNig	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.
15. MG27-HEV-swNig	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.
16. MG28-HEV-swNig	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.
17. PK18-HEV-swNig	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
18. SH36-HEV-swNig	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	Q	.	.	.	.	.	.	.	.
19. SH50-HEV-swNig	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	Q	.	.	.	.	.	.	.	.
20. M73218 Bur-82 Human Burma 1a	.	.	L	C	L	.	A	.	.	.	.	.	.	.	.	L	V	A	S	C	S	T	.	.	P	.	.	Q	.	.	.	.	.	.	.	S
21. M80581 Sar-55 Macaca Pakistan 1b	.	.	P	C	L	.	.	Y	.	.	.	.	.	.	.	L	V	A	S	C	S	T	L	A	P	S	.	Q	.	.	.	.	.	.	.	S
22. M74506 M1 Human Mexico 2a	.	.	R	S	.	.	G	S	.	.	.	.	.	.	.	L	A	G	.	C	.	.	.	A	R	.	.	Q	.	.	.	.	Q	P	*	S
23. AB097812 HE-JA1 Human Japan 4c	.	.	R	.	.	.	G	S	.	.	.	.	.	.	.	L	V	A	S	Y	.	T	L	A	P	S	.	Q	.	.	.	.	.	Q	*	S
24. AY594199 swCH25 Pig China 4d	.	I	R	C	.	.	G	F	.	.	.	.	.	.	.	L	.	A	S	Y	.	T	.	A	P	.	.	Q	.	.	.	.	.	Q	*	.
25. AF060668 HEV-US1 Human USA 3a	.	I	R	.	L	V	.	Y	.	.	.	.	.	.	.	L	G	.	.	C	.	T	.	A	L	.	.	.	.	.	.	.	Q	Q	*	S
26. AB189070 JBOAR1-Hyo04 Boar Japan 3	.	.	R	.	.	V	.	.	.	.	.	.	.	.	.	L	G	A	S	C	S	T	.	A	L	S	.	Q	.	.	.	.	.	.	*	S

VIId: Amino acid analysis of the translated 459-nt partial sequence of ORF2; variable sites are indicated in yellow

Name	S	R	L	L	N	Y	T	L	P	S	R	M	H	S	K	?	R	A	S
1. BK20-HEV-swNig	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	?	.	.	.
2. BK22-HEV-swNig	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.
3. BK23-HEV-swNig	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.
4. BL37-HEV-swNig	.	.	.	.	.	.	.	.	.	.	.	.	.	.	R	I	.	V	L
5. BL49-HEV-swNig	.	.	.	.	.	.	.	.	.	.	.	.	.	.	R	I	.	V	L
6. JN119-HEV-swNig	.	.	.	.	.	.	.	.	.	.	.	.	.	.	R	I	.	V	L
7. LS9-HEV-swNig	.	.	.	.	.	.	.	.	L	.	.	.	.	.	R	I	.	.	L
8. LS17-HEV-swNig	.	.	.	.	.	.	.	.	L	.	.	.	.	.	R	I	.	.	L
9. LS22-HEV-swNig	.	.	.	.	.	.	I	.	L	.	.	T	.	.	R	T	.	.	L
10. LS23-HEV-swNig	.	.	.	.	.	.	I	.	L	.	.	T	.	.	R	T	.	.	L
11. LS25-HEV-swNig	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.
12. MG7-HEV-swNig	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.
13. MG8-HEV-swNig	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.
14. MG18-HEV-swNig	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.
15. MG27-HEV-swNig	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.
16. MG28-HEV-swNig	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.
17. PK18-HEV-swNig	.	.	.	.	.	.	.	P	.	.	.	.	.	.	.	I	.	.	L
18. SH36-HEV-swNig	.	.	.	.	.	.	.	.	.	.	.	.	.	.	R	I	.	.	L
19. SH50-HEV-swNig	.	.	.	.	.	.	.	.	.	.	.	.	.	.	R	I	.	.	L
20. M73218 Bur-82 Human Burma 1a	.	.	.	.	S	C	I	H	L	*	.	.	L	.	R	I	.	V	L
21. M80581 Sar-55 Macaca Pakistan 1b	.	.	.	.	S	C	I	H	L	W	.	.	L	.	R	I	.	V	L
22. M74506 M1 Human Mexico 2a	.	Q	P	*	S	S	I	H	Q	W	.	.	L	.	R	I	.	V	L
23. AB097812 HE-JA1 Human Japan 4c	.	.	Q	*	S	F	.	.	Q	.	.	T	P	.	R	I	.	V	*
24. AY594199 swCH25 Pig China 4d	.	.	Q	*	.	F	.	P	Q	.	.	.	L	.	R	T	.	V	*
25. AF060668 HEV-US1 Human USA 3a	.	Q	Q	*	S	.	.	H	L	L	.	.	R	.	.	T	.	.	.
26. AB189070 JBOAR1-Hyo04 Boar Japan 3b	.	.	.	*	S	.	I	H	L	L	.	.	R	.	R	I	.	V	.

APPENDIX VIII

VIIIa: Amino acid analysis of the translated 459-nt partial sequence of ORF2; parsimony informative sites are indicated in yellow

Name	Group	P	?	I	L	T	P	I	R	P	I	P	G	R	W	G	F	L	T	S	R	S	S	L	N	L	G	I	*	P	P	G	I	L	T
1. BK20-HEV-swNig		.	?	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
2. BK22-HEV-swNig		.	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.
3. BK23-HEV-swNig		.	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.
4. BL37-HEV-swNig		.	L	.	P	I	.	.	.	.	.	L	.	H	.	.	.	.	I	L	H	L	.	.	.	.	.	.	.	.	L	.	T	.	.
5. BL49-HEV-swNig		.	L	.	?	I	.	.	.	.	.	L	.	H	.	.	.	.	I	L	H	L	.	.	.	.	.	.	.	.	L	.	T	.	.
6. JN119-HEV-swNig		.	L	.	.	I	.	.	.	L	.	L	.	H	.	.	.	.	I	L	H	L	.	.	.	.	.	.	.	.	L	.	T	.	I
7. LS9-HEV-swNig		.	L	.	?	I	.	.	.	.	.	.	.	.	.	.	.	.	I	L	H	L	.	.	.	.	.	T	.	.	L	.	T	.	.
8. LS17-HEV-swNig		.	?	?	?	I	.	.	.	.	.	.	.	.	.	.	.	.	I	L	H	L	.	.	.	.	.	T	.	L	L	.	T	.	.
9. LS22-HEV-swNig		.	*	T	.	I	.	.	.	.	.	L	E	H	.	.	.	.	I	L	H	L	.	.	.	.	.	T	.	.	L	.	T	.	.
10. LS23-HEV-swNig		.	*	T	.	I	.	.	.	.	.	L	E	H	.	.	.	.	I	L	H	L	.	.	.	.	.	T	.	.	L	.	T	.	.
11. LS25-HEV-swNig		.	L	.	P	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	H	.	.	.	.	.	.	T	.	.	L	.	.	.	.
12. MG7-HEV-swNig		.	L	.	P	.	.	.	.	.	.	L	.	.	*	.	.	.	.	L	H	.	.	.	.	S	.	T	.	.	L	.	T	.	.
13. MG8-HEV-swNig		.	L	.	P	.	.	.	.	.	.	L	.	.	*	.	.	.	.	L	H	.	.	.	.	S	.	T	.	.	L	.	T	.	.
14. MG18-HEV-swNig		?	?	.	P	.	.	.	.	.	.	L	.	.	*	.	.	.	.	L	H	.	.	.	.	S	.	T	.	.	L	.	T	.	.
15. MG27-HEV-swNig		.	L	.	?	.	.	.	.	.	.	L	.	.	*	.	.	.	.	L	H	.	.	.	.	S	.	T	.	.	L	.	T	.	.
16. MG28-HEV-swNig		.	L	.	P	.	.	.	.	.	.	L	.	.	*	.	.	.	.	L	H	.	.	.	.	S	.	T	.	.	L	.	T	.	.
17. PK18-HEV-swNig		.	L	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	H	.	.	.	.	.	.	T	.	.	.	.	T	.	.
18. SH36-HEV-swNig		.	L	.	.	.	.	.	.	.	.	?	.	.	.	.	.	.	.	L	H	L	.	.	.	.	.	T	.	.	L	.	T	.	.
19. SH50-HEV-swNig		.	L	.	.	.	.	.	.	.	.	L	.	.	.	.	.	.	.	L	H	L	.	.	.	.	.	T	.	.	L	.	T	.	.
20. M73218 Bur-82 Human Burma 1a		S	*	.	P	I	L	.	H	.	.	.	V	P	S	.	C	W	.	L	P	L	.	.	S	F	A	T	L	.	.	V	T	P	I
21. M80581 Sar-55 Macaca Pakistan 1b		L	*	.	.	I	L	.	H	.	.	.	V	P	S	.	C	W	.	L	P	.	N	.	S	S	A	T	S	.	.	V	.	P	I
22. M74506 M1 Human Mexico 2a		Q	L	T	P	I	.	.	P	L	.	.	V	P	L	A	Y	W	.	L	P	*	.	.	S	F	A	.	S	.	.	V	T	P	I
23. AB097812 HE-JA1 Human Japan 4c		L	*	T	.	.	L	.	H	L	.	L	V	L	L	A	C	S	I	.	H	L	N	.	S	S	A	.	.	H	.	V	T	R	I
24. AY594199 swCH25 Pig China 4d		L	*	.	P	.	L	.	.	.	T	.	V	P	S	A	Y	S	.	.	H	L	.	S	S	S	V	.	.	H	L	V	T	.	.
25. AF060668 HEV-US1 Human USA 3a		L	L	T	P	.	L	.	H	L	T	.	V	H	.	.	.	.	I	L	H	*	N	.	.	.	E	.	.	H	.	.	T	.	.
26. AB189070 JBOAR1-Hyo04 Boar Japan 3		Q	S	.	H	I	Q	.	H	L	.	L	.	H	.	.	.	.	I	L	H	W	.	.	.	.	.	.	.	H	L	.	T	P	I

VIIIb: Amino acid analysis of the translated 459-nt partial sequence of ORF2; parsimony informative sites are indicated in yellow

Name	Group	P	A	T	A	Y	A	V	A	P	T	G	L	L	S	L	?	L	L	Q	P	R	A	S	*	R	T	C	I	S	P	G	R	M	A
1. BK20-HEV-swNig		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	?	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
2. BK22-HEV-swNig		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
3. BK23-HEV-swNig		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
4. BL37-HEV-swNig		.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	R	.	P	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
5. BL49-HEV-swNig		.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	R	.	P	.	.	.	.	.	.	.	.	.	.	.	L	.	.	.	.
6. JN119-HEV-swNig		.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	R	P	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
7. LS9-HEV-swNig		.	.	.	.	.	.	.	.	.	M	.	.	.	.	S	R	.	.	.	.	.	.	L	.	.	.	.	.	.	.	.	.	.	.
8. LS17-HEV-swNig		.	.	.	.	.	.	.	.	.	M	.	.	.	.	S	R	.	.	.	.	.	.	L	.	.	.	.	.	.	.	.	.	.	.
9. LS22-HEV-swNig		L	V	.	.	.	V	.	.	.	.	.	.	.	.	.	R	P	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Q	.	.
10. LS23-HEV-swNig		L	V	.	.	.	V	.	.	.	.	.	.	.	.	.	R	P	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Q	.	.
11. LS25-HEV-swNig		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.
12. MG7-HEV-swNig		.	.	.	.	.	V	A	.	.	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
13. MG8-HEV-swNig		.	.	.	.	.	V	A	.	.	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
14. MG18-HEV-swNig		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.
15. MG27-HEV-swNig		.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.
16. MG28-HEV-swNig		.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.
17. PK18-HEV-swNig		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
18. SH36-HEV-swNig		.	.	.	V	.	.	.	.	.	M	.	.	P	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
19. SH50-HEV-swNig		.	.	.	V	.	.	.	.	.	M	.	.	P	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
20. M73218 Bur-82 Human Burma 1a		L	.	.	.	F	V	A	V	R	.	.	.	P	.	S	P	P	R	L	L	P	.	L	.	.	.	S	.	L	L	V	L	.	V
21. M80581 Sar-55 Macaca Pakistan 1b		.	V	.	.	F	V	A	V	Q	M	.	.	P	.	S	P	P	R	L	L	L	.	.	.	.	.	S	.	L	L	V	L	.	V
22. M74506 M1 Human Mexico 2a		L	V	.	?	R	?	E	G	.	.	.	.	R	.	*	P	Q	.	.	.	P	G	.	.	K	I	S	T	L	.	A	L	.	G
23. AB097812 HE-JA1 Human Japan 4c		R	.	.	S	.	V	.	G	.	.	V	.	.	.	*	P	P	.	L	L	H	G	L	.	K	I	F	T	.	Q	.	P	.	V
24. AY594199 swCH25 Pig China 4d		H	V	I	N	C	.	.	G	L	M	V	.	*	N	*	L	.	.	L	.	H	V	L	.	K	.	S	T	L	R	.	L	.	V
25. AF060668 HEV-US1 Human USA 3a		.	.	.	G	C	.	A	V	L	M	.	P	.	.	S	P	P	Q	.	.	H	.	.	.	.	I	.	.	L	L	V	.	T	.
26. AB189070 JBOAR1-Hyo04 Boar Japan 3		.	V	.	G	C	.	.	V	.	M	.	P	.	.	.	P	.	R	R	.	H	V	L	.	.	.	.	.	.	.	V	.	.	E

VIIIc: Amino acid analysis of the translated 459-nt partial sequence of ORF2; parsimony informative sites are indicated in yellow

Name	Group	*	L	Y	S	T	S	L	T	H	F	S	A	V	C	R	Q	N	*	F	R	R	P	E	V	N	S	F	I	P	V	Q	L	S	R
☑1. BK20-HEV-swNig		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
☑2. BK22-HEV-swNig		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
☑3. BK23-HEV-swNig		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
☑4. BL37-HEV-swNig		.	P	.	.	.	L	.	I	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	A	.	.	.	Q
☑5. BL49-HEV-swNig		.	P	.	.	.	L	.	I	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	A	.	.	.	Q
☑6. JN119-HEV-swNig		.	.	.	.	.	.	.	I	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	A	.	.	.	.
☑7. LS9-HEV-swNig		.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	A	.	.	.	.	.	A	R	.	.	Q
☑8. LS17-HEV-swNig		.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	A	.	.	.	.	.	A	R	.	.	Q
☑9. LS22-HEV-swNig		.	P	.	.	I	L	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	A	.	.	.	.
☑10. LS23-HEV-swNig		.	P	.	.	I	L	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	A	.	.	.	.
☑11. LS25-HEV-swNig		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
☑12. MG7-HEV-swNig		.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	S	.	.
☑13. MG8-HEV-swNig		.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	S	.	.
☑14. MG18-HEV-swNig		.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.
☑15. MG27-HEV-swNig		.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.
☑16. MG28-HEV-swNig		.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.
☑17. PK18-HEV-swNig		.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.
☑18. SH36-HEV-swNig		.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	Q
☑19. SH50-HEV-swNig		.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	Q
☑20. M73218 Bur-82 Human Burma 1a		S	P	C	.	.	L	.	.	L	C	L	.	A	.	.	.	.	.	.	.	.	L	V	A	S	C	S	T	.	.	P	.	.	Q
☑21. M80581 Sar-55 Macaca Pakistan 1b		L	P	C	L	.	L	.	.	P	C	L	.	.	Y	.	.	.	.	.	.	.	L	V	A	S	C	S	T	L	A	P	S	.	Q
☑22. M74506 M1 Human Mexico 2a		.	H	.	L	.	L	.	.	R	S	.	.	G	S	.	.	.	.	.	.	.	L	A	G	.	C	.	.	.	A	R	.	.	Q
☑23. AB097812 HE-JA1 Human Japan 4c		.	.	C	L	I	L	.	.	R	.	.	.	G	S	.	.	.	.	.	.	.	L	V	A	S	Y	.	T	L	A	P	S	.	Q
☑24. AY594199 swCH25 Pig China 4d		.	P	C	L	I	L	.	I	R	C	.	.	G	F	.	.	.	.	.	.	.	L	.	A	S	Y	.	T	.	A	P	.	.	Q
☑25. AF060668 HEV-US1 Human USA 3a		.	.	C	L	I	L	.	I	R	.	L	V	.	Y	.	.	.	.	.	.	.	L	G	.	.	C	.	T	.	A	L	.	.	.
☑26. AB189070 JBOAR1-Hyo04 Boar Japan 3		.	H	C	L	I	L	.	.	R	.	.	V	.	.	.	.	.	.	.	.	.	L	G	A	S	C	S	T	.	A	L	S	.	Q

VIIId: Amino acid analysis of the translated 459-nt partial sequence of ORF2; parsimony informative sites are indicated in yellow

☑ Name	Group	E	V	N	S	F	I	P	V	Q	L	S	R	P	M	A	S	R	L	L	N	Y	T	L	P	S	R	M	H	S	K	?	R	A	S
☑ 1. BK20-HEV-swNig		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	?	.	.	.
☑ 2. BK22-HEV-swNig		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.
☑ 3. BK23-HEV-swNig		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.
☑ 4. BL37-HEV-swNig		.	.	.	.	.	T	.	A	.	.	.	Q	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	R	I	.	V	L
☑ 5. BL49-HEV-swNig		.	.	.	.	.	T	.	A	.	.	.	Q	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	R	I	.	V	L
☑ 6. JN119-HEV-swNig		.	A	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	R	I	.	V	L
☑ 7. LS9-HEV-swNig		G	A	.	.	.	.	.	A	R	.	.	Q	.	.	.	.	.	.	.	.	.	.	.	L	.	.	.	.	.	R	I	.	.	L
☑ 8. LS17-HEV-swNig		G	A	.	.	.	.	.	A	R	.	.	Q	.	.	.	.	.	.	.	.	.	.	.	L	.	.	.	.	.	R	I	.	.	L
☑ 9. LS22-HEV-swNig		.	.	.	.	.	T	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	L	.	.	T	.	.	R	T	.	.	L
☑ 10. LS23-HEV-swNig		.	.	.	.	.	T	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	L	.	.	T	.	.	R	T	.	.	L
☑ 11. LS25-HEV-swNig		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.
☑ 12. MG7-HEV-swNig		.	.	.	.	.	.	.	A	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.
☑ 13. MG8-HEV-swNig		.	.	.	.	.	.	.	A	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.
☑ 14. MG18-HEV-swNig		.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.
☑ 15. MG27-HEV-swNig		.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.
☑ 16. MG28-HEV-swNig		.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.
☑ 17. PK18-HEV-swNig		.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	P	.	.	.	.	.	.	.	I	.	.	L
☑ 18. SH36-HEV-swNig		.	.	.	.	.	.	.	A	.	.	.	Q	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	R	I	.	.	L
☑ 19. SH50-HEV-swNig		.	.	.	.	.	.	.	A	.	.	.	Q	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	R	I	.	.	L
☑ 20. M73218 Bur-82 Human Burma 1a		V	A	S	C	S	T	.	.	P	.	.	Q	.	.	.	.	.	.	.	S	C	I	H	L	*	.	.	L	.	R	I	.	V	L
☑ 21. M80581 Sar-55 Macaca Pakistan 1b		V	A	S	C	S	T	L	A	P	S	.	Q	.	.	.	.	.	.	.	S	C	I	H	L	W	.	.	L	.	R	I	.	V	L
☑ 22. M74506 M1 Human Mexico 2a		A	G	.	C	.	.	.	A	R	.	.	Q	.	.	.	.	Q	P	*	S	S	I	H	Q	W	.	.	L	.	R	I	.	V	L
☑ 23. AB097812 HE-JA1 Human Japan 4c		V	A	S	Y	.	T	L	A	P	S	.	Q	.	.	.	.	.	Q	*	S	F	.	.	Q	.	.	T	P	.	R	I	.	V	*
☑ 24. AY594199 swCH25 Pig China 4d		.	A	S	Y	.	T	.	A	P	.	.	Q	.	.	.	.	.	Q	*	.	F	.	P	Q	.	.	.	L	.	R	T	.	V	*
☑ 25. AF060668 HEV-US1 Human USA 3a		G	.	.	C	.	T	.	A	L	.	.	.	.	.	.	.	Q	Q	*	S	.	.	H	L	L	.	.	R	.	.	T	.	.	.
☑ 26. AB189070 JBOAR1-Hyo04 Boar Japan 3		G	A	S	C	S	T	.	A	L	S	.	Q	.	.	.	.	.	.	*	S	.	I	H	L	L	.	.	R	.	R	I	.	V	.