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MOLECULAR DETECTION OF HEPATITIS E VIRUS IN SWINE FARMS IN AND AROUND ADDIS ABABA, AND IN CAMELS FROM WESTERN HARARGHE

MSC THESIS

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MOLECULAR DETECTION OF HEPATITIS E VIRUS IN SWINE FARMS IN AND AROUND ADDIS ABABA, AND IN CAMELS FROM WESTERN HARARGHE

A Thesis Submitted to College of Veterinary Medicine and Agriculture, Addis Ababa University, in partial fulfilment of the requirements for the degree of Masters of Veterinary Science in Veterinary Microbiology.

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September, 2020

Bishoftu, Ethiopia

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DEDICATION

This thesis is dedicated to Ethiopian citizens who sacrificed their life in 2012E.C. in their home country.

STATEMENT OF AUTHOR

First, I declare that this thesis is my authentic work and that all sources of materials used for this thesis have been properly acknowledged. This thesis has been submitted in partial fulfilment of the requirements for MVSc degree at Addis Ababa University, College of Veterinary Medicine and Agriculture and it is deposited at the College library to be made available to borrowers under rules of the library. I seriously declare that this thesis is not submitted to any other institution anywhere for the award of any academic certificate, diploma or degree. So it is the first molecular attempt to detect HEV genome in our country.

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ABBREVIATIONS

cDNA	Complementary Deoxy Nucleic Acid
DcHEV	Dromedary Camel Hepatitis E Virus
dNTP	Deoxyribo-Nucleotide Triphosphate
eHEV	Enveloped Hepatitis E virus
ER	Endoplasmic Reticulum
ET-NANBH	Enterically Transmitted Non-A, Non-B Hepatitis
HEV	Hepatitis E Virus
IRES	Internal Ribosome Entry Site
NSP	Non-Structural Protein
ORF	Open Reading Frame
RdRp	RNA Dependant RNA Polymerase
RNA	Ribose Nucleic Acid
UTR	Un-Translated Region
WHO	World Health Organization

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ABSTRACT

Hepatitis E disease, caused by hepatitis E virus (HEV), is a worldwide human disease that is endemic in many developing countries. Hepatitis E virus is becoming an emerging infectious agent causing mainly acute infection worldwide and a major cause of water-borne hepatitis epidemic in tropical and subtropical countries in areas with poor sanitary conditions. It is a zoonotic disease of public health concern with high seroprevalence in pregnant women in Addis Ababa, transmitted primarily via the faecal-oral route. The presence of HEV specific antibodies were also reported in dromedary camels in Ethiopia; however, the infectious virus or a viral genome has not been demonstrated so far. To address this gap, a nested broad-spectrum RT-PCR protocol technique that is capable of detecting HEV in faecal samples including those derived from pigs and camels was used. A total of 95 faecal samples collected from both apparently healthy animals, 50 from pigs and 45 faecal samples from camels, were screened molecularly that resulted in the detection of HEV in four (8%)pig faecal samples collected from Burayu, Ethiopia. Three camel samples (6.7%) gave doubtful results while the rest samples did not result amplification of detectable viral gene fragments at the expected band size. Therefore, these results indicate that HEV is present in pigs and they could be a source of infection to humans and could be an unrecognized public health concern. Further confirmation of the presence of the viral genome in both the detected and doubtful samples via gene sequence generation is recommended to institute control and/or preventive measures.

Key words: *Burayu, Dromedary Camels, Hepatitis E virus, nested PCR*, *Pigs*

1. INTRODUCTION

Hepatitis E was recognized for the first time during an epidemic of hepatitis disease, which occurred in Kashmir Valley in 1978 as the main cause of non-A, non-B enterically transmitted hepatitis (Khuroo, 2011). It is the only hepatitis virus that has animal reservoir (Khuroo, 2011). Globally, HEV is becoming an emerging infectious agent causing mainly acute infection and a major cause of water-borne hepatitis epidemic in tropical and subtropical countries in areas with poor sanitary conditions. The infection is endemic in many countries or continents including southeast and central Asia, the Middle East, and Africa (Aggarwal and Jameel, 2011).

The causative agent was firstly known as enterically transmitted non-A, non-B hepatitis virus that was subsequently named hepatitis E virus (HEV), based on its enteric transmission and association with hepatitis epidemics (Aggarwal and Jameel, 2011). The etiological agent is a small, non-enveloped, single stranded RNA virus and is the only member of the genus *Hepevirus*, in the family of *Hepeviridae* (Pauli *et al*., 2009). Two major species of the virus are recognised: avian HEV and mammalian HEV. The mammalian HEV causes disease in human beings while the avian HEV causes enlargement of liver and spleen in chickens, but not in humans. HEV has considerable genomic diversity and four major genotypes of the virus have been identified (Khuroo, 2011) namely genotype 1, 2, 3 and 4. Hepatitis E is major cause of enterically transmitted hepatitis and an important public health concern globally (Khuroo, 2011).

The occurrence of hepatitis E disease is characterized by large scale water-borne epidemics of jaundice in regions of the world with contaminated water supplies and low sanitary conditions. For example, Kashmir recorded four major epidemics of hepatitis E from 1978 to 1982 involving estimated 52,000 cases of icteric hepatitis with around 1700 deaths (Khuroo, 1991). Every year, there are an estimated 20 million HEV infections worldwide, leading to an estimated 3.3 million symptomatic cases of hepatitis E. WHO,8 July 2019 estimated that hepatitis E caused approximately 44000 deaths in 2015 accounting for 3.3% of the mortality due to viral hepatitis (WHO, 2019).

Among humans in worldwide, HEV is the most common cause of acute viral hepatitis. The disease is generally self-limiting; however, mortality rates are high among pregnant women

and young infants. Chronic HEV infection is a problem for immunocompromised patients, such as those who have received a solid organ transplant and those with HIV infection(Woo *et al*., 2014). In addition to humans, HEV has been found in the other mammals: pigs, boar, deer, rodents, ferrets, rabbits, mongoose, bats, cattle, sheep, foxes, minks, and horses(Nakamura *et al*., 2006; Kenney, 2019). Human infections with HEV genotype 3 (HEV3) and HEV genotype 4 (HEV4) have been associated with consumption of raw or undercooked pork meat (Meng, 2011). In general HEV infection is mainly transmitted through contaminated water with infected faeces. Since water supplies and sanitary infrastructures have been improved, animals have become a major source of human HEV infection. It is also detected in faecal samples from dromedary camels in the Middle East (Woo *et al*., 2014).

Therefore the disease caused by HEV infection is a major public health problem in the world, especially in resource limited countries. In African countries, a number of HEV outbreaks were reported including in Ethiopia, Somalia, Uganda, Democratic republic of Congo, Sudan and South Sudan in different periods (Tsega, 1991; Nicand *et al*., 2005). The highest seroprevalence (50.01%) was reported in North Africa followed by East Africa (35%) (Dagnew *et al*., 2019). In Ethiopia there is a report of high seroprevalence (31.6%) of HEV in pregnant women (Abebe *et al*., 2017) in single hospital found in Addis Ababa. A single study carried out in camels in Ethiopia (Li *et al*., 2017) reported serological evidence of the virus. However, there is no report of researches done on the virus and its main source of infection such as camel, pig, and people rather than serological investigation in people and camels.

Therefore, the objective of this research was

- To detect the HEV from faecal samples of extensive swine farms in and around Addis Ababa, and in dromedary camels collected from western Hararghe

2. LITRATURE REVIEW

Hepatitis E was initially designated as enterically transmitted non-A, non-B hepatitis disease (ET-NANBH) due to similar clinical presentations to hepatitis A and B in patients, but the causative agent was firstly unknown (Balayart, *et al*.,1983). Early researches implied that an RNA virus was the prospective pathogen for the ET-NANBH. A portion of a highly conserved RNA dependent RNA-polymerase (RdRp) motif which is commonly found in RNA viruses was identified by analysis of a cDNA library from infectious bile sample(Reyes *et al*., 1983). This new virus was designated as hepatitis E virus (HEV) which was responsible for the outbreak of ET-NANBH (Nan and Zhang, 2016). Therefore, hepatitis E is a disease caused by infection with hepatitis E virus (HEV), an RNA virus that exists in both enveloped and naked forms and was first recognized in the early 1980s (Balayart *et al*.,1983).

2. 1. Hepatitis E Virus

Since the discovery of HEV in 1983 (Balayart*et al*., 1983), the first report of HEV genomic sequence was reported eight years later (Tam *et al*., 1991). The HEV virus was classified into the family *Hepeviridae* and genus *Hepevirus*. The recent agreement has divided this family into two genera: genus *Orthohepevirus,* which includes HEV strains from mammals and birds (Purdy *et al*., 2017) and genus *Piscihepevirus,* which consists solely of the species *Piscihepevirus A* and its single member, cutthroat trout HEV. Genus *Orthohepevirus* is further divided into four species such as: *Orthohepevirus A, Orthohepevirus B, Orthohepevirus C* and *Orthohepevirus D* (Purdy *et al*., 2017). HEV is the most common cause of acute viral hepatitis in humans globally (Woo *et al*., 2014). The genus *Hepevirus* consists of two species: (i) mammalian HEV, which causes human disease and infects several other mammalian species, in particular pigs; and (ii) avian HEV, which is responsible for big liver and spleen disease in chicken. The virus is known to infect other birds such as turkeys. The zoonotic importance of avian HEV is not reported so far (WHO, 2010).

HEV is identified as a non-enveloped, icosahedral shaped and spherical (Aggarwal and Jameel, 2011), approximately 27-34nm in diameter, and consisting of a single-stranded, positive sense RNA molecule about 7.5 kilobases (kb) in length. The outer surface of the particle consists of indentation and projections (spikes) (Figure 4), and it is often found in faeces of infected individuals (Doceul *et al*., 2016).

It has seven genotypes, of these 4 of them are known mammalian genotypes levelled as genotype 1, 2, 3 and 4 which belong to a single serotype (Bradley, 1995; Nair *et al*., 2016). Genotype 1 and 2 HEV remain endemic in the absence of non-primate animal reservoirs without a known reason, where as genotypes 3 and 4 have also been found in several mammalian species, particularly highly prevalent in pig herds (Clemente-Casares *et al*., 2003; Doceul *et al*., 2016; Kenney and Meng, 2019). Up to now, only a single report of genotype 1 HEV infecting outside of a primate species exists (Kenney and Meng, 2019). Genotype5, 6 and 7 viruses are known to infect animals such as wild boar and camel, respectively (Nair *et al*., 2016) (Figure 1). The viral genome contains three partially overlapping open reading frames (ORF 1-3). Of these, ORF2 codes for the viral capsid protein which is the target of neutralizing antibodies against HEV (Bradley, 1995). The virus is relatively stable in the environment (Clemente-Casares *et al*., 2003), and is sensitive to heat, chlorination and ultraviolet light (Girones *et al*., 2014).

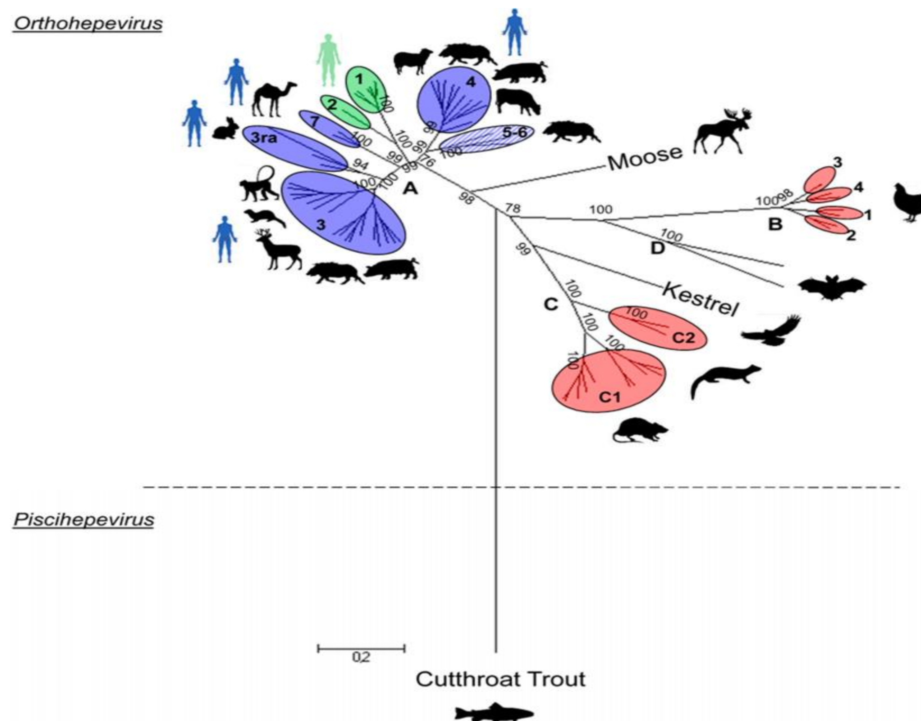

Figure 1: Phylogenetic tree of members of the family Hepeviridae.

Source: (Doceul *et al*., 2017)

2.2. Genomic organization of HEV

The mammalian HEV genome is a single stranded, positive-sense RNA ~7200 nucleotides in length; whereas the avian HEV genome is ~6650 nucleotides long (Huang *et al*., 2004; Sun

et al., 2019). HEV genome RNA possesses a 5' 7-methylguanosine cap structure followed by a short 5' untranslated region (UTR) of 26 nucleotides, three major open reading frames namely ORF1, ORF2, and ORF3, and a 3'UTR (Zhang *et al*., 2001). The three open reading frames (ORFs) partially overlapped in an order of sequences encoding non-structural proteins (NSPs) followed by structural proteins (Tam *et al*., 1991; Tsarev *et al*., 1992).

Open reading frame1 is the largest ORF in the HEV genome approximately with 5082 nucleotides that can be translated directly from the viral genome to produce a poly-protein containing the viral non-structural proteins (Tsarev *et al*., 1992; Emerson *et al*., 2001). There is an RNA structural element in the upstream region of ORF1 that binds to the capsid protein and is thought to be the RNA packaging signal (Surjit *et al*., 2004). ORF1 is the largest, comprising ~5 kB of the virus and encoding enzymes required for genomic replication including the methyl transferase (Met), RNA helicase (Hel), a putative papain-like cysteine protease (PCP), and the RNA-dependent RNA polymerase (RdRp) (Robert *et al*., 2019). The genome additionally contains 'X', 'Y', and 'hyper variable region (HVR)' domains whose precise functions are not understood but that are known to play crucial roles in viral replication (Robert *et a*l., 2019).

The viral capsid protein is encoded by ORF2 which is the major structural component of the virion. In genotype1 (gt1) HEV, ORF2 is 1983 nucleotides (nt) in length, beginning 37 nucleotides downstream from the ORF1 stop codon and overlapping all but 14 nucleotides of ORF3 (Reyes *et al*., 1993).

While ORF3 (340nt) overlaps the 5' end of ORF2 by 300 nt and encodes a phosphoprotein that interacts with cellular signalling proteins (Smith and Purdy, 2013). ORF3 is the smallest ORF of the HEV genome, which translated from a sub-genomic RNA into a protein of 113–115 amino acids. Previous studies showed that ORF3 is bound to viral particles found in patient sera (Takahashi *et al*., 2008) and produced in cell culture (Takahashi *et al*., 2008; Emerson *et al*., 2010). Although in cultured cells ORF3 has not appeared essential for HEV infection, RNA replication, viral assembly or, it is required for viral particle release (Emerson *et al*., 2010). There is a junction region between ORF1 and ORF3 containing cis-active elements thought to control the expression of a sub-genomic bicistronic messenger RNA (mRNA) (Fig. 2) (Graff *et al*., 2006; Huang *et al*., 2007). Both the sequence and the stem-loop structure in the junction region play important roles in HEV replication (Cao *et al*., 2010).

A fourth open reading frame (ORF4), reported by a single study which is only present in HEV genotype 1and is translated into a protein that increases the activity of the RNA-Dependent RNA Polymerase (RdRp) (Nair *et al*., 2016). Overlapping the X and helicase (Hel) domains in gt1 HEV is a small frame shifted ORF4 that is controlled by an internal ribosome entry site (IRES)-like RNA structure that directs translation of ORF4 under endoplasmic reticulum (ER) stress (Kenney and Meng, 2019).

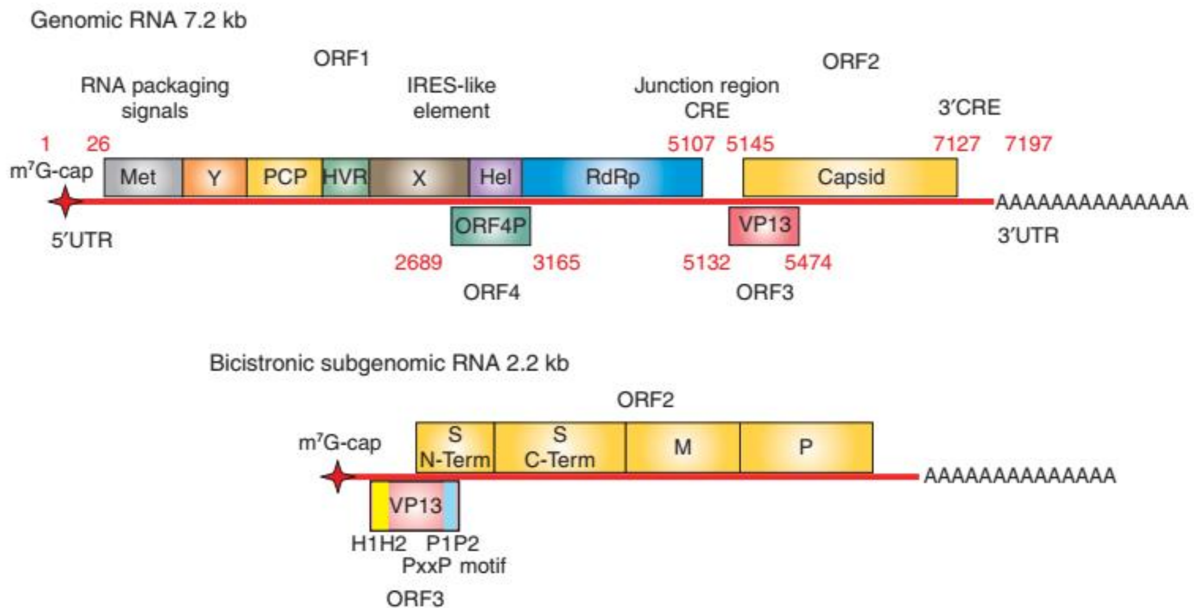

Figure 2: Genomic organization of HEV

Source: (Kenney and Meng, 2019)

2. 3. Viral Transmission

The major way of HEV transmission is primarily by the faecal–oral route and has been reported to occur as large water borne epidemics and small outbreaks in developing countries. So its infection was first described as water borne disease, transmitted through drinking of faecally contaminated water (WHO, 2019). However, current investigations have not consistently found well-defined water sources of HEV, suggesting other possible modes of transmission (Teshale *et al*., 2010). These other transmission modes may be related to the level of sanitary conditions, population immunity, living conditions, and other factors. In sporadic hepatitis E, modes of transmission are even less clear and are generally not identified. Transmission by blood transfusion has been reported in humans (Khuroo *et al*., 2004).

In different geographical areas, frequent and continuous detection of specific HEV types in the same species clearly indicates a true animal reservoir as represented by domestic pigs, wild boars, chickens, and rats. In other animal species where HEV is detected sparsely, this suggests spill-out infections rather than a true reservoir host (Kenney, 2019). Therefore Hepatitis E is considered a zoonotic infection with pig and wild boar serving as the main reservoir for human infections (Mirazo *et al.*, 2014). Zoonotic transmission through direct contacts with infected animals has also been reported. Farmers, veterinarians, and workers attending animals comprise highly at-risk and exposed group for HEV infection (Meng *et al.*, 2002). Moreover, a number of sporadic autochthonous cases have been related to the consumption of raw or undercooked pork products, especially liver-based products (Colson *et al.*, 2010; Moal *et al.*, 2012). Incidence and mortality rate of hepatitis A undergone sharp declines in China due to rapid industrialization and socioeconomic development, leaving hepatitis E as the most common cause of acute viral hepatitis (Xiang *et al.*, 2017). Such an epidemiological shift is likely driven by ongoing zoonotic food-borne transmission of HEV, which has been demonstrated in swine populations across the country (Sridhar *et al.*, 2017).

Moreover vertical (maternal-foetal) transmission is also considered the main routes of HEV transmission from pregnant women. Maternal to foetal transmission of HEV infection has been reported (Aggarwal and Naik, 2009). HEV-RNA or immunoglobulin (Ig) M anti-HEV antibodies have been detected in seven of eight babies born to mothers with acute hepatitis E in the third last of pregnancy (Khuroo*et al.*, 1995).

2.4. Virus Replication

The HEV genome resembles mRNA, with a 5′ cap and a 3′ poly-A tail structure (Figure 2). A small non-coding region (NCR; 1-25bp) is located at the 5′ end of the genome and is followed by open reading frame 1 (ORF1), encoding the non-structural proteins used for replication (5109bp) with a predicted molecular mass of ~185kDa (Tsarev *et al.*, 1992). ORF1 and the two bicistronic overlapping reading frames, ORF2 and ORF3 are separated by a small cis reactive element (CRE site) (Graff *et al.*, 2006). The 3′ end also contains an untranslated region (UTR) and a poly (A) tail. After the polyprotein is translated (Figure 3B), the replication of the viral RNA by RdRp proceeds with the synthesis of the negative strand RNA (Figure 3C). Based on the negative strand, two different RNAs are synthesized, the full-length genomic RNA (D) and a 2.2 kb subgenomic RNA (D) (Graff *et al.*, 2006). The

genomic RNA serves as a template for ORF1 translation and is packaged into viral particles or serves as a template for the synthesis of additional negative strand RNA, whereas the subgenomic RNA serves as a template for the translation of the capsid protein (72 kDa) and the ORF3 protein (E) (13 kDa) (Himmelsbach *et al*., 2018).

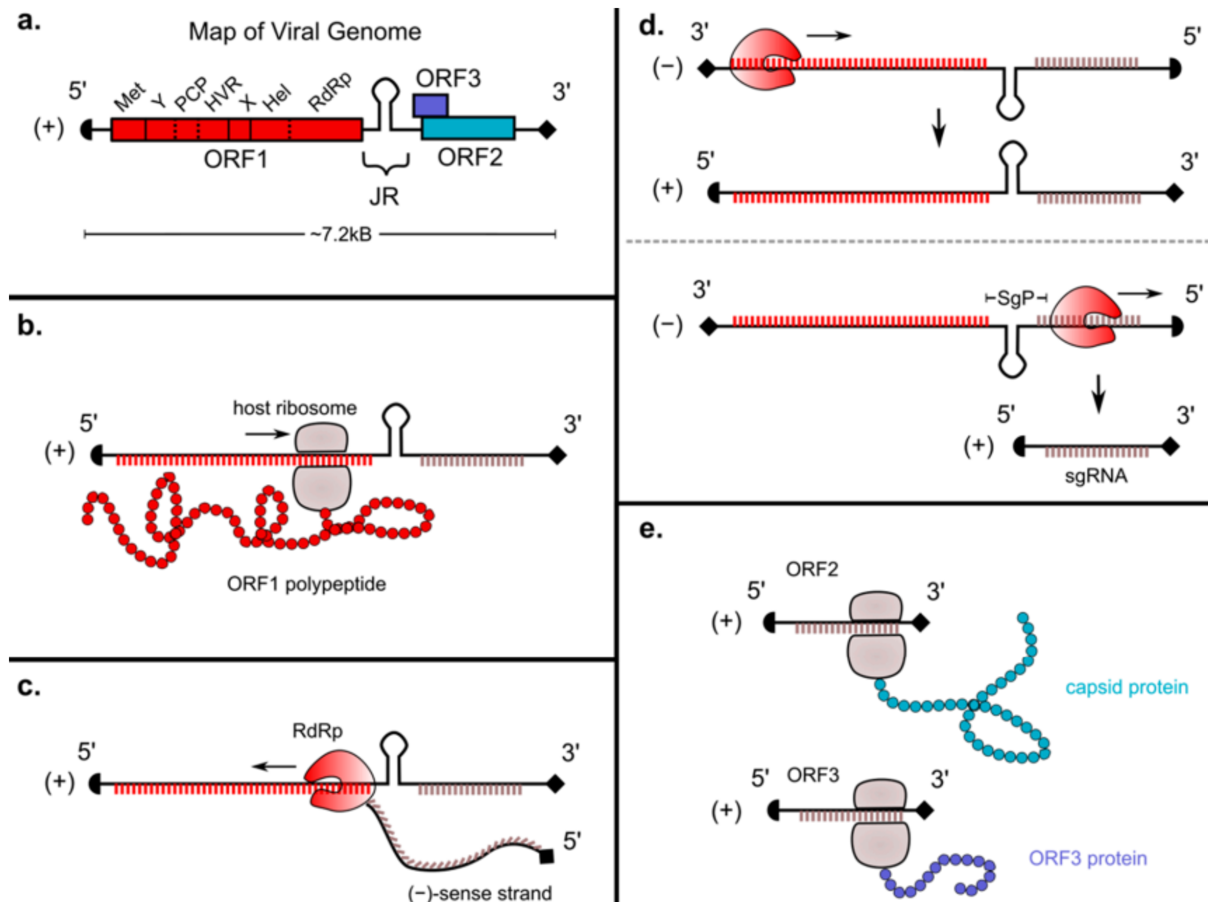

Figure 3: Genomic organization and replicative mechanism of hepatitis E virus

Source: (Robert *et al*., 2019)

Hepatocytes are polarized epithelial cells in vivo, which have a uniquely organized polarity with distinct apical (facing the bile canaliculi) and basolateral (facing the hepatic sinusoid) domains in physiological conditions (Gissen and Arias, 2015). The progeny virions can release at both the apical and basolateral membranes of infected hepatocytes. Studies have found that ORF3 is mainly located close to the bile canaliculi of hepatocytes in vitro (Emersonet *et al*., 2010; Capelli *et al*., 2019) and in vivo (Allweiss *et al*., 2016). Most infectious HEV particles as enveloped (eHEV) form are released from the hepatocyte via its apical domain into the bile canaliculi, where they enter the biliary tract and are subsequently shed into faeces, while a small fraction of HEV particles (as eHEV form) are released from

the basolateral domain into the blood, where they can spread throughout the host. Enveloped (eHEV) released from the apical domain enters the bile, and the eHEV membrane is degraded by the detergent action of bile, resulting in non-enveloped HEV in faeces (Yin *et al.*,2016).

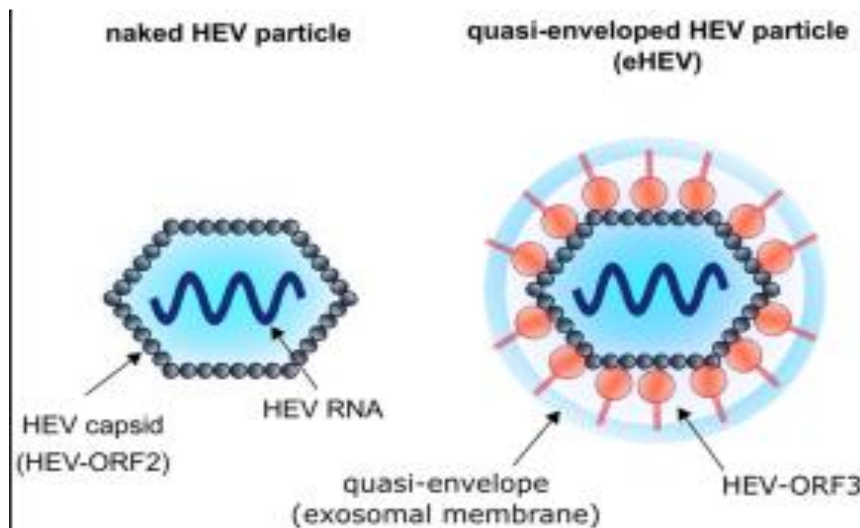

Figure 4: Representation of enveloped and naked hepatitis e virus

Source: (Himmelsbach *et al*., 2018)

2.5. Host Range

Swine/pig are becoming an important species for fighting poverty in many of the African countries (Thomas *et al*., 2013; Goraga *et al*., 2017) and serve as a cheap source of animal protein in many countries except in Ethiopia. Although in Ethiopia pig production is at its infant stage; nowadays, it is getting better attention and emerging as a new business area. The situation in Ethiopia is being changed nowadays, where pork products started getting market attention. This can be associated with an ever increasing human population, immigration of several tourists and other foreigners who come to Ethiopia for business leads to higher pork meat demand in the country (Birhan *et al*., 2015; Goraga *et al*., 2016). However, the number of swine herds per region in Ethiopia is not many; the production is expanding across regions, especially in major towns such as Addis Ababa, Bahirdar, Gondar, Mekele, Bishoftu (Debre Zeit) and Adama (Nazareth) (Tekle *et al*., 2013; Birhan *et al*., 2015). Although pigs kept under all production systems can be the host of a variety of pathogens, allowing pigs to roam freely increases the risk of disease transmission to other pigs, to other wild and domestic animals, and to humans (Thomas *et al*., 2013).

The other potential HEV animal host is camels. The camel rearing sub-sector in Ethiopia has been an integral part of agricultural activity. It has been contributing to the household food via provision of milk, meat, income and poverty alleviation, transportation and national economy through export of live camels. Previous report indicated that the country has huge camel resources that made it the sixth leading country in camel population in Africa (Sisay and Awoke, 2015). The ability of camels to act as a point source or reservoir and source of HEV is a concern due to increasing human demands for camel products like meat and milk, lack of biosafety and biosecurity protocols in many regions. According to Zhu *et al.,* (2019) report, 65% of camel-born zoonotic diseases focused on Middle East respiratory syndrome (MERS), brucellosis, *Echinococcus granulosis*, and Rift Valley fever.

2.5. 1. Transmission

Available evidence indicate that meat, milk and faeces from camels might pose a risk of HEV transmission to humans (Li *et al*., 2016). Zoonotic disease transmission could occur through a number of ways, such as direct contact with livestock or manure or from contaminated food/meat, milk, or water. Sixty-four percent of herder households reported using dried manure for fuel. Unprocessed river water was often the primary source (52.7%) of drinking water in Mongolian herder, which is potentially contaminated with manure-borne pathogens (Sack *et al*., 2018).

2. 6. Pathogenesis and Clinical Presentation of the Virus

The virus affects mainly young adults (15–30 years) in developing countries, but pregnant women are particularly vulnerable. The clinical symptoms are typical of acute viral hepatitis and include jaundice, malaise, anorexia, nausea, abdominal pain, fever and hepatomegaly; an icteric hepatitis is also observed (Smith, 2001). The Hepatitis E has a mortality rate of 0.2–1% in the general populations (Chandra *et al*., 2008), but it may reach 30% during the last trimester of pregnancy (Navaneethan *et al*., 2008). Pregnant women die of obstetric complications such as haemorrhage or eclampsia. Fulminant liver failure can also occur (Khuroo *et al*., 2009). Still births are common, as is vertical transmission to infants which leads to increased neonatal morbidity and mortality (Khuroo *et al*., 2009).

The pathogenesis of hepatitis E is poorly understood. Since HEV is mainly transmitted by the faecal-oral route, it is unclear how the virus reaches the liver. Perhaps there is an extra-

hepatic site of virus replication. The virus could replicate in the intestinal tract before reaching the liver. Negative sense strands of HEV RNA have been detected in the small intestine, lymph nodes, colon, and liver of pigs which is feature of replication, indicating extra-hepatic HEV replication (Williams *et al*., 2001). HEV then replicates in the cytoplasm of hepatocytes and is released into both blood and bile. The liver damage induced by HEV infection may be immune-mediated by cytotoxic T cells and natural killer (NK) cells since HEV is not cytopathic (Knipe *et al*., 2013). Endotoxin mediated hepatocyte injury was proposed (Jameel, 1999), but the precise cellular/ molecular mechanisms are not clear (Chandra *et al*., 2008). A shift in the Th1/Th2 balance towards Th2 has been observed in pregnant women infected with HEV compared to non-pregnant women (Pal *et al*., 2005), but how this influences the severity of HEV infection is not clear (Chandra *et al*., 2008).

Pregnant women with jaundice and acute viral hepatitis due to HEV showed higher mortality rates and worse obstetric and foetal outcomes than those with other types of viral hepatitis (Patra *et al*., 2007). There were increased levels of estrogen, progesterone and β human chorionic gonadotropin (βHCG) in HEV-positive pregnant patients with fulminant hepatitis compared to HEV-negative patients and controls (Jilani *et al*., 2007). Selective suppression of nuclear factor kappa B (NF*κ*B) p65 in pregnant compared to non-pregnant fulminant hepatitis patients has also been proposed to cause liver degeneration, severe immunodeficiency, and multi-organ failure (Prusty *et al*., 2007).

2.7. Epidemiology

2.7.1. Geographic distribution of the virus

Hepatitis E is hyper endemic in many countries located in southeast Asia (Burma, Cambodia, Indonesia, Thailand, Vietnam and Laos), southern Asia (India, Bangladesh, Bhutan, Nepal, Pakistan and Sri Lanka), central Asia (Kazakhstan, Tajikistan and Uzbekistan); east Africa (Kenya, Uganda and Burundi), north Africa (Algeria, Morocco, Sudan and Tunisia), west Africa (Ivory Coast, Liberia, Nigeria and Mali) and some countries in North America (Mexico) (Khuroo, 2011). It is endemic in many countries of Middle East (Turkey, Saudi Arabia, Yemen, Libya, Oman, Bahrain, Iran, Kuwait and the United Arab Emeritus), some regions of Southeast Asia (Singapore) and South America (Brazil, Argentina, Ecuador and Uruguay). Hepatitis E is responsible for more than one-fourth of all cases of acute sporadic hepatitis and fulminant hepatitis (Ghabrah *et al*., 1995). Hepatitis E is caused mainly by

genotypes 1 (G1) to 4 (G4) hepatitis E virus (HEV) infection, and genotype 3 (G3) and G4 HEV are responsible for sporadic and zoonotic infections in both humans and other animal species worldwide (Meng, 2010).

The different HEV genotypes show a distinct geographical distribution (Lu *et al*., 2006). Genotype 1 hepatitis E virus strains are mainly isolated from hepatitis E patients in Asian and African countries, both from sporadic cases as well as outbreak-cases. Genotype 2HEV-strains have been reported during outbreaks in Mexico, Nigeria and Chad. Genotype 3 HEV-strains are commonly associated with locally acquired hepatitis E cases in North-America, Europe, Africa, Japan and China. Genotype 4 strains are also studied mostly in sporadic cases of hepatitis E in developed countries in Asia, such as Japan and Taiwan, but also in other countries such as Indonesia, China, and Vietnam (van der Poel and Rzezutka, 2017).

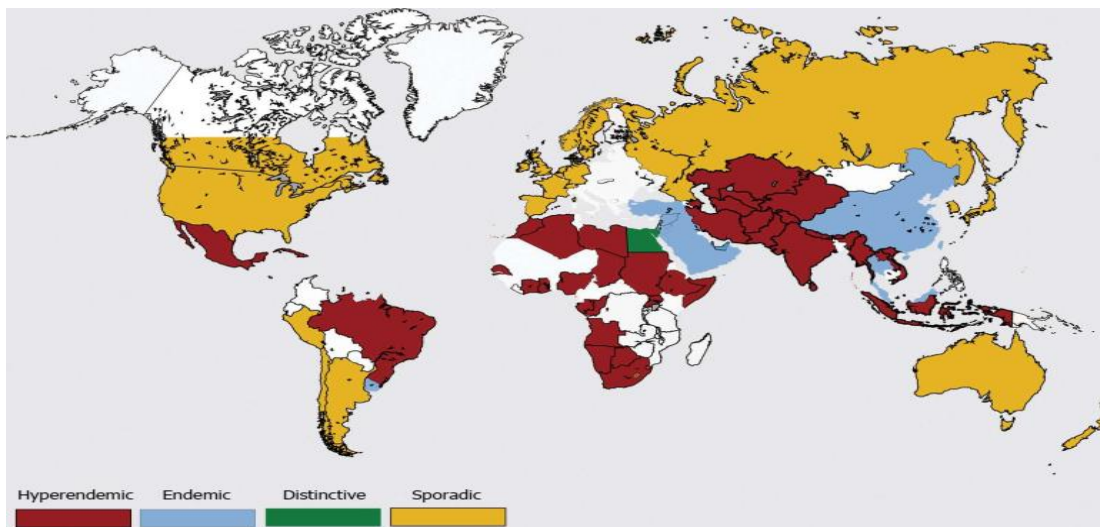

Figure 5: Geographic distribution of the hepatitis e virus

Source: (Khuroo *et al*., 2016)

2.7.2. Virus status in Ethiopia

Different seroprevalence reports of human HEV in different times have been reported. During Ethiopia and Eritrea were not separated countries, there was a report of HEV from October 1988 to March 1989 in military camps found in Eritrea northern provinces of Ethiopia as 28 of 30 (93%) patients had anti-HEV antibodies due to waterborne outbreaks of acute hepatitis (Tsega, 1991; WHO, 2010).

In a study done on 32 pregnant and 34 non pregnant Ethiopian women between 15 and 45 years of age with sporadic acute viral hepatitis 19 (59%) pregnant women had seroprevalence of the hepatitis E virus (HEV) infection (Tsega *et al*., 1993). Seroprevalence of HEV in pregnant women is also reported as 31.6% in Addis Ababa (Abebe *et al*., 2017) and 43.4% in Tigray (Niguse *et al*., 2018).

In animals there is a report which revealed that 22.4% of Ethiopian dromedary camels were positive for anti-DcHEV IgG (Li *et al*., 2017), suggesting that the dromedary camel HEV (DcHEV) infection is circulating in the dromedary camels in Ethiopia. In addition to camels studies indicated that swine and human HEV strains are genetically very close, and cross-species transmission has been proved (Meng *et al*., 1998). Moreover, there is a report of seroprevalence of HEV in pig in developing countries such as Cameron as 43.2% (Modiyinji *et al*., 2018). However, there is no a report of HEV studies in Ethiopian pigs. Therefore, it is crucial to fully understand the conditions related to swine farm and dromedary camels infection and HEV transmission dynamics within the swine and camel population in order to limit the risk of introducing contaminated products into the food chain and water.

3. MATERIALS AND METHODS

3.1. Study Area

The study was conducted from November to April in 2020 in Burayu and Kolfe Keranio swine farms and camel faecal samples collected from western Hararghe during 2019. Western Hararghe is located to the eastern part of Ethiopia and Oromiya, 317km far from Addis Ababa (CSA, 2007). It is located between 7^0 52' 15''- 9^0 28' 43'' N latitude and 40^0 03' 33''– 40^0 34'' 13'' E longitude with an altitude of 1200-3600m above sea level (WHZANRO, 2016). The other study area, Burayu town is located in Oromiya national regional state and in the western direction of Addis Ababa at a distance of 15km near to Kolfe Keranio sub-city found in Addis Ababa. The town is a high land area located at an altitude of 2580m above sea level with an area of 66.5km^2. Burayu town is bounded in the East by Finfinne, in West by Walmera district, in North by Sululta district and in South by Sebata Hawas district (Hordofa *et al*., 2018).

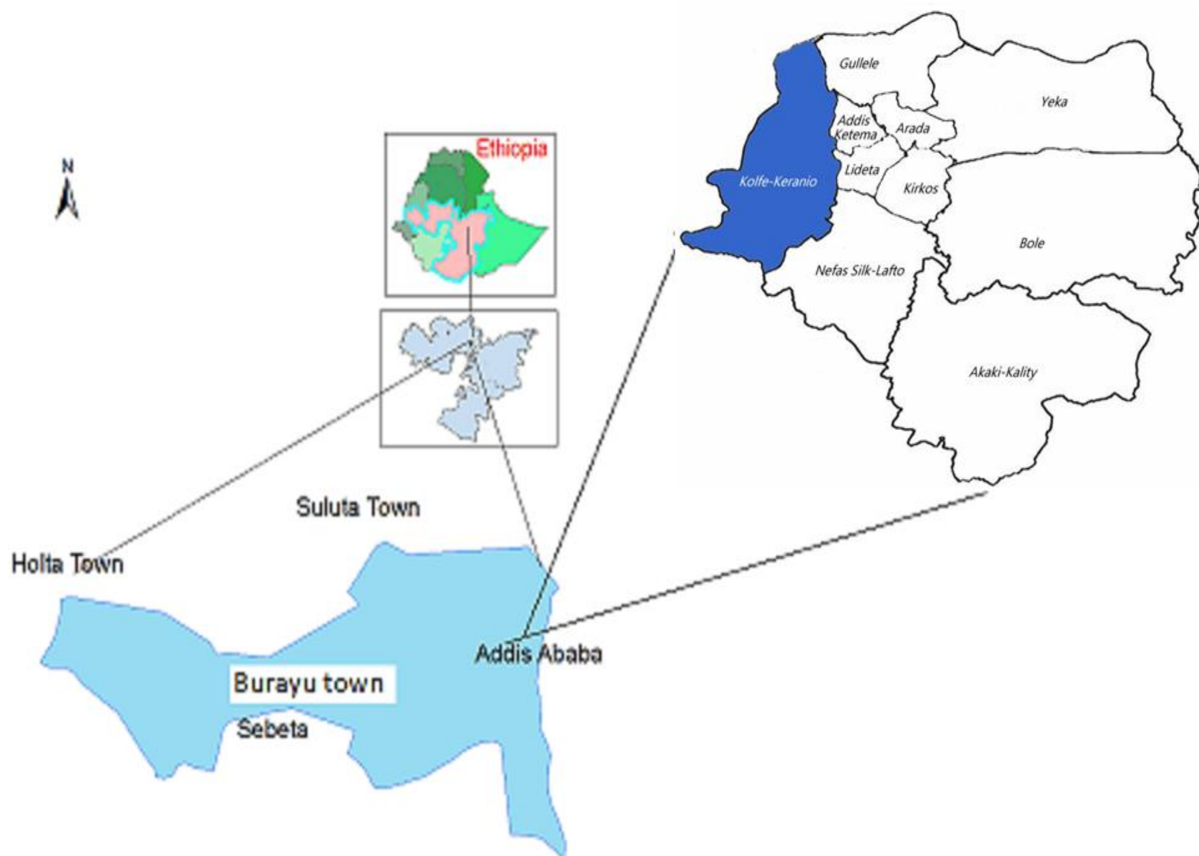

Figure 6: Location of study area (Burayu town and Kolfekeranio)

Source: (Legese, 2015)

3.2. Study Population

The target populations for the study were swine and camels of all age groups and both sexes (males and females).The swine are found in and around Addis Ababa, Ashewa Media, and Burayou towns. There was no known record of swine farms and population from office of livestock and fisheries of Burayu district. However, in Burayu district there were three extensive swine farms and one extensive farm in Kolfe Keranio with 50 to 100 heard size. All farms were sampled; and of the total swine faecal samples (50) 41 were rectal samples directly from rectum and 9 samples collected freshly voided faeces from the barn.

Western Hararghe zone has 91 948 camel populations (CSA, 2017), and they are important component of the prevailing crop-livestock mixed farming systems of the study Zone. Small holder farmers of the study area owned various livestock species such as cattle, sheep, goat, chicken, camel and equines (CSA, 2017) table 3-1. Out of the total camel population, 26 samples were collected from Chiro town and 29 were collected from Mieso town.

3.3. Study Design

A cross sectional study design was conducted in three extensive pig farms found in Burayu and one farm in Kolfe keranio sub-city, which are situated in the western part of Addis Ababa, Ethiopia. This study design was also conducted during camel faecal sampling, collected from western Hararghe in 2019 for identification of hepatitis E virus from camel faeces stored at -80°C.

3.4. Sample Collection, Transportation and Storage

Swine rectal faecal samples and freshly voided faeces from swine farms were collected in a sterile universal bottle from three farms in Burayu district and one Kolfe Keranyo extensive swine farming system. Rectal faeces were collected by inserting two fingers into the rectum wearing sterile glove during when the pigs were recumbent (Figure7). All samples were collected hygienically and placed in sterile universal tubes. The samples were coded with permanent marker and managed properly until laboratory activities conducted. These samples were labeled and transported in ice box at 4^{0}C to National Animal Health Disease Investigation Centre (NAHDIC) virology laboratory, Sebeta. The samples were stored at -80^{0}C until molecular detection was conducted in NAHDIC molecular laboratory. The camel

faecal samples which had been collected during 2019 from western Hararghe were already stored at -80^0C until the molecular detection commenced.

Figure 7: Pig rectal sampling at the farm

3.5. Molecular Detection of Hepatitis E Virus

3.5.1. HEV RNA extraction

Hepatitis E virus RNA was extracted from the faecal samples using the QIAamp viral RNA extraction mini kit (QIAGEN, Hilden, Germany) (Takahashi *et al*., 2007; Johne *et al*., 2010). Briefly, 0.5gm of faecal samples were first suspended in 5ml of phosphate-buffered saline (PBS) to form a 10% suspension and then centrifuged at 8000 revolution per minute (rpm) for 5 minutes. The supernatant obtained was collected and used to extract viral RNA according to the QIAamp® Viral RNA Mini kit, 2018 instructions.

560µl prepared Buffer AVL containing carrier RNA was Pipette into a 1.5ml microcentrifuge tube and mixed with 140µl of the sample. The mixture incubated at room temperature for 10 min followed by brief centrifugation. 560µl of ethanol (96–100%) added to the solution and mixed by vortex. Then 630µl of this solution had been centrifuge at 6000 x *g* (8000 rpm) for 1min. It was followed by adding 500µl Buffer AW1 and centrifugation at 6000 x *g* (8000 rpm) for 1min. The filtrate was discarded and 500µl Buffer AW2 added. The cap closed and centrifuged at full speed revolution (20,000 x *g*; 14,000 rpm) for 3 min. The QIAamp Mini column placed in new collection tube and full speed revolution repeated for 1min to remove droplets and made RNA concentrated. The QIAamp Mini column placed in 1.5ml microcentrifuge tube and 60µl Buffer AVE added. The solution then centrifuged at 6000 x *g*

(8000 rpm) for 1 min for RNA elution (Annex 1).The Viral RNA was frozen at -80^{0}C until further use.

3.5.2. Amplification and detection of HEV RNA

The RNA was amplified with full nested conventional reverse transcriptase polymerase chain reaction (nRT-PCR) in two consecutive amplification rounds. The amplification targeted to amplify about 469bp in the first round and 325bp in the second round PCR in the 5' end of ORF1of HEV. The first round conventional PCR amplification was conducted with forward and reverse external primers (HEV-CS) and (HEV-CAS) with primer set: HEV-CS 5'TCG CGC ATC ACM TTY TTC CAR AA 3' and HEV-CAS 5' GCC ATG TTC CAG ACD GTR TTC CA 3' respectively, followed by the second RT-PCR (Zaki *et al*., 2009). The initial RT-PCR was performed with an One-Step RT-PCR Kit (Qiagen, Hilden, Germany) in a 25µL volume using 5µL of total extracted RNA, 2µL of 100pM/µL of the external primers, and 1µL Qiagen one step RT-PCR enzyme mix, 1µL of 10mM each dNTP mix, 0.5µL RNase out RNA inhibitor and 5µL of 5x Q solution and 5µL of 5x PCR buffer (Zaki *et al*., 2009).

The thermal cycling conditions included one step of reverse transcription for 30 min at 50^{0}C and an initial PCR activation step for 15 min at 95^{0}C. This was followed by 40 cycles of denaturation for 30 seconds (sec) at 94^{0}C, annealing for 30sec at 50^{0}C, extension for 1min and 15 sec at 72^{0}C and a final incubation for 10 min at 72^{0}C (Zaki *et al*., 2009).

The second amplification was performed with internal primers: (HEV-CSN) 5' TGT TGC CCT GTT TGG CCC CTG GTT TAG 3' and (HEV-CASN) 5' CCA GGC TCA CCR GARTGYTTC TTG CA 3'. ATaqDNA PCR Kit (Qiagen, Hilden, Germany) was used to amplify the cDNA in a 50µL reaction mixture volume using 5µL of the RT-PCR product (cDNA), 5µL of Taq Buffer 5×, 5µL of Mg^{2+} ($MgCl_2$), 1µL of 10-mM dNTPs, 0.25µL of 5-U/L Taq polymerase, and 3µL of each 10pM primer. The amplification program was performed as follows: initial denaturation at 94^{0}C for 5min; 38 cycles of denaturation at 95^{0}C for 30sec, alignment at 55^{0}C for 45sec, and elongation at 72^{0}C for 60 sec; followed by a final extension cycle at 72^{0}C for 5min and at 4^{0}C for 5min (López-Santaella *et al*., 2019). A Flex Cycler^2 thermal Cycler was used for genome amplification. The amplification product was separated by gel electrophoresis on a 2% agarose gel for 40min at 100 voltages. SYBR Safe Dye (Invitrogen, Carlsbad, USA) staining was used to reveal the amplification product. The bands of positive results were aligned at 325pb with 100bp differences with 600bp PCR

marker (ladder) (Qiagen, gen pilot) and the amplification was stored at -20^{0}C or -80^{0}C for DNA sequencing and gel documented. However; due to the occurrence of Corona virus disease (covid-19) pandemic outbreak in the world, the sequence was not generated to date.

3.6. Data Management

Laboratory results were recorded accurately in a recording format and saved in Microsoft Excel for data analysis using and proportion was calculated manually.

4. RESULT

4.1. Faecal Contamination Watering Points

The figure below (Figure 8) shows pigs feeding around the watering river in Burayu. Feeding in such location could be accompanied by defection of the pigs that could be taken to the river by wind and rain to contaminate the river. In grazing point contamination could also lead to other susceptible pig population infection as they are feeding together. The watering point contamination via letting the pigs to feed around the water points and/rivers could be one route of virus transmission to the community in the study area (Figure 8). Therefore, the probable route of contamination of the environment with faeces originating from pigs could lead to HEV infection of susceptible hosts (Figure 8).

Figure 8*:* Rooting and feeding of the pigs around the farm near to the river.

The figure shows likely contamination of the water with dust and pig faecal materials in Burayu district, Tatek kebele.

4.2. Viral RNA Detection

A total of 50swine and 45 dromedary camel faecal samples collected in extensive farms located in Burayu district, Kolfe Keraniyosub-city and western Hararghe, respectively. These samples were screened using the full nested broad-spectrum conventional RT-PCR for the presence of HEVRNA. Of the total 50 pig samples, 4 samples (8%, 4/50) showed clear evidence of HEV gene amplification based on the band size that gave expected amplification of 325bp (Figure 9and 10) upon separating the amplicon on agarose gel electrophoresis. In Figure 9 and 10 below, lanes 5 and 8 respectively gave strong band, suggestive genome amplification of observed while in two pig samples, light bands of expected 325bp size (Figure 10, lanes 11 and 12) gave positive results. Therefore, the four bands aligned perfectly with the expected band of 325bp upon running fully nested RT-PCR.

Three camel faecal samples (Figure 10; lanes 2, 4 and 5) showed doubtful results as the band generated gave slightly lower than the expected 325bp.

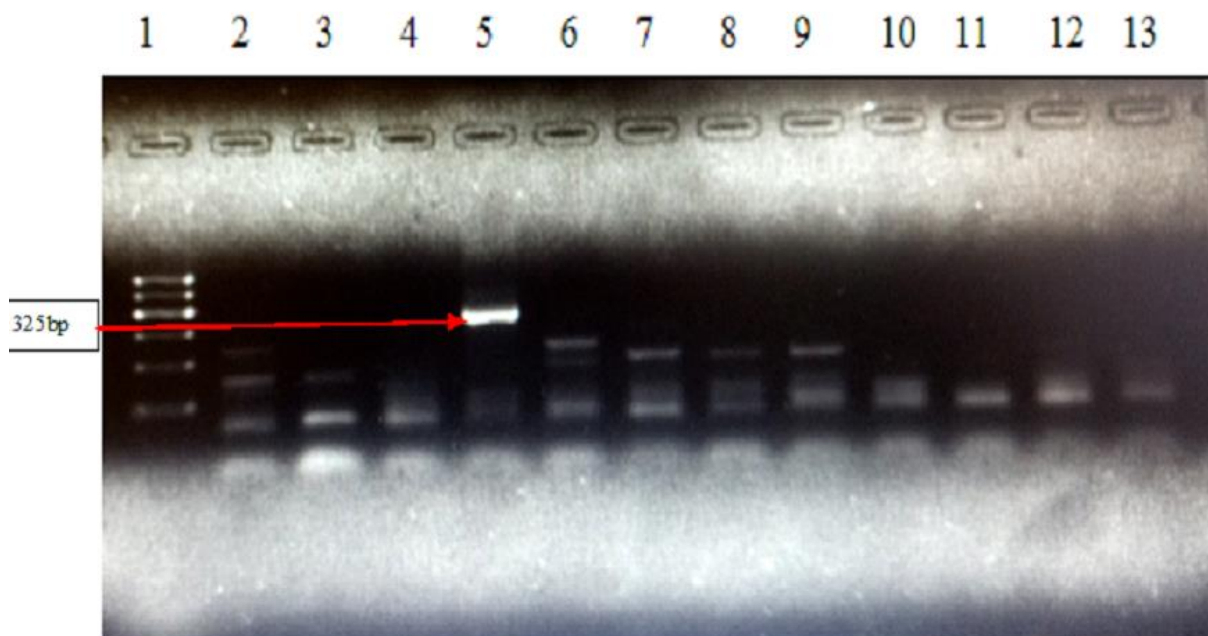

Figure 9**:** Amplification of HEV cDNA from ORF1 gene from pig faecal samples.

Where lane 1: Molecular marker of 600 base pair with 100bp difference ladder, lane 5 (Sample 5) gave expected band size of 325bp which is strong positive, lane 2, 3, 4, 6- 13 are negative results.

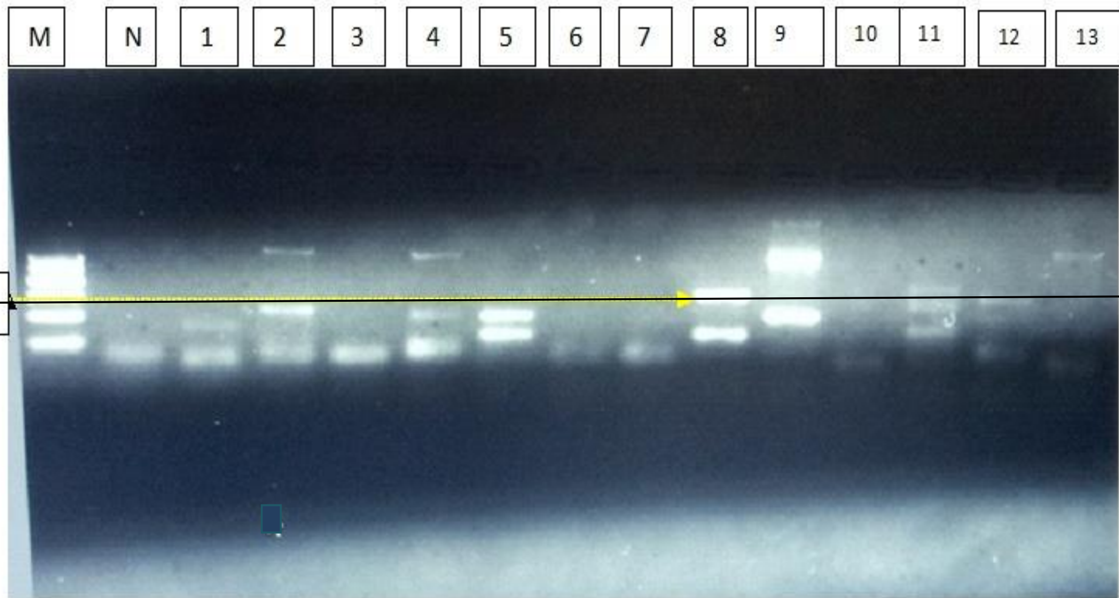

Figure 10: Agarose gel electrophoresis pattern of HEV partial ORF1 gene PCR products.

Where M=600bp DNA molecular markers (ladder) with 100bp ladder differences, N= negative control, 1-7 camel faecal samples, 8-13 pig faecal samples. Sample 8 gave expected band size of 325bp. Camel samples 2, 4, and 5 were considered as doubtful because they gave band size slightly lower than expected band of 325bp.

5. DISCUSSION

In this study, HEV genome was successfully amplified in pig faecal samples (8%; Figure 9and Figure10) and molecular evidence of the HEV gene was detected for the first time in Ethiopia. In addition, the three doubtful results of camel faecal samples showed likely evidence of the virus circulation in camel population in Ethiopia. The current PCR finding is in agreement with the previous report of John *et al*., (2010) who reported similar PCR product band size obtained using same amplification forward and reverse primers of the first round (HEV-cs TCG CGC ATC ACM TTY TTC CARAA and HEV-cas GCCATGTTCCAG ACDGTRTTCCA) respectively, except that the second round primers they used has little modification (HEV-csn TGT GCT CTG TTT GGC CCN TGG TTY CDG andHEV-casn CCA GGC TCA CCR GAR TGY TTC TTC CA). The nested broad spectrum conventional RT-PCR analysis successfully amplified the virus gene from the four pig samples revealed that the virus is circulating in the pig population. In addition, this study also agreed with Widén *et al*., (2014) nested PCR results done on detection and analysis of potentially zootic HEV in French rats with primers same with primers used for this research.

Moreover this study is agreed with Milojevic *et al*., (2019) done on screening and molecular characterization of hepatitis E virus in slaughter pigs in Serbia with similar primer sequences to this study with little modifications.

Many swine viruses are known to infect humans (Meng *et al*., 1998). Some studies proved that swine HEV also has the ability to cross species barriers and therefore might infect humans (Meng *et al*., 1998). The role of pigs as potential HEV reservoirs is well documented in many parts of the world. For example, the incidence of swine HEV was reported in United Kingdom, China, Japan, and Canada, and over 20% of swine faecal samples have been confirmed positive for HEV RNA in South Africa (Kanayama *et al*., 2015; Yan *et al*., 2008; Wilhelm *et al*., 2017; Adelabu *et al*., 2017). Also, the widespread distribution of HEV has been reported in Nigeria, with prevalence rate of 76.7% of genotype 3 (Buisson et al., 2000). Actually, grazing, rooting, and nosing, as well as wallowing in mud is natural behaviour of the pigs. Behaviours associated with wallowing include feeding, drinking, defecating, and urinating in the mud (Bracke and Spoolder 2011) and water bodies suggests the probable route of contamination of the environment with HEV (Figure 8). In Ethiopia, there is no former report of prevalence of HEV in pigs.

The serological evidence of HEV infection in pregnant women in Addis Ababa hospital (Abebe *et al*., 2017) suggests the high significance and public health threat of the disease. As stated before in literature review part, the disease is transmitted primarily via the faecal-oral route. Extensive pig production is carried out in Burayu, and Ashewa meda areas of Oromiya special zone around Finfinnee (Addis Ababa) and in the city of Addis Ababa. The contamination of watering points and other materials that could expose the virus to susceptible hosts including humans are likely and the observation of high seropositive evidence in pregnant women is expected. In Abebe *et al*. (2017) study, the exact locations of the pregnant women other than evidence of detection in hospital settings were not reported to correlate source of acquiring infection of the virus by the women and other susceptible host populations.

In dromedary camels, the observation of three doubtful results is small but interesting in that previous serological report indicated the detection of anti-HEV IgG antibody in more than 20% of Ethiopian dromedary camels (Li *et al*., 2017). Such serological detection suggests the HEV infection has likely spread among dromedary camels in Ethiopia. Li *et al* (2017) reported the report from Afar region while the current report is from Oromiya region which are different in their locations. From the serologically positive areas of Afar, no further study in dromedary camels were conducted to grow the virus and/or to molecularly detect the virus genome. This study is the only molecular evidence available in the country that could show likely detection of the virus genome in camel faecal samples. Both the pig and the camel results need further confirmation by gene sequencing and sequence analysis to elucidate the evolutionary relationship and emergence of the virus in current hosts and other host species. Similar serological study conducted in different countries like Kenya, UAE, Somalia, Sudan, Pakistan, and Egypt reported detection of 31% to 63% (Rasche *et al*., 2016) which is much higher than the report from Ethiopia.

Previous reports indicated that many swine viruses are known to infect humans and has the ability to cross species barriers (Meng *et al*., 1998). Various studies have reported the incidence of swine HEV from different parts of the world as described above in the discussion part such as China (Kanayama *et al*., 2015), and Canada (Wilhelm *et al*., 2017). In African countries like Nigeria, the widespread distribution of HEV reported with prevalence rate of 76.7% of genotype 3 (Adelabu *et al*., 2017). All these are pointing to increased responsiveness, surveillance and prevalent nature of this virus in the environment. In Ethiopia

there is no serological and molecular report of prevalence of HEV in pigs and this report is the first molecular evidence regarding HEV gene detection.

Usually, large numbers of camels and other domestic animals from many different herds/flocks congregate at watering sites, and this may create a perfect condition for disease transmission and spread among susceptible animals. During milking, washing of hands, milking vessels, the udder and teats is not practised by many milkers' prior to milking the camels. Besides, the milking area is generally full of dust and dung and without shade. This affects the quality and safety of the produced milk (Mirkena *et al*., 2018) and suggesting good way of HEV contact with susceptible hosts and acquiring of infection.

HEV RNA has been detected in diverse food products ranging from meat and seafood, to fruits and vegetables (Doceul *et al*., 2016). According to Kokkinos *et al*., (2016) and (2012) HEV was reported in 5% of the irrigation water samples and 3.2% of fresh lettuce respectively. Raw and undercooked fruits and vegetables are normally sold to the consumer in a ready to eat form in Addis Ababa. Most of vegetations in and around Addis Ababa used irrigation water containing sewages released from the city, which may be the source of contamination. Therefore, one route of virus transmission to the community in the study area may be through drinking of contaminated water and the irrigation of vegetables such as cabbage with HEV-contaminated water.

6. CONCLUSION AND RECOMMENDATIONS

In this study, molecular detection of the HEV from faecal samples of pigs and doubtful results from camel samples using a nested RT-PCR is reported for the first time. The observation of pigs grazing in the field and around watering points, suggest possible environmental contamination of the virus that could lead to infection of susceptible animal species including humans. The virus is the leading cause of enterically-transmitted viral hepatitis in humans globally. These viruses are transmitted by the faecal-oral route and many of the environmental and socio-economic factors foster the transmission routes. Although, HEV gene is successfully detected in this study, the gene of the virus was not sequenced because of global problem of Covid-19 pandemic. Therefore, it needs to be further confirmed by HEV gene sequencing and sequence analysis to arrive on final conclusion.

Based on the above conclusions the following recommendations are forwarded

- Further study should be conducted to confirm by gene sequencing of the HEV virus in animals and humans and trace roots of transmission in the country.
- Evolutionary relationship of the virus detected in different animals and humans need to be established and correlated with possible environmental sources like water bodies.
- Apparently, multidisciplinary efforts are needed to characterize in detail the impact of HEV in animals on human health in country.
- Pigs should be prevented from spreading the virus via faecal contamination of the environment, chlorinate adequately or boil drinking water, and repeatedly give health education about personal and environmental hygiene.
- Awareness creation and prevention disease transmission need to be initiated regarding the role of pigs in the transmission of the disease in the future.

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8. ANNEXES

Annex 1. QIAamp Viral RNA Mini Kit

QIAamp Viral RNA Mini Kit principle and procedures

QIAamp Viral RNA Mini Kits provide the fastest and easiest way to purify viral RNA for reliable use in amplification technologies. It represents a well-established technology for general-use viral RNA preparation. The kit combines the selective binding properties of a silica-based membrane with the speed of microspin or vacuum technology and is highly suited for simultaneous processing of multiple samples. QIAamp Viral RNA spin protocols can be fully automated on the QIAcube®. The sample is first lysed under highly denaturing conditions to inactivate RNases and to ensure isolation of intact viral RNA. Buffering conditions are then adjusted to provide optimum binding of the RNA to the QIAamp membrane, and the sample is loaded onto the QIAamp Mini spin column. The RNA binds to the membrane, and contaminants are efficiently washed away in two steps using two different wash buffers. High-quality RNA is eluted in a special RNase-free buffer, ready for direct use or safe storage. The purified RNA is free of protein, nucleases, and other contaminants and inhibitors.

Annex 2. Viral RNA extraction Procedures (Spin Protocol)

1. Pipet 560 µl prepared Buffer AVL containing carrier RNA into a 1.5 ml microcentrifuge tube.
Note: If the sample volume is larger than 140 µl, increase the amount of Buffer AVL–carrier RNA proportionally (e.g., a 280 µl sample will require 1120 µl Buffer AVL–carrier RNA) and use a larger tube.
2. Add 140 µl plasma, serum, urine, cell-culture supernatant or cell-free body fluid to the Buffer AVL–carrier RNA in the microcentrifuge tube. Mix by pulse-vortexing for 15 s. Note: To ensure efficient lysis, it is essential that the sample is mixed thoroughly with Buffer AVL to yield a homogeneous solution. Frozen samples that have only been thawed once can also be used.

3. Incubate at room temperature (15–25°C) for 10 min.

Note: Viral particle lysis is complete after lysis for 10 min at room temperature. Longer incubation times have no effect on the yield or quality of the purified RNA.

4. Briefly centrifuge the tube to remove drops from the inside of the lid.

5. Add 560µl of ethanol (96–100%) to the sample, and mix by pulse-vortexing for 15 s. After mixing, briefly centrifuge the tube to remove drops from inside the lid.

Note: Use only ethanol, since other alcohols may result in reduced RNA yield and purity. Do not use denatured alcohol, which contains other substances such as methanol or methylethylketone. If the sample volume is greater than 140 µl, increase the amount of ethanol proportionally (e.g., a 280 µl sample will require 1120 µl ethanol). To ensure efficient binding, it is essential that the sample is mixed thoroughly with the ethanol to yield a homogeneous solution.

6. Carefully apply 630 µl of the solution from step 5 to the QIAamp Mini column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Mini column into a clean 2 ml collection tube, and discard the tube containing the filtrate.

Note: Close each spin column to avoid cross-contamination during centrifugation. Note: Centrifugation is performed at 6000 x *g* (8000 rpm) to limit microcentrifuge noise. Centrifugation at full speed will not affect the yield or purity of the viral RNA. If the solution has not completely passed through the membrane, centrifuge again at a higher speed until all of the solution has passed through.

7. Carefully open the QIAamp Mini column, and repeat step 6. If the sample volume was greater than 140 µl, repeat this step until all of the lysate has been loaded onto the spin column.
8. Carefully open the QIAamp Mini column, and add 500 µl Buffer AW1. Close the cap, and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Mini column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.

Note: It is not necessary to increase the volume of Buffer AW1 even if the original sample volume was larger than 140 µl.

9. Carefully open the QIAamp Mini column, and add 500 µl Buffer AW2. Close the cap and centrifuge at full speed (20,000 x *g*; 14,000 rpm) for 3 min. Continue directly with step 11, or to eliminate possible Buffer AW2 carryover, perform step 10 and then continue with step 11.

Note: Residual Buffer AW2 in the eluate may cause problems in downstream applications. Some centrifuge rotors may vibrate upon deceleration, resulting in flowthrough, containing Buffer AW2, contacting the QIAamp Mini column. Removing the QIAamp Mini column and collection tube from the rotor may also cause flow-through to come into contact with the QIAamp Mini column. In these cases, the optional step 10 should be performed.
10. Recommended: Place the QIAamp Mini column in a new 2 ml collection tube (not provided), and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.

11. Place the QIAamp Mini column in a clean 1.5 ml microcentrifuge tube (not provided). Discard the old collection tube containing the filtrate. Carefully open the QIAamp Mini column and add 60 µl Buffer AVE equilibrated to room temperature. Close the cap, and incubate at room temperature for 1 min.

12. Centrifuge at 6000 x *g* (8000 rpm) for 1 min. A single elution with 60 µl Buffer AVE is sufficient to elute at least 90% of the viral RNA from the QIAamp Mini column. Performing a double elution using 2 x 40 µl Buffer AVE will increase yield by up to 10%. Elution with volumes of less than 30 µl will lead to reduced yields and will not increase the final concentration of RNA in the eluate. Viral RNA is stable for up to one year when stored at –30 to –15°C or at –90 to –65°C.

Annex 3. Principle and procedure of nested RT-PCR

In the nested PCR, the specificity of the PCR reaction is enhanced by reducing the non-specific binding with the help of the two sets of primers. The specificity is the main aim of any of the PCR reaction. Every PCR modifications are mean to increase the specificity as well as the sensitivity of the reaction. Two sets of primers are used to achieve high sensitivity in the nested PCR. Here both primers have different and unique properties. The first set of primer binds outside of our target DNA and amplifies larger fragment, this set of primer is referred to as *an outer primer or external primer.*

Another set of primer binds specifically at the target site and in the second round of amplification, it amplifies only the target DNA, this set of primer is referred to as *an inner primer.* Here, in the nested PCR, our template DNA is the primary binding site for the outer set of primers while the amplicon of the first set of the PCR is the site for binding for the inner set of primers or internal primers.