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Zoonotic Hepatitis E and Enteric Protozoa in Vietnam: Epidemiological Evidence from Animal Reservoirs to Human Health Risk

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1. INTRODUCTION

1.1 Overview of Zoonotic Diseases

Zoonotic diseases are infections naturally transmitted between animals and humans. These infections can be caused by a range of pathogens including bacteria, viruses, parasites, fungi, and prions and account for approximately 60% of emerging infectious diseases worldwide, posing a significant threat to public health and global economy (Jones et al., 2008). While prominent zoonotic pathogens such as *Salmonella* spp., *Escherichia coli*, *Campylobacter spp.* rabies virus, Ebola virus, coronaviruses, *Plasmodium* spp. and avian influenza are routinely monitored by many nationwide surveillance systems, underrecognized pathogens such as the HEV and certain zoonotic protozoa are often neglected in monitoring (European Food Safety et al., 2023). The clinical severity of zoonotic diseases varies widely, for instance seasonal influenza typically results in mild illness, whereas coronaviruses such as SARS-CoV and SARS-CoV-2 have demonstrated severe illness with pandemic potential (Rahman et al., 2020).

Zoonotic pathogens can be transmitted to humans by multiple means, such as direct contact with infected animals, indirect exposure to contaminated environments, vector-borne transmission via mosquitoes or ticks, consumption of contaminated animal products or inhalation of aerosolized pathogen particles (Rahman et al., 2020). The complexity of these transmission pathways necessitates effective surveillance systems, particularly for pathogens emerging from wildlife reservoirs (Sharan et al., 2023). Wild animals such as bats, rodents, and primates often serve as natural reservoirs for a wide range of pathogens, facilitating the spillover events, especially when intensified by human activities like hunting, wildlife trade, environmental and habitat disruption (Ellwanger & Chies, 2021). Domestic animals, including livestock and pets, can also act as amplifying hosts in intensive farming systems where close human-animal contact is common.

To control the spread of zoonotic diseases, several preventive measures have been adopted. These include stricter regulations on wildlife trade, improved biosecurity in livestock farming, and strengthened disease surveillance systems (Authored by the members of the One Health High-Level Expert et al., 2023). Many countries now follow the One Health framework,

fostering collaboration across public health, veterinary and environmental sectors. In addition, complementary actions such as regulating illegal wildlife markets, and controlling deforestation and promoting vaccination programs, education and rapid response mechanisms remain essential to minimize the risk of zoonotic spillovers and enhancing pandemic preparedness (Authored by the members of the One Health High-Level Expert et al., 2023; Rahman et al., 2020).

Vietnam has emerged as a hotspot for zoonotic and emerging infectious diseases in Southeast Asia. With around half of the population residing in rural areas and heavily engaged in livestock farming, the risk of animal to human transmission and subsequent community spread of zoonotic diseases is imminent (Dinh et al., 2006; My et al., 2014; Rabaa et al., 2015). Key zoonotic pathogens of concern in Vietnam include rotaviruses, Leptospirosis, *Streptococcus suis*, hepatitis E virus (HEV), influenza A viruses and enteroviruses, all of which pose substantial public health risks (Rabaa et al., 2015). A recent modelling study have identified several provinces in central and southern Vietnam as high-risk regions for zoonotic spillover, underscoring the urgent need to enhance disease surveillance and control efforts in these regions (Meisner et al., 2025).

1.2 HEV

1.2.1 Epidemiology

HEV is one of the leading causes of acute hepatitis worldwide. According to the World Health Organization (WHO), an estimated 20.1 million people are infected with HEV each year, of whom an estimated 3.4 million develop symptomatic disease. These infections result in more than 70,000 deaths and approximately 3,000 stillbirths annually (World Health Organization, 2023). While most of the HEV cases are often asymptomatic and self-limiting in healthy individuals, it can lead to severe course in high-risk groups, including immunocompromised patients (organ transplant recipients), pregnant women and individuals living with HIV. In these populations, HEV infection can progress to fulminant hepatitis, including life-threatening cirrhosis (Horvatits et al., 2019; Nimgaonkar et al., 2018). HEV is primarily transmitted by the faecal-oral route, which makes the virus more prevalent in low-and

middle-income countries (LMICs). Inadequate access to clean water, poor sanitation and hygiene, and weaker healthcare infrastructure significantly contribute to the outbreaks and transmission of the virus in these regions (Khuroo et al., 2016). HEV infections occur sporadically in industrialized countries, primarily through zoonotic transmission typically via consumption of raw or undercooked meat from wild boar, pigs or deer (Arends et al., 2014). Less common transmission routes include vertical transmission from mother to foetus during pregnancy and through blood transfusion (Meng, 2013; Singson et al., 2024).

1.2.2 Taxonomy and HEV genome organization

Taxonomy and animal reservoir

HEV belongs to the *Hepeviridae* family and the *Orthohepevirinae* subfamily, which comprises four genera: *Avihepevirus*, *Chirohepevirus*, *Rocahepevirus* and *Paslahepevirus*. The genus *Paslahepevirus*, is phylogenetically distinct from other members of the subfamily, and exhibits a broad host range which includes humans as well as domestic and wild mammals (Purdy et al., 2022). Within this genus, *Paslahepevirus balayani* is classified into eight different genotypes (HEV-1 to HEV-8) (Smith et al., 2020). Human infections are mainly caused by HEV genotypes HEV-1 to HEV-4. While HEV-1 and HEV-2 are restricted to humans and are associated with waterborne outbreaks in regions with poor sanitation, HEV-3 and HEV-4 are zoonotic with a broad host range infecting both humans and animals. These genotypes are frequently detected in pigs and deer across Europe, the Americas and Asia (Meng, 2010; Velavan et al., 2021). Recent studies have further expanded the host range of HEV-3 to include goats, rats and bottlenose dolphins (Di Martino et al., 2016; Kanai et al., 2012; Montalvo Villalba et al., 2017), while HEV-4 has been reported in cattle and sheep (Huang et al., 2016; Wu et al., 2015). HEV-5 and HEV-6 have so far only been identified in wild boars in Japan (Takahashi et al., 2014). Two additional genotypes, HEV-7 and HEV-8, have been identified from camels (Woo et al., 2016; Woo et al., 2014). Notably, HEV-7 has been shown to cause chronic hepatitis in a liver transplant patient, highlighting its potential clinical relevance in immunocompromised individuals (Lee et al., 2016).

Structure and genome organization

HEV is a positive-sense, non-enveloped, single-stranded RNA virus with a diameter of 27-35 nm and a genome length of 7.2 kb (Purdy et al., 2022). The genome consists of a short methylguanine-capped 5′ untranslated region (UTR), three open reading frames (ORF1-3), and a polyadenylated 3′ UTR. ORF1 produces a non-structural polyprotein with seven domains: methyltransferase (Met), Y domain, papain-like cysteine protease (PCP), proline-rich hypervariable region (HVR), X domain, helicase (Hel), and RNA-dependent RNA polymerase (RdRp) (Koonin et al., 1992). While the functions of all ORF1 domains are not fully understood, the Met, Hel and RdRp domains have been well characterized and are involved in HEV replication (Pallerla et al., 2020). ORF2 encodes the capsid protein, which contains three potential N-glycosylation sites including S, M, and P domains (Zafrullah et al., 1999). ORF2 is the crucial target for neutralizing antibodies and plays a essential role in diagnostics and vaccine development (Zhou et al., 2005). ORF3 encodes a small multifunctional protein (MFP) with hydrophobic (D1, D2) and proline-rich (P1, P2) regions. ORF-3 plays a role in intracellular signalling, immune system evasion and the protection of infected cells. In addition, ORF3 contributes to the release of infectious particles (Ding et al., 2017). Another identified ORF4, which is specific to HEV-1, encodes a 158-amino acid protein that is involved in HEV replication by interacting with viral proteins and host factors (Van Tong et al., 2016).

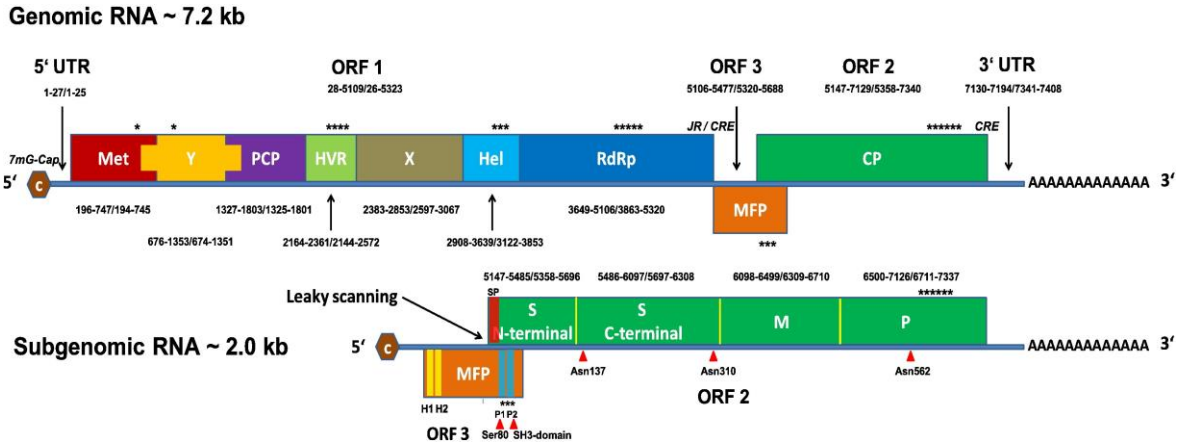

Figure 1. Overview of HEV Genome Structure and Protein Composition. The figure illustrates the ~7.2 kb RNA genome of HEV. Open reading frame 1 (ORF1) encodes several

domains, including the methyltransferase (Met), the Y domain, the papain-like cysteine protease (PCP), the hypervariable region (HVR), the macrodomain (X), the RNA helicase (Hel) and the RNA-dependent RNA polymerase (RdRp). ORF2 encodes the capsid protein (CP), consisting of S, M and P domains, as well as glycosylation sites at Asn137, Asn310 and Asn562. ORF3 encodes a small multifunctional protein (MFP) with hydrophobic (H1, H2) and proline-rich (P1, P2) regions. The region where ORF2 and ORF3 overlap comprises the intergenic junction region (JR), a cis-reactive element (CRE) and a signal peptide (SP). Asterisks (*) indicate mutational hotspots reported in literature. The figure is from a study by Tong et al. from our working group and is reproduced with permission (Van Tong et al., 2016).

1.2.3 Outbreaks of hepatitis E

HEV outbreaks are often documented in LMICs and are primarily caused by the waterborne transmission of genotypes HEV-1 and HEV-2. The first documented outbreak of HEV was reported in India in 1955 (Viswanathan, 2013). Since then, numerous outbreaks have been documented in tropical and subtropical regions, particularly in Asian and African countries, with more than 320,000 cases documented (Pallerla et al., 2020). In recent years, outbreaks have increasingly occurred in conflict zones and humanitarian crisis settings, such as war affected areas and across camps for refugees or internally displaced persons, where clean water and sanitation are limited (Guerrero-Latorre et al., 2011; Guthmann et al., 2006; Teshale et al., 2010). In particular, the 2018 HEV outbreak in South Sudan documented a high case fatality rate exceeding 10% among 169 confirmed cases, with 45% of these cases involving pregnant women and associated with HEV genotype 1e (Aumuller, 2024; Orf et al., 2024). In Namibia, between 2017 and 2020, 7,247 confirmed cases were reported, although the overall case fatality rate was lower at 0.8% (Bustamante et al., 2020). These concerning outbreaks highlight the urgent need for a targeted vaccination strategies in high-endemic regions.

Zoonotic transmission in Europe is driven by consumption of contaminated animal products where HEV genotype 3 predominates (Aspinall et al., 2017). Reported HEV cases in Europe have increased tenfold, from around 500 cases in 2005 to more than 5,000 cases in 2015, with the majority being autochthonous infections (Aspinall et al., 2017). Germany, France

and the United Kingdom accounted for more than 80 % of reported cases (Aspinall et al., 2017). Similarly, sporadic HEV infections occur in Southeast Asia, where genotype 3 being the most prevalent, followed by genotype 4, indicating that zoonotic transmission is equally dominant in this region (Raji et al., 2021). The first outbreak in Vietnam occurred in 1996 in the southwestern region and was likely linked to water-borne transmission via the Hau River, although the specific genotype was not characterized (Corwin et al., 1996). Since then, no major outbreaks of HEV have been reported. Vietnam, known for its extensive pig farming and pork consumption, is considered a hotspot for swine-associated HEV. However, surveillance studies have confirmed the circulation of HEV genotypes 3 and 4 in domestic pigs across northern and southern regions of Vietnam (Berto et al., 2018; Lee et al., 2020). In addition, seroprevalence data further highlight the extent of HEV exposure. Studies have reported HEV-IgG positivity ranging from 8-24% in pregnant women (Huy et al., 2021; Thi Hong Van et al., 2025) to 27% in blood donors (Cao et al., 2023), and up to 53% in individuals exposed to pigs (Hoan et al., 2019), indicating a significant and widespread zoonotic risk within the Vietnamese population.

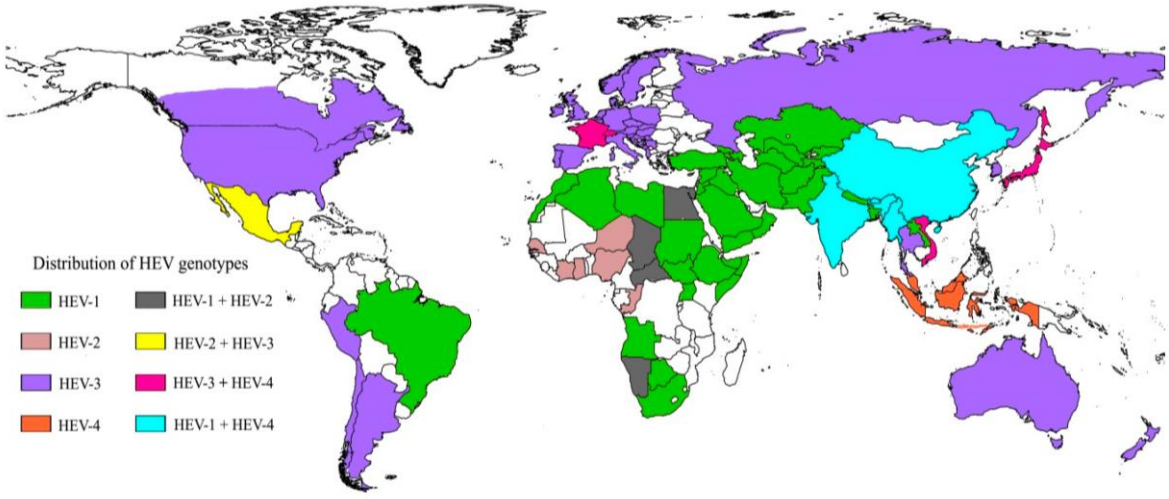

Figure 2. **Worldwide Distribution of HEV Genotypes 1 to 4**. The figure was created from our working group using SimpleMappr and has been approved for use in this thesis (Pallerla et al., 2020).

1.2.4 Clinical manifestation and differential diagnosis

The incubation period of HEV infection can reach to two months. While most cases are asymptomatic or acute or mild symptoms, approximately 5% of patients may develop acute hepatitis characterized by jaundice, which is usually accompanied by malaise, anorexia, nausea, vomiting, abdominal pain, fever and hepatomegaly. Less common clinical features include diarrhoea, arthralgia, pruritus and urticarial rash (Aslan & Balaban, 2020). Some patients may experience extrahepatic complications such as membranous glomerulonephritis and neurological diseases (aseptic meningitis or Guillain-Barré syndrome) (Fousekis et al., 2020). Rarely, some patients can progress to hepatic encephalopathy, with elevated liver enzymes and impaired synthetic liver function. Without intensive care or liver transplantation, these cases are associated with high mortality rates (Choi et al., 2022). Chronic infections develop if HEV RNA is detectable in the blood or faeces for longer than six months. This often occurs in immunocompromised patients, such as those individuals living with HIV, or who have undergone solid organ or bone marrow transplantation (Murali et al., 2015). Chronic infections have primarily associated with HEV genotypes 3, 4, and 7 (Chen et al., 2022; Lee et al., 2016; Ying et al., 2023), whereas no chronic cases with genotypes 1 and 2 have been reported.

HEV infection is often misdiagnosed, as many cases are asymptomatic, resulting in substantial under recognition of the disease (Davern et al., 2011). Screening for HEV is particularly important in high risk groups such as solid organ transplant recipients and patients with haematological malignancies (Pawlotsky, 2014). Diagnosis of HEV infection is usually based on detecting anti-HEV antibodies, HEV antigens, or HEV RNA in serum and stool samples (Velavan et al., 2021). Serological tests, particularly enzyme-linked immunosorbent assays (ELISA), are the most used. Anti-HEV IgM is produced early during infection, acting as an indicator of acute or recent infection and typically declines within four to five months. Anti-HEV IgG emergers thereafter and can persists for years, indicating past exposure (Al-Sadeq et al., 2018). HEV antigen appears in serum within one to three weeks following infection and remains detectable for up to seven weeks (Aggarwal et al., 2000). The detection of HEV RNA in serum or faeces provides a definitive confirmation of infection.

Viral RNA can be detected in faeces approximately one week before the onset of symptoms and persist for up to two weeks. In serum, HEV RNA is usually detected two to six weeks after infection and may persist up to four weeks after resolution of acute symptoms (Balayan et al., 1983; Clayson et al., 1995). While viremia is typically short-lived in acute infections, it can persist for years in chronically infected and among immunosuppressed patients (Velavan et al., 2021). Currently, the detection of HEV RNA in urine is not recommended due to limited evidence and low diagnostic sensitivity, estimated at less than 50% (Marion et al., 2019).

1.2.5. Treatment and Prevention

Most patients with acute HEV infection require only supportive care. However, in severe cases such as fulminant liver failure, a liver transplantation may be necessary (Wedemeyer et al., 2012). Acute HEV infection is usually self-limiting, and thus antiviral therapy is not recommended in these cases. In contrast, chronic HEV infection requires both a reduction in immunosuppressive therapy and antiviral treatment to achieve viral clearance (European Association for the Study of the Liver, 2018). A 12-week course of ribavirin monotherapy is generally recommended for non-pregnant patients with chronic HEV infection, as the use of ribavirin in pregnant women is controversial due to limited data and concerns about its teratogenicity (Wu et al., 2020). Monitoring HEV RNA levels in both stool and serum samples at the end of treatment is essential to assess therapeutic response to antiviral treatment (European Association for the Study of the Liver, 2018). For patients who do not respond to ribavirin, alternative antiviral agents such as pegylated interferon-alpha or sofosbuvir, may be considered (European Association for the Study of the Liver, 2018).

Vaccination against HEV plays an essential role in preventing infection and reducing disease severity. The recombinant HEV 239 (Hecolin), based on the capsid protein of HEV genotype 1 (ORF2, amino acids 368–606), was developed and licensed in China in 2011 and in Pakistan in 2020. Hecolin provides cross-genotype protection against HEV genotypes 1-4 (Huang et al., 2024; Wu et al., 2016). Although Hecolin has demonstrated high efficacy and safety in clinical trials, the WHO emphasizes the need for epidemiological data in general

and high risk populations, before issuing recommendations (Peron et al., 2023). The vaccine is beneficial for high-risk groups, including women of childbearing age, patients with chronic liver disease and immunocompromised individuals (Dudman et al., 2025). The vaccine has also played a role in controlling a prolonged HEV genotype 1 outbreak in southern Sudan (Nesbitt et al., 2025). In addition, the immunogenicity and safety of Hecolin in children are currently being evaluated in a placebo-controlled trial in South Africa (WHO, March 2024). In addition to vaccination, preventive measures are essential to reduce of HEV transmission. These include avoid drinking water of unknown purity and refraining from consuming raw or undercooked seafood, pork, wild boar sausages, and meat from wild animals (Melgaco et al., 2018).

1.2.6 High-risk population

Pregnant woman

In endemic regions, pregnant women infected with HEV are more likely to experience severe symptoms, which can lead to acute liver failure. The associated mortality and morbidity rates are unusually high, ranging from 15 to 60% (Kumar et al., 2004; Patra et al., 2007). HEV infection in pregnancy, especially during the third trimester is associated with high rates of adverse pregnancy outcomes including premature births, miscarriages and stillbirths (Perez-Gracia et al., 2017). HEV genotype 1 is the main cause of HEV infection in pregnant women in developing countries, primarily spreading through contaminated water. Surveillance data from Bangladesh (2001-2007) show that HEV-1 is the leading cause of acute hepatitis in pregnant women, accounting for around 10% of pregnancy-related deaths (Labrique et al., 2012). Although the mechanism leading to severe liver complications in pregnant women with hepatitis E remains unclear, it is hypothesized that hormonal changes during pregnancy such as fluctuations in oestrogen and progesterone levels and altered immune responses (Navaneethan et al., 2008) may contribute to adverse outcomes. In addition, poor nutrition and inadequate medical care may contribute to increased mortality among pregnant women with HEV in LMICs (Lagare et al., 2018). In contrast, HEV infections by zoonotic genotypes

HEV-3 and HEV-4 during pregnancy are often milder and self-limiting, as observed in non-pregnant individuals (Huy et al., 2021; Navaneethan et al., 2008; Stoszek et al., 2006).

Individuals with pre-existing liver disease or malnutrition

Co-infection with HEV has been shown to exacerbate the progression of underlying liver disease. A previous study from Vietnam suggests that HEV infection may significantly affect the course of liver disease in patients with chronic hepatitis B virus (HBV) infection, with the highest seroprevalence of HEV observed in cirrhotic patients (anti-HEV IgG at 52% and anti-HEV IgM at 19%) (Hoan et al., 2015). In addition, there is increasing evidence that HEV infection in HBV-infected individuals increases the risk of developing decompensated cirrhosis (LC) and is associated with an increased mortality rate (Tseng et al., 2020; Zhao et al., 2023). In a phase 3 clinical trial, the HEV vaccine Hecolin showed an estimated efficacy of 72% in reducing the risk of hepatitis E in HBsAg-positive individuals (Zhuang et al., 2025). The clinical burden of HEV superinfection has also been observed in patients with other liver diseases, including hepatitis C virus (HCV) infection and various chronic liver diseases (Marascio et al., 2022; Wong et al., 2021). In addition, a high seroprevalence of anti-HEV IgG has been documented in patients with hepatocellular carcinoma (HCC) in a Cameroonian cohort, suggesting a possible role of past HEV infection in the progression of chronic liver disease to malignancy (Amougou Atsama et al., 2017).

Solid organ transplant recipients and other immunocompromised individuals

HEV infection in solid organ transplant (SOT) recipients often progress to chronic hepatitis with rapid development of liver fibrosis and cirrhosis. Systematic studies have reported a high chronic HEV infection rates of 47% to 65% among transplant recipients, with immunosuppression as a risk factor for chronicity (Kamar et al., 2011; Legrand-Abravanel et al., 2011). The use of certain immunosuppressive drugs such as mTOR (mammalian target of rapamycin) may worsen chronic HEV infection (Behrendt et al., 2014; Debing & Neyts, 2014). Several factors have been associated with chronic progression of HEV infection in immunocompromised individuals, including decreased lymphocyte counts (in particular, CD2, CD3 and CD4 T-cell counts,) as well as increased viral diversity, with chronicity

observed in approximately 40-70 % of cases (Kamar et al., 2011). In SOT recipients in Western Europe, the prevalence of HEV infection is between 0.7 % and 1.5 %, with a high incidence of 3.2 % observed in lung transplant recipients (Haagsma et al., 2009; Pas et al., 2012). A significant risk factor for acute HEV infection in this group appears to be foodborne transmission through consumption of undercooked game meat or pork products (Legrand-Abravanel et al., 2010). Chronic HEV infections have also been reported in individuals with congenital immunodeficiencies and among those receiving immunosuppressive therapy for autoimmune diseases or malignancies (Honer zu Siederdissen et al., 2014). Although HEV exposure appears higher in HIV-infected individuals compared to the general population, the prevalence of chronic HEV infection in this group remains relatively low (Abravanel et al., 2017).

1.3. Zoonotic Enteric Protozoa

Zoonotic enteric protozoa such as *Cryptosporidium*, *Entamoeba* and *Giardia* pose a major threat to human and animal health, especially in countries with poor sanitation and inadequate water quality. These parasites are mainly transmitted by the faecal-oral route, commonly through the ingestion of contaminated water, food or through direct contact with infected animals (Daniels et al., 2015). While their prevalence is highest in LMICs, waterborne outbreaks have also been documented in high-income settings. Livestock, wildlife and domestic animals are important reservoirs (Ma et al., 2022). The burden of these infections are especially severe among immunocompromised individuals and young children with high rates of morbidity and mortality (Fletcher et al., 2012).

1.3.1 *Entamoeba* spp.

Taxonomy, epidemiology and life cycle

The genus *Entamoeba*, which includes parasitic amoebae, is taxonomically classified within the kingdom *Protozoa*, the phylum *Amoebozoa*, class *Lobosa*, order *Amoebida,* and family *Entamoebidae*. To date, more than 50 *Entamoeba* species have been identified in various hosts, including animals and humans (Hooshyar et al., 2015; Royer & Petri, 2014). Among these, *E. histolytica* is the only species that has been clearly identified as pathogenic to

humans. *E. histolytica* is morphologically indistinguishable from the non-pathogenic species *E. dispar* and *E. moshkovskii*, requiring the use of molecular diagnostic techniques to accurately distinguish the species (Cui et al., 2019).

The human gastrointestinal tract can harbour multiple of *Entamoeba* species. Of these, only *E. histolytica* is recognized as pathogenic and can cause disease in humans, while other species are generally considered non-pathogenic in the intestinal lumen (Dos Santos Zanetti et al., 2021). *Entamoeba* exists in two morphological forms: as a cyst and as a trophozoite. The cyst is typically found in formed stools and are the infectious form responsible for transmission, whereas the trophozoites are usually found in diarrheal stools. Infection begins with the ingestion of mature cysts from contaminated food, water or objects in contact with faeces. Once in the small intestine, cysts release trophozoites that colonize the colon, multiply by binary fission, and encyst to form infectious cysts excreted in faeces. While cysts are resistant and can survive for days to weeks in the environment, trophozoites are usually destroyed by stomach acid if ingested (Centers for Disease Control and Prevention, 6 Nov. 2023). Unlike other *Entamoeba* species, *E. histolytica* can invade the intestinal mucosa and penetrate blood vessel, enabling the spread to extra intestinal sites such as the liver, lungs and brain (Centers for Disease Control and Prevention, 23 Oct. 2023).

Intestinal and extra-intestinal amoebiasis

While most infections are asymptomatic, a minority of cases can progress to acute fulminant colitis. Symptomatic cases typically present with amoebic dysentery or extraintestinal manifestations, with severity influenced by factors such as age, malnutrition, pregnancy, immunosuppression, and coexisting illnesses (Singh & Banerjee, 2022). Symptoms range from mild diarrhoea to severe dysentery characterized by abdominal pain and bloody stools. Chronic non-dysenteric colitis is the most common form in all age groups (Moran et al., 2023) and often resembles inflammatory bowel disease with persistent diarrhoea, abdominal discomfort, and weight loss. Localized infections may also lead to formation of an ameboma, a mass like lesion that can radiologically resemble like a colon carcinoma (Misra et al., 2006). Diagnosis is made by stool microscopy, antigen testing or PCR, however, microscopy alone cannot differentiate between morphologically identical species such as *E. histolytica*, *E.*

dispar and *E. moshkovskii*. Colonoscopy with histopathological examination and serologic testing can be helpful, but may not reliably differentiate between active and previous infections (Saidin et al., 2019).

Amoebic liver abscess is the most common manifestation of extraintestinal amoebiasis. It is caused by the spread of trophozoites via the bloodstream and is responsible for around 50,000 deaths per year worldwide. Involvement of the lungs and brain is rare (Kumanan et al., 2020). Diagnosis of extraintestinal amoebiasis is supported by imaging techniques, such as ultrasound or computed tomography scans, and along with serologic tests. Detection of *E. histolytica* antigen or PCR analysis of aspirated abscess fluid can provide confirmatory evidence, although stool microscopy and PCR analysis are usually negative in extraintestinal cases (Moran et al., 2023). The prevention of amoebiasis primarily depends on improving hygiene, sanitation, and living conditions, especially in endemic regions. Effective community-level measures include ensuring access to clean water, implementing proper waste disposal systems, and promoting safe food handling practices. Public health education is also crucial in reducing faecal-oral transmission. At the individual level, preventive measures such as regular hand washing, avoiding consumption of untreated water and unsafe foods, and early detection and treatment of infections are essential to control the spread of the disease. In addition, screening and treating asymptomatic carriers can significantly reduce the risk of transmission (Li et al., 2021).

1.3.2 *Cryptosporidium* spp.

Taxonomy, life cycle and epidemiology

Cryptosporidium belongs to the phylum *Apicomplexa*, class *Conoidasida*, subclass *Coccidiasina*, order *Eucoccidiorida* and family *Cryptosporidiidae*. The genus includes over 40 recognized species and more than120 genotypes, with a broad host range, including fish, reptiles, birds, mammals and humans (Ryan et al., 2021). Among them, *C. hominis* (formerly *C. parvum* genotype 1) and *C. parvum* (formerly *C. parvum* genotype 2) are the primary species causing diarrheal disease in humans, often leading to outbreaks associated with contaminated drinking water, animal contact, or travel (Gharpure et al., 2019).

Cryptosporidium infections are transmitted via the faecal-oral route, mainly through the ingestion of oocysts in contaminated water or food, or through direct contact with infected people or animals. After ingestion, the oocysts excyst and release sporozoites, which invade the epithelial cells of the gastrointestinal tract. Both asexual and sexual reproduction occur within the host, resulting in resulting in thick-walled, sporulated oocysts that are immediately infectious upon release, facilitating direct faecal-oral transmission (Centers for Disease Control and Prevention, 23 October 2023). Both zoonotic and non-zoonotic *Cryptosporidium* species and genotypes are circulated worldwide. In the United States, outbreaks have been linked to recreational areas such as swimming pools, water playgrounds, unpasteurized cider, and contaminated food in childcare centers and camps (Gharpure et al., 2019). Prevalence varies by settings, affecting 1-3% of immunocompetent patients with diarrhoea in high-income countries compared to over 20% in low-resource settings (Dabrowska et al., 2023). Reported prevalence also depends on the diagnostic methods, with microscopy detecting 7-8% and molecular methods revealing 9-21% in the Americas (Jann et al., 2022).

Cryptosporidiosis

In humans, cryptosporidiosis is most often caused by *C. parvum* or *C. hominis*, and it can present a wide range of symptoms. In healthy individuals, the infection usually results in self-limiting diarrhoea that resolves within two to three weeks. However, in immunocompromised individuals and malnourished children, the disease can lead to severe, prolonged diarrhoea, resulting in significant morbidity and mortality (Bouzid et al., 2013). *Cryptosporidium* infection can also be severe in children under five years of age, leading to growth faltering and weight loss. In patients with AIDS, the cryptosporidiosis can persist as a chronic, debilitating disease with a more severe clinical course (Ahmadpour et al., 2020). *Cryptosporidium* infection can be diagnosed using modified acid-fast stool microscopy, faecal immunoassays, or PCR. Of these, faecal microscopy is the least sensitive, detecting only about 30% of cases from a single specimen. Immunofluorescence tests that target *Cryptosporidium* antigens can significantly improve diagnostic sensitivity. PCR is the most sensitive and specific method, making it particularly valuable for genotyping in outbreaks and epidemiological investigations. Although serological tests, such as ELISA or IFA, are

available for detecting antibodies, they are not routinely used in clinical practice (Adeyemo et al., 2018). Protecting water sources and using effective filtration systems are critical in limiting transmission (Hossain et al., 2023). *Cryptosporidium* oocysts are resistant to standard water purification methods, including chlorination. However, they can be inactivated by freezing, boiling, filtration, or exposure to high concentrations of ammonia or formalin (Hoepelman, 1996).

1.3.3 *Entamoeba* and *Cryptosporidium* in the context of one health

Entamoeba species can occur as pathogenic or non-pathogenic organisms in humans, animals or as free-living species in water or soil. To date, seven species have been detected in humans: *E. histolytica*, *E. dispar*, *E. coli*, *E. hartmanni*, *E. bangladeshi*, *E. moshkovskii* and *E. polecki*. In addition, various *Entamoeba* species have been reported in domestic and wild animals, including pigs, cattle, sheep, goats, horses, deer, rodents, reptiles and Asian elephants. Pigs are an important reservoir, with *E. suis* and *E. polecki* are commonly associated with diarrheal disease (Cui et al., 2019). *E. polecki* is further divided into four subtypes (ST1-ST4), with ST1 and ST3 being the most common in pigs, while all four subtypes have also been detected in humans (Ji et al., 2019). Although experimental studies have demonstrated an ability of *E. histolytica* to infect pigs, natural infections in livestock have not been reported (Girard-Misguich et al., 2011). *E. histolytica* has been detected in sewage and irrigation water in various regions, underscoring its public health significance (Cui et al., 2019). Furthermore, the cysts have been found in environmental samples near cattle farms (Matsubayashi et al., 2018), emphasizing the importance of studying non-human reservoirs to improve our understanding of this protozoan parasite's transmission dynamics.

To date, approximately 20 species of *Cryptosporidium* have been identified in humans and animals, highlighting the zoonotic potential of this protozoan parasite (Bujila et al., 2024). In particular, the highly virulent zoonotic species *C. parvum* and *C. hominis* are prevalent in humans and non-human hosts worldwide, indicating a potential occupational health risk for individuals in close contact with animals (McDaniel et al., 2014). Pigs are also an important reservoir, with 13 different species and genotypes identified. *C. scrofarum* (formerly

Cryptosporidium porcine genotype II) and *C. suis* are particularly important, as they are the main causative agents of cryptosporidiosis in pigs and have occasionally been reported in humans, indicating the zoonotic potential of these species (Kvac et al., 2009; Moore et al., 2016; Wang et al., 2021). Currently, there are no vaccines available to prevent cryptosporidiosis in humans or livestock. In pigs, the infection is often asymptomatic or mildly symptomatic, while the pathogenicity and infectivity of these species in humans are poorly understood and pose a potential public health threat.

1.4 Research scope and thesis objectives

This thesis investigates on the epidemiological patterns of underrecognized zoonotic pathogens in Vietnam, with a particular emphasis on HEV and protozoan parasites in domestic pigs and farmed wild boars, both of which present potential transmission risks to humans. Additionally, the thesis assesses the prevalence of HEV infection and serological exposure in the general population, placing a special focus on high-risk groups, including individuals with chronic liver disease such as patients with chronic hepatitis B and hepatitis of unknown etiology. Given the limited data currently available on these topics in Vietnam, the thesis is structured into three main chapters, each incorporating peer-reviewed articles published in international journals. The specific objectives of this study are to:

1. Determine the distribution and perform molecular characterization of circulating zoonotic HEV strains among domestic pigs and wild boars in southern and central Vietnam.
2. Investigate HEV seroprevalence and RNA positivity in blood donors, individuals living with chronic hepatitis B, and patients with hepatitis of unknown etiology in central Vietnam.
3. Assess the distribution and species diversity of circulating *Entamoeba* and *Cryptosporidium* parasites in domestic pigs and wild boars across northern, central and southern Vietnam.

2. RESULTS

Chapter 1: Distribution and molecular characterization of zoonotic HEV genotypes in domestic pigs and wild boars

Publication No.1

Characterization of zoonotic hepatitis E virus in domestic pigs and wild boar in Vietnam: Implications for public health

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Characterization of zoonotic hepatitis E virus in domestic pigs and wild boar in Vietnam: Implications for public health

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ABSTRACT

Vietnam's unprecedented demand for meat from livestock, including pigs and farmed wildlife, underscores the importance of understanding zoonotic reservoirs for hepatitis E virus (HEV). This study aimed to identify and characterize circulating zoonotic HEV in domestic pigs and wild boar to understand genotype frequencies, transmission dynamics, and associated human health burdens. Rectal swabs, feces, and liver samples from 415 pigs and 102 wild boars were collected across various farms and slaughterhouses in central and southern Vietnam and screened for HEV RNA using nested PCR. HEV RNA-positive samples underwent sanger sequencing and genotyping. Overall, 10% (n = 54/517) of samples were HEV RNA-positive, with wild boars exhibiting the highest HEV positivity rate at 25%, followed by domestic pigs at 7%. Southern Vietnam showed a higher HEV RNA positivity rate (20%) compared to central Vietnam (7%). Notably, rectal swabs demonstrated the highest positivity rate (15%), followed by feces (8%) and liver (4%). HEV-3a was the predominant genotype at 85%, followed by HEV-4b at 9% and HEV-3f at 6%. While HEV-3a was distributed across both central and southern Vietnam, HEV-3f was exclusively detected in central Vietnam, and HEV-4b was identified in wild boar in southern Vietnam. These findings underscore the substantial prevalence of HEV in wild boars, emphasizing their potential as crucial zoonotic reservoirs alongside domestic pigs. Further investigations involving occupationally exposed individuals in high-prevalence areas are warranted to evaluate the human health impact of zoonotic hepatitis E and inform preventive measures. Regular epidemiological studies are imperative for assessing the prevalence and transmission of zoonotic HEV infections among common reservoirs, thereby aiding in the prevention of spillover events within the community.

1. Introduction

Hepatitis E virus (HEV) is a leading cause of acute viral hepatitis, particularly in low and middle-income countries, with limited access to basic sanitation and hygiene. Globally, an estimated 20 million infections and 3.3 million symptomatic cases of hepatitis E occur annually, resulting in around 56.600 deaths [1]. Hepatitis E typically resolves itself in certain population depending on the HEV genotype but poses a greater risk to high-risk groups in developing countries, such as pregnant women. In industrialized countries, organ transplant recipients, HIV-infected individuals and those with underlying liver disease also face significant risk regardless of the HEV genotype. Equally, the

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infections may lead to serious extrahepatic manifestations such as neurological sequelae, acute pancreatitis, kidney injury and thyroiditis [2].

HEV is a small single-stranded RNA virus with a genome length of approximately 7.2 kb. The virus exists in two forms: quasi-enveloped particles found in the blood of the infected hosts, and non-enveloped virions shed in the patient's stool. Belonging to the family *Hepeviridae* and the subfamily *Orthohepevirinae,* it comprises four genera: *Avihepevirus*, *Chirohepevirus*, *Rocahepevirus* and *Paslahepevirus*. Members of the *Paslahepevirus* genus (hepatitis E virus) are phylogenetically distinct and exhibit a broad host range, infecting humans, domestic and wild mammals [3]. Currently, eight HEV genotypes (HEV-1 to HEV-8) have been identified. Genotypes 1 and 2 exclusively infect humans, while genotypes 3, 4 and 7 infect both humans and animals, and genotypes 5, 6 and 8 solely infect animals [4].

Genotypes 1 and 2 are more prevalent in developing countries and primarily spread through the faecal-oral route, often causing waterborne HEV outbreaks, particularly in Africa and Asia [4]. Notably, the most recent HEV outbreak occurred in April 2023 in Wau, South Sudan, with a mortality rate of 5.5%, likely attributed by HEV-1 [5]. In contrast, infections with genotypes 3 and 4 primarily occur through zoonotic transmission, either via close contact with infected animals or by consuming contaminated food such as raw or undercooked meat [4]. These genotypes are common in developed countries and were initially thought to be imported solely through travel to endemic regions. However, the rising number of autochthonous human hepatitis E cases, sharing high similarity with swine HEV isolates, indicates evidence of zoonotic transmission in these regions [6].

HEV genotypes are associated with distinct clinical manifestations of the disease. Acute hepatitis typically results from infection with HEV-1 and HEV-2, whereas HEV-3, HEV-4, and HEV-7 can induce chronic hepatitis, particularly in immunocompromised patients [4]. Studies indicate variations in the pathogenicity of genotypes 3 and 4 [7,8]. Notably, HEV-3f is likely associated with higher viral loads and increased hospitalization rates compared to subgenotype 3c [7]. However, the clinical manifestations of HEV did not correlate with HEV-3 or its subtypes, as shown in patients with acute hepatitis E [9]. Additionally, patients infected with HEV-4 tend to exhibit higher alanine aminotransferase activity than those infected with HEV-3, potentially heightening the risk of fulminant hepatitis [8]. Over recent years, Southeast Asia and China have witnessed a surge in sporadic cases of HEV genotype (HEV-3 and HEV-4) [10,11], indicative of an emerging zoonotic HEV in these regions. Consequently, surveillance of zoonotic HEV infections in common reservoirs is imperative in these areas.

Since the initial identification of HEV strains in domestic pigs in the United States in 1997, these strains have been detected worldwide both in domestic and wild boar populations, displaying widely varying hosts [12,13]. Vietnam, renowned for its significant pig production and consumption, stands as a potential hotspot for swine hepatitis E virus. The first outbreak in Vietnam occurred in 1996 in the Southwestern region, suspected to have spread via the Hau River, although the genotype remain unidentified [14]. Since then, no further outbreaks of HEV have been reported. Several surveillance campaigns for HEV in animals and high-risk groups have been conducted in the Southern and Northern regions, revealing the circulation of genotypes 3 and 4 in domestic pigs [15,16]. Seroprevalence studies have reported HEV- IgG positivity ranging from 8% in pregnant women [11] to 27% in blood donors [17] and 53% in individuals exposed to pigs [18], highlighting a significant exposure in the Vietnamese population. Pigs comprised the largest proportion of livestock in Vietnam, accounting for 67% (23 million) in 2021 and 74% (25 million) in 2022 [19]. Additionally, around 217 farms in Vietnam raised approximately 7500 wild boars in 2021 [20], underscoring the importance of evaluating the risk of zoonotic disease transmission in this specific population.

Given the substantial burden of HEV infection in Vietnam, routine surveillance of the virus in the animal reservoir is imperative. This molecular epidemiological study aims to evaluate the distribution and genetic diversity of zoonotic HEV in domestic pigs and wild boars in Southern and Central Vietnam, regions.

2. Material and methods

2.1. Ethics statement

The study was approved by the ethics committee of Hue University of Medicine and Pharmacy, Hue University, Vietnam (H2022/020) and the animal ethics committee of the International University (IU) - Vietnam National University - Ho Chi Minh City (VNUHCM- August 2022).

2.2. Study design and sampling

From April to June 2022, 517 samples were collected from pigs and wild boars across Central and Southern Vietnam. In Thua Thien Hue province - Central Vietnam, liver samples ($n = 199$), rectal swabs ($n = 92$) and faecal samples ($n = 77$) were obtained from domestic pigs at seven study sites (six slaughterhouses and one farm). Additionally, two wild boar farms were sampled, resulting in six rectal swabs and eleven faecal samples were collected. In Ho Chi Minh City - Southern Vietnam, pig liver samples ($n = 47$) were collected from eight wet markets, while rectal swabs ($n = 60$) and faecal samples ($n = 25$) were obtained from wild boars from two different farms. Study locations are detailed in Fig. 1 and Table 1, and sampling adhered to standard operating procedures for One Health surveillance [21]. Liver tissue (approx. 1–2 g) was collected immediately post-slaughter, rectal swabs were individually labelled to prevent duplication, an. 2–3 faecal samples were collected from various locations within each pigpen. All samples were stored with DNA/RNA shield (Zymoresearch, Irvine, CA, USA), a solution that preserves genetic integrity, expression profiles and inactivates infectious agents at room temperature. The collected samples were then stored at -20 °C for subsequent analysis.

2.3. RNA extraction and cDNA synthesis

100 mg of liver tissue which was preserved in a DNA/RNA shield was washed twice with phosphate-buffered saline (PBS), that can help to stabilize the tissue and maintain the pH, creating an optimal environment for subsequent extraction procedures (Thermo Fisher Scientific, Frederick, USA) before RNA isolation. This step is essential to remove blood, extracellular proteins, and other contaminants that could interfere with the extraction process. Total RNA was extracted using TRIzol™ LS reagent (Thermo Fisher Scientific, Carlsbad, CA, USA) following the method described by Mendez et al. [22]. Samples were homogenised using a FastPrep 24™ homogeniser (MP Biomedicals, Santa Ana, CA, USA) by adding five glass beads to each tube (4–7 cycles of 30 s at 5 m/s) prior to isolate RNA according to the manufacturer's instructions. For the other sample types, 40 µl of rectal swab and 200 µl of faecal sample were resuspended in 100 µl PBS and 800 µl PBS respectively. A total of 140 µl of the rectal swab mixture was used for RNA isolation using QIAamp Viral RNA Kits (Qiagen GmbH, Hilden, Germany). 200 µl of the supernatant from faecal samples was used for RNA isolation after centrifugation at 10,000*g* for 2 min, using the same procedure as for the rectal swabs. The quality and quantity of 1 µg of extracted RNA were assessed using the Nanodrop (absorbance: 260/280 nm ratio) and the Qubit™ 4 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). The RNA was then transcribed into complementary DNA using the LunaScript RT SuperMix Kit (New England BioLabs, Ipswich, MA, USA).

2.4. Screening HEV using nested polymerase chain reaction (PCR)

All samples were tested for HEV RNA by nested PCR targeting the viral ORF1 and ORF2 regions as described by Hoan et al. [18]. For ORF1, the outer primer pairs were HEV-38 (sense) 5′-

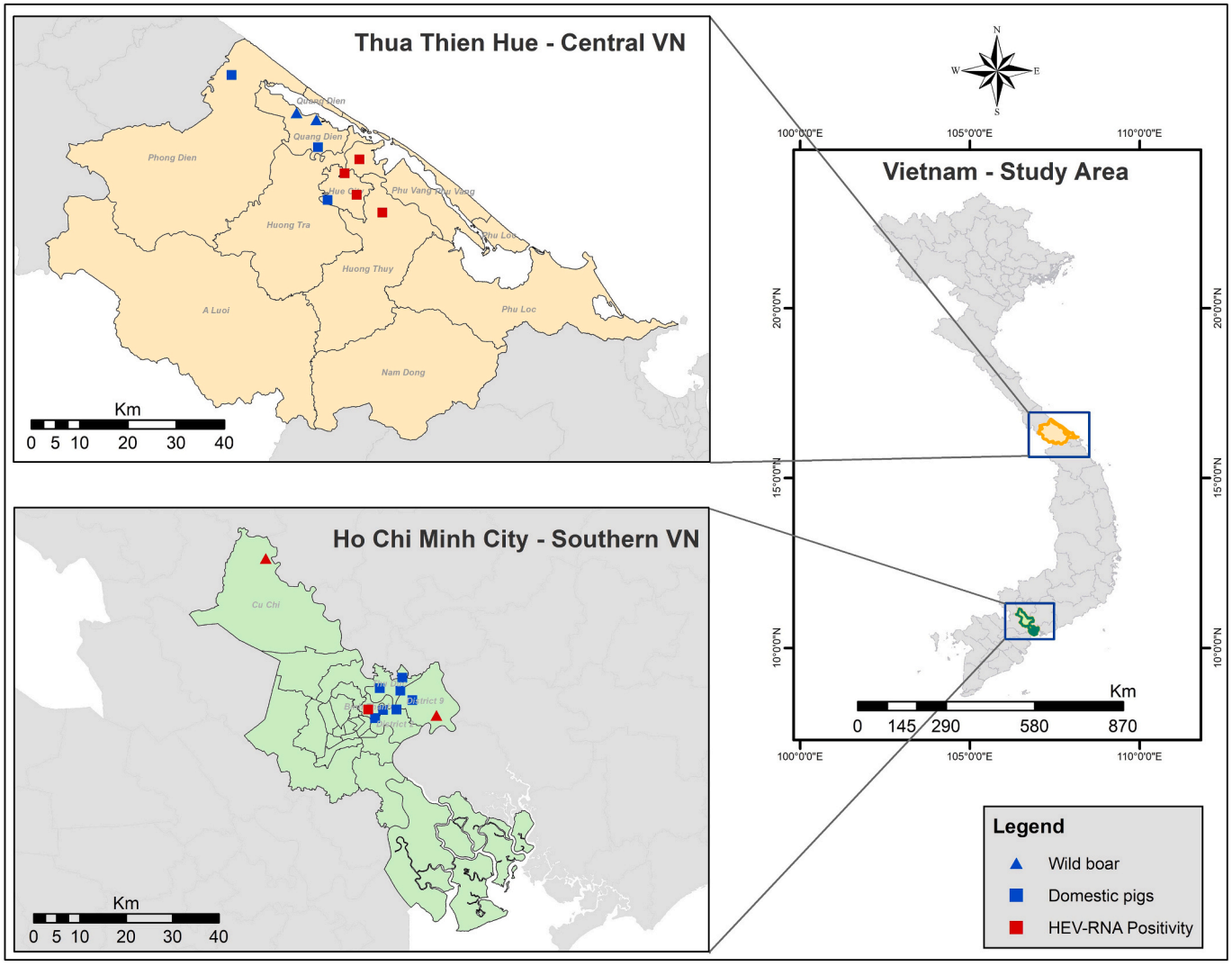

Fig. 1. Zoonotic HEV screening from two study sites (Central and Southern Vietnam): The sampling sites for pigs are represented by squares, while the traingle indicate wild boar, which include slaughter houses, farms and wet markets. Sites where HEV positive samples were detected are highlighted in red. The map was created using ArcGIS 10.8 software.

GAGGCYATGGTSGAGAARG-3′ and HEV-39 (antisense) 5′-GCCATGTTCCAGACRGTRTTCC-3′; while the inner primers were designated HEV-37 (sense) 5′- GGTTCCGYGCTATTGARAARG-3′ and HEV-27 (anti-sense) 5'-TCRCCAGAGTGYTTCTTCC-3′. For ORF2, the outer primers were HEV-34 (sense) 5'-CCGACGTCYGTYGAYATGAA-3′ and HEV-36 (anti-sense) 5'-TTRTCC TGCTGAGCRTTCTC-3′; inner primers were HEV-35 (sense) 5'-AAGTGAGCGCCTACAYTA YCG-3′ and HEV-29 (anti-sense) 5'-CTCGCCATTGGCTGAGAC-3′. The PCR amplification was performed in a 25 μL volume containing 50 ng viral cDNA, 1× PCR buffer, 0.4 mM dNTPs, 0.4 mM MgCl2, 0.6 μM specific primer pairs, and 1 unit of Taq polymerase (Qiagen GmbH, Hilden, Germany). The thermocycling parameters for the outer ORF1-PCR were an initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation (95 °C for 30 s), annealing (56 °C for 30 s) and extension (72 °C for 30 s), followed by a final extension at 72 °C for 10 min. The thermocycling parameters for the inner ORF1-nested PCR were an initial denaturation at 94 °C for 5 min, followed by 36 cycles of denaturation (95 °C for 30 s), annealing (54 °C for 30 s) and extension (72 °C for 30 s), followed by a final extension at 72 °C for 10 min. For ORF2, the thermal cycling program was similar to ORF1, but the annealing temperature for PCR and nested PCR was 54 °C and 56 °C, respectively. A plasmid containing HEV cDNA served as a positive control. The amplicons (307 bp for ORF1/489 bp for ORF2) were visualized on 1.1% agarose gels stained with SYBR Green. Sample positive for ORF1 or ORF2 was considered positive for HEV RNA. All positive samples were replicated for confirmation.

2.5. HEV genotyping and phylogenetic analysis

PCR products were purified using the Exo-SAP-IT kit (USB, Affymetrix, Santa Clara, CA, USA) and utilized as templates for Sanger sequencing (Bigdye Terminator v3.1 Cycle Sequencing Kit; Applied Biosystems, Foster City, CA, USA) with the ABI 3130XL sequencing system. Sequence correction and alignment were conducted using DNASTAR-Lasergene v6 software (www.dnastar.com). Phylogenetic analysis of the ORF1 and ORF2 regions was carried out using MEGA 11 software (www.megasoftware.net) [23], employing the Maximum Likelihood method and the General Time Reversible (GTR) plus Gamma Distribution model. The statistical robustness and reliability of the branching order were confirmed via bootstrapping with 1000 replicates. The resulting phylogenetic tree was annotated and visualized using the online tool iTOL v6 (itol.embl.de) [24].

Table 1
HEV RNA positivity and genotypes distribution across sampling sites in Central and Southern Vietnam.

Region	Commune -District or City - Province	Source of samples #ID	Sample type	HEV RNA positivity	HEV RNA positivity (%); HEV genotypes
Central Vietnam (n = 385)	Phu Duong- Phu Vang-Thua Thien Hue	Slaughterhouse #1	Pig liver Pig rectal swab Pig feaces	0/21 1/20 0/2	(2%; n = 1/43); HEV3a
	Thuy Bieu-Hue city-Thua Thien Hue	Slaughterhouse #2	Pig liver Pig rectal swab Pig feaces	0/4 0/7 0/3	(0%; n = 14)
	Thuy Duong-Hue city-Thua Thien Hue	Slaughterhouse #3	Pig liver Pig rectal swab Pig feaces	2/126 2/18 2/19	(4%; n = 6/163) HEV3a; HEV3f,
	Thuy Chau-Huong Thuy-Thua Thien Hue	Slaughterhouse #4	Pig liver Pig rectal swab Pig feaces	6/48 5/33 2/19	(13%; n = 13/100); HEV3a
	Phong Hoa-Phong Dien-Thua Thien Hue	Slaughterhouse #5	Pig rectal swab Pig feaces	0/3 0/1	(0%; n = 04)
	Phu Hau-Hue city-Thua Thien Hue	Slaughterhouse #6	Pig rectal swab Pig feaces	6/11 2/20	(26%; n = 8/31); HEV3a
	Quang Tho-Quang Dien-Thua Thien Hue	Pig farm #1	Pig feaces	0/13	(0%; n = 13)
	Quang Loi-Quang Dien-Thua Thien Hue	Wild boar farm #1	Wild boar rectal swab Wild boar feces	0/4 0/9	(0%; n = 13)
	Quang Loi-Quang Dien-Thua Thien Hue	Wild boar farm #2	Wild boar rectal swab Wild boar feces	0/2 0/2	(0%; n = 04)
	An Phu-Cu Chi-Ho Chi Minh city	Wild boar farm #3	Wild boar rectal swab Wild boar feces	13/25 0/5	(43%; n = 13/30); HEV3a
	Binh Trung Dong-District 9-Ho Chi Minh city	Wild boar farm #4	Wild boar rectal swab Wild boar feces	6/35 6/20	(22%; n = 12/55) HEV3a; HEV4b
Southern Vietnam (n = 132)	Linh Trung-Thu Duc District-Ho Chi Minh city	Wet market #1	Pig liver	0/5	(0%; n = 05)
	Le Van Chi-Thu Duc-Ho Chi Minh city	Wet market #2	Pig liver	0/5	(0%; n = 05)
	Vo Van Ngan-Thu Duc-Ho Chi Minh city	Wet market #3	Pig liver	0/4	(0%; n = 04)
	Thao Dien-District 2-Ho Chi Minh city	Wet market #4	Pig liver	0/1	(0%; n = 01)
	Thao Dien-District 2-Ho Chi Minh city	Wet market #5	Pig liver	0/11	(0%; n = 11)
	Commune 1-Binh Thanh-Ho Chi Minh city	Wet market #6	Pig liver	1/8	(13%; n = 1/8) HEV3a
	Xo Viet Nghe Tinh- Binh Thanh-Ho Chi Minh city	Wet market #7	Pig liver	0/10	(0%; n = 10)
	Phuoc Long- District 9 —Ho Chi Minh city	Wet market #8	Pig liver	03	(0%; n = 03)

2.6. Data analysis

All analyses were conducted using GraphPad Prism (version 9.5.1). A p-value <0.05 was deemed statistically significant. Demographic data were depicted as mean values with corresponding ranges for quantitative variables, and as absolute numbers and percentages for categorical variables. Categorical data were assessed using Chi-square or Fisher's exact tests, while continuous variables were evaluated using t-tests or Kruskal-Wallis tests, as appropriate.

3. Results

3.1. Demographic and study population characteristics

This study was conducted in central and southern Vietnam, analyzing a total of 517 samples. In central Vietnam, 385 samples were collected from adult pigs and wild boars at six slaughterhouses, one pig farm, and two wild boar farms. The ages of the adult pigs and wild boars differed: pigs were 5–6 months old, while wild boars were 12–24 months old. In southern Vietnam, 132 samples were collected from adult pigs and wild boars at eight wet markets and two wild boar farms. The pigs had a body mass ranging from 60 to 100 kg. Sex determination was performed only on rectal swabs, with 98 samples from central Vietnam (34 females and 64 males) and 60 samples from southern Vietnam (33 females and 27 males). In southern Vietnam, 64% (85/132) of the samples were from wild boar population, including wild-boar piglets sampled between 24 and 160 days after birth (mean: 100 days ±54), with weights ranging from 4 to 35 kg each (mean: 17.2 kg ± 30.5).

3.2. HEV-RNA positivity

A total of 10% (n = 54/517) of samples were positive for HEV RNA with wild boars exhibited the highest HEV positivity rate at 25%, followed by domestic pigs at 7%. In central Vietnam, slaughterhouse Nr. 6 displayed the highest HEV positivity at 26% (n = 8/31), followed by slaughterhouse Nr. 4 at 13% (n = 13/100), slaughterhouse 3 at 4% (n = 6/163), and slaughterhouse Nr. 1 at 2% (n = 2/43). Similarly, in southern Vietnam, wild boar farm Nr. 3 showed the highest HEV positivity at 43% (n = 13/30), followed by wild boar farm Nr. 4 at 22% (n = 12/55) (Table 1). Additionally, a higher HEV RNA positivity rate was observed in southern Vietnam (20%) compared to central Vietnam (7%) (Fig. 2A). Among the sample types from domestic pigs, rectal swabs exhibited the highest positivity rate at 15%, followed by feces at 8%, and liver at 4%. Differences in RNA positivity were noted between rectal and liver samples. While wild boar rectal swabs showed a higher RNA positivity compared to feces (29% vs. 17%), this difference was not statistically significant (p = 0.17) (Fig. 2B and C).

3.3. Phylogenetic analysis

The phylogenetic analysis of both HEV-ORF1 and ORF2 revealed HEV-3a as the predominant genotype at 85%, followed by HEV-4b at 9% and HEV-3f at 6% (Figs. 3 and 4). HEV 3a genotype was found distributed across both central and southern Vietnam, regardless of the studied slaughterhouses, pig farms, wild boar farms, or wet markets in the region. However, HEV 3f was only detected in slaughterhouse Nr. 3 in central Vietnam, while HEV 4b was identified in wild boar farm Nr. 4 in

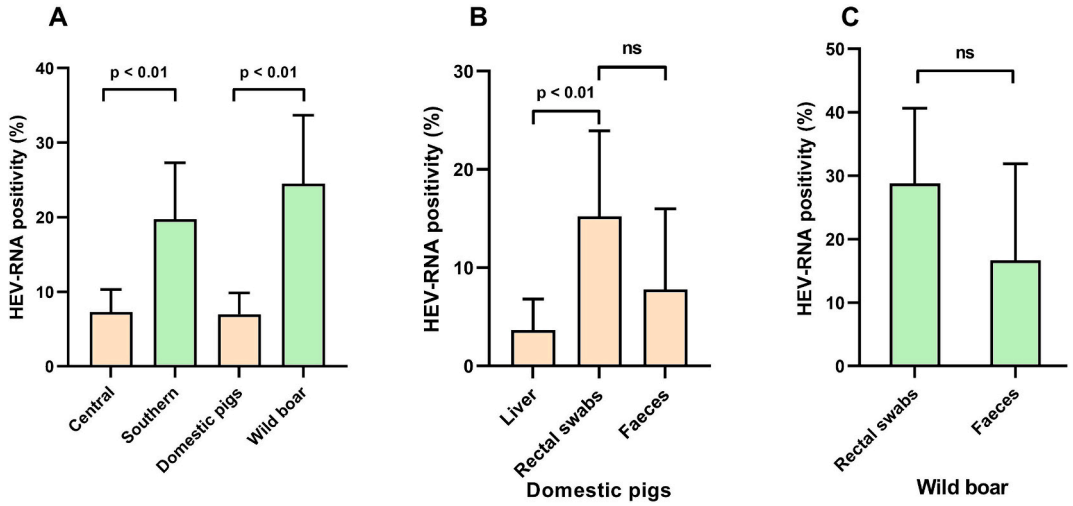

Fig. 2. HEV positivity rates observed in this study: (A):Categorised by region and animals investigated; (B): Categorised by sample type in domestic pigs; (C): Cateogarised by sample type in wild boar; ns: non-significant

southern Vietnam (Table 1). The nested PCR results for both HEV ORF-1 and ORF-2 were consistent, with samples positive for HEV ORF-1 also testing positive for HEV ORF-2. A total of 87 successfully sequenced samples were submitted to the NCBI GenBank database, with accession numbers for ORF1 ranging from PP504786 to PP504831 and PP150468 to PP150473 (n = 52), and for ORF2 ranging from PP531178 to PP531212 (n = 35).

4. Discussion

While zoonotic transmission of HEV and autochthonous HEV cases are well-documented in high-income countries, a growing body of research from Southeast Asia increasingly reports human HEV infections originating from animals. This trend suggests that the virus is emerging as a significant pathogen in the region [25,26]. In this study, the HEV RNA positivity rate of 10% aligns closely with rates observed in neighboring countries such as Laos (11.6%) [27], the Philippines (7.4%) [28], and Thailand (3%) [29]. Previous investigations in northern Vietnam revealed a 12% HEV positivity rate in pig livers [18], while southern Vietnam exhibited a higher rate of 19% [15], indicating geographical variation in HEV prevalence. Notably, no prior studies have explored the prevalence of HEV in the wild boar population in Vietnam. Wild boars are commonly hunted game animals, and HEV prevalence has been noted to be higher in European countries (8.7%) [30]. Germany, in particular, reported the highest prevalence with 56% RNA positivity in bile sample [30].

Our study reveals a high prevalence of HEV RNA positivity in wild boars, particularly notable in southern Vietnam at 29%, with wild boar farm Nr. 4 recording up to 43% positivity. These findings underscore the significance of wild boars as a reservoir for HEV alongside domestic pigs in Vietnam, highlighting the predominance of HEV-4 genotypes among the wild boar population. Despite wild boars often being raised for breeding alongside domestic pigs, which primarily carry HEV genotype 3, the cohabitation raises the potential for cross-transmission via the faecal-oral route. This scenario heightens the risk of HEV recombination, potentially leading to the emergence of new genotypes.

When comparing various sample types from the same animal, rectal swabs and faecal samples exhibited higher HEV positivity rates compared to liver samples, indicating their potential suitability for HEV monitoring in animals. A study in southern Vietnam reported a higher HEV RNA positivity in faecal samples (19%), than in rectal swabs (8.2%) [15]. Another systematic study on wild boar populations revealed the highest HEV positivity in bile (17%), followed by liver (10%), serum (7%), feces (5%) and meat (3%) [30]. HEV RNA is more frequently detected in stool samples than in liver samples due to several factors. HEV sheds into feces as non-enveloped virions, whereas in blood, it circulates in a quasi-enveloped form [31]. Although HEV primarily replicates in the liver and infects hepatocytes, a significant amount of the virus is excreted into bile and subsequently into the intestine [32–35]. This results in a high concentration of the virus in stool, increasing the likelihood of RNA detection compared to liver samples, which may have lower and more localized viral quantities, thereby enhancing detection rates. Equally, liver biopsies or tissue samples may not capture all areas where the virus is present, potentially resulting in lower detection rates compared to stool samples, which are more uniform and likely to contain higher concentrations of the virus. This underscores that the choice of screening method using different sample types can significantly impact the observed prevalence of HEV infection in animals.

In our study, PCR primers targeting both ORF1 and ORF2 were utilized for detecting and characterizing HEV. The sensitivity for ORF1 was notably higher than for ORF2 (96% vs. 64%). This finding aligns with a comparative study by La Rosa et al., which demonstrated that nested PCR targeting ORF1 detects more HEV cases than ORF2 and ORF3 [36]. However, our study also revealed that while the ORF1 assay was effective for HEV identification at the genotype level, subtype characterization necessitated sequencing of ORF2 (capsid region). Notably, HEV3a was the most common subgenotype (85%), followed by HEV4b (9%) and HEV3f (6%). While HEV3a and HEV4b prevalence has been documented in pig populations in northern Vietnam [16,18], our study is the first to report the circulation of HEV4b in wild boar and HEV3f in domestic pigs in Vietnam. The emergence of the new subgenotype 3f in Vietnam suggests that the import and export of pigs may facilitate the movement of HEV strains and the introduction of new subtypes from Thailand and Cambodia. Farms in central Vietnam have imported breeding pigs from these regions, facilitating the spread of HEV subtypes. Genotypes 3 and 4 are widespread in pigs and wild boars globally, yet the distribution of subgenotypes varies regionally. In European countries, subgenotype 3e is predominant, followed by 3f and 3c, while in Asia, subtypes 3a, 3b, and 3f are prevalent, particularly in Indonesia, Thailand, Japan, and Vietnam [37]. Notably, genotype 4, previously limited to Asian countries, is now widespread across other continents [38].

The HEV3a sample from this study (#PP531208) showed 95% nucleotide identity with the HEV3a isolate (#OK129292) observed in a pregnant Vietnamese woman [11], suggesting potential zoonotic transmission. Furthermore, the growing evidence of acute HEV infection linked to the consumption of liver-containing sausages, as reported in

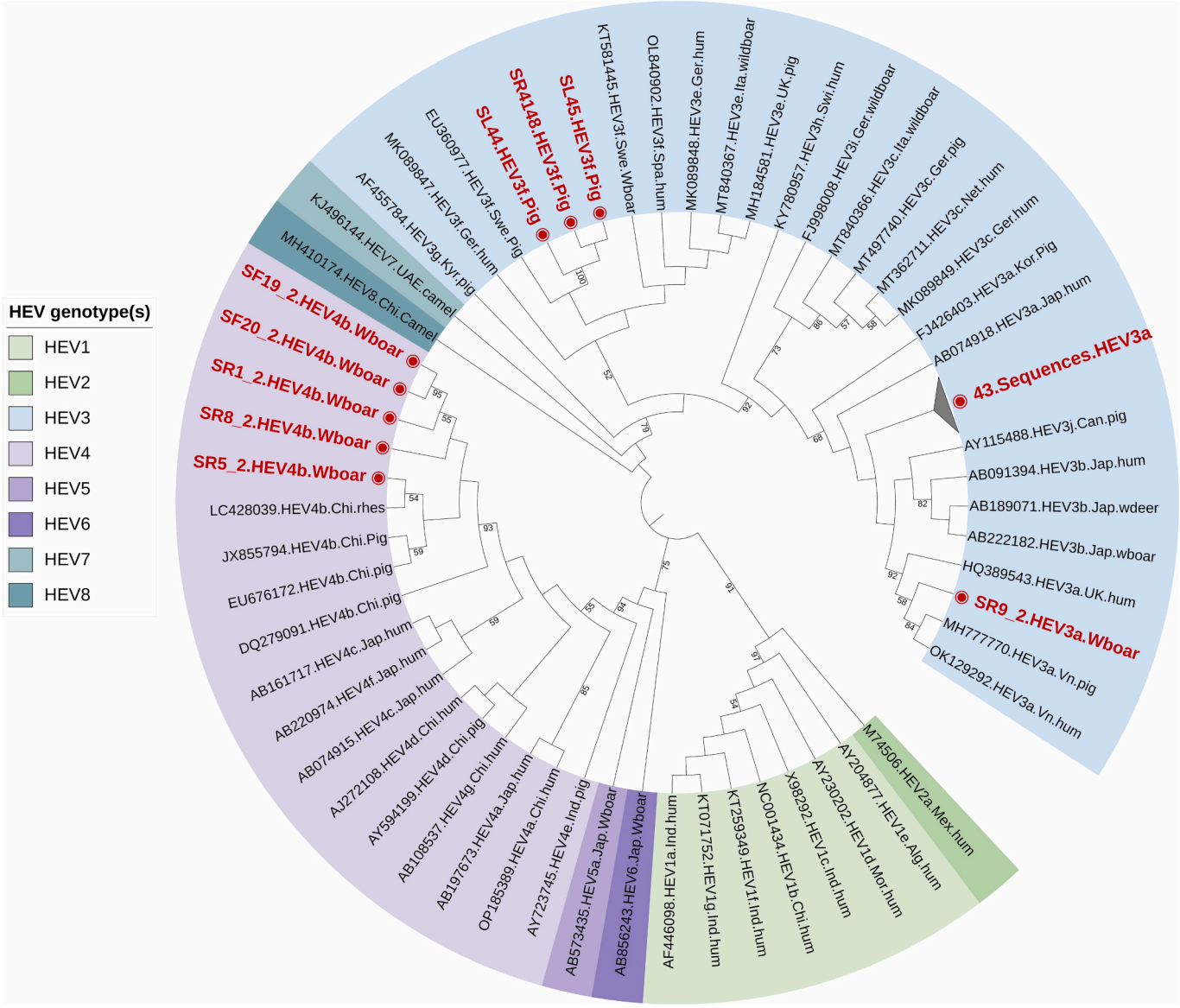

Fig. 3. Phylogenetic analysis of HEV ORF-1 specific sequnces (n=52) obtained in this study from domestic pigs and wildboars. All positive (n=52) sequences are highlighted in red.

Finland in 2024 [39], underscores the potential risk of HEV transmission in Vietnam. The increasing popularity of liver sausages in the Vietnam also raises concerns about foodborne HEV transmission if these products are not properly cooked. Our studies have primarily investigated HEV transmission in pig farms. However, pig farmers and slaughterhouse workers likely serve as reservoirs for HEV transmission within the community and contribute to spillover between the animal-human compartments. This hypothesis is supported by our previous findings, which indicated elevated IgG and IgM seroprevalence among individuals engaged in these occupations. Specifically, we identified significantly higher levels of anti-HEV IgG and anti- HEV IgM antibodies in pig farmers and slaughterhouse workers compared to pork meat vendor [18].

Growing evidence of genotype 4b infections in Vietnam, and Cambodia, indicate a potential emerging public health concern [40], while no clinical study has compared the severity of genotype 3 and 4 infections in hepatitis patients. A study by Schemmerer et al. suggested that infections with genotype 3e and 3f might be linked to more severe disease and higher mortality compared to other HEV3 subtypes [41]. The HEV 239 vaccine, known as Hecolin, was licensed in China in 2011, and gained authorization in Pakistan in 2020. It is also undergoing clinical studies in other countries, including India, Bangladesh, Nepal, and Indonesia. Despite there being four genotypes of HEV (HEV1–4), the vaccine, which is based on genotype 1 (ORF-2 capsid protein), is expected to offer cross-genotype protection against all four genotypes since they are characterized as one serotype in humans. A study evaluating the immunogenicity and safety of Hecolin in children is currently underway in South Africa through a placebo-controlled trial [42].

Our study had limitations: Firstly, unequal sample sizes of domestic and wild pigs in central and southern Vietnam hindered direct prevalence comparisons between regions. Secondly, the efficacy of our RNA extraction method could have been better assessed by incorporating internal or external controls, to provide a clearer evaluation of RNA recovery from liver tissues and stool samples. The HEV RNA recovery rates, as demonstrated in the study by Wang et al. [43], also ranged from 1.27% to 100%. Thirdly, due to the inability to collect serum samples, we were unable to determine HEV seroprevalence in animals. Lastly the lack of samples from occupationally exposed groups precluded an assessment of zoonotic transmission within the Vietnamese population.

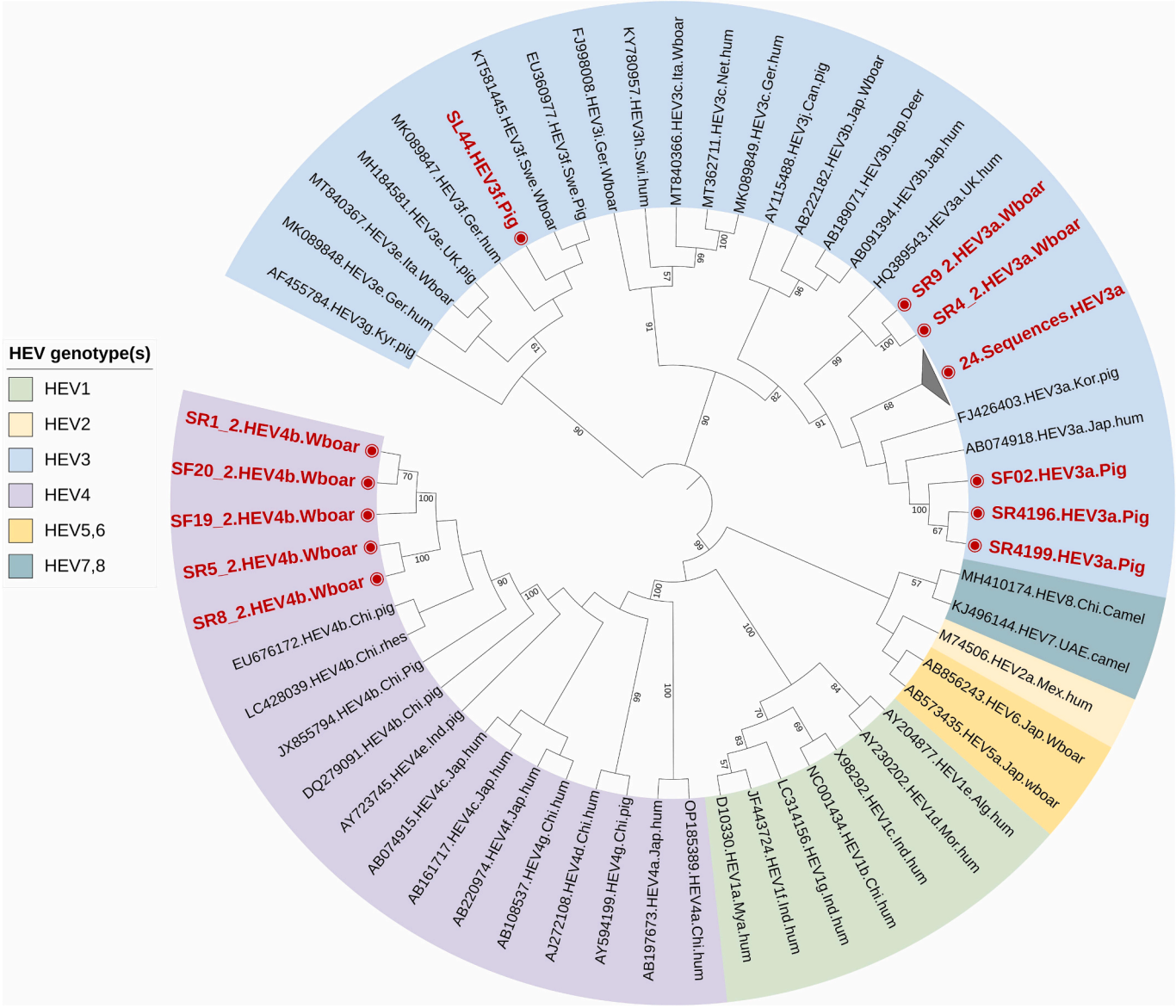

Fig. 4. Phylogenetic anaylsis of HEV ORF-2 specific sequences (n=35) obtained in this study from domestic pigs and wild boars. All positive (n=35) sequences are highlighted in red.

5. Conclusion

Our study highlights the significant prevalence of HEV in wild boars in Vietnam, suggesting their potential role as an important zoonotic reservoir alongside domestic pigs. Further investigations involving occupationally exposed individuals in areas with high HEV prevalence are crucial to evaluate the human health impact of zoonotic hepatitis E. These epidemiological studies play a vital role in regularly assessing the prevalence and transmission of zoonotic HEV infections among common reservoirs, aiding in the prevention of spillover events within the community.

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Institutional review board statement

The study was approved by the ethics committee of Hue University of Medicine and Pharmacy, Hue University, Vietnam (H2022/020), and the animal ethics committee of the International University (IU) - Vietnam National University - Ho Chi Minh City (VNUHCM- August 2022).

CRediT authorship contribution statement

Le Chi Cao: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Le Nguyen Nhat Ha:** Methodology, Investigation. **Tran Thi Giang:** Methodology, Investigation. **Vo Minh Tiep:** Methodology, Investigation. **Ngo Thi Minh Chau:** Project administration, Methodology. **Ton Nu Phuong Anh:** Project administration, Methodology. **Pham Khanh Duy:** Resources, Methodology. **Le Phuc Nhan:** Resources, Methodology. **Nguyen Thi Thu Hoai:** Supervision, Resources, Project administration. **Le Thi Kieu Linh:** Methodology, Investigation. **Nourhane Hafza:** Methodology, Investigation. **C. Thomas Bock:** Writing – review &

editing, Supervision, Resources, Methodology. **Truong Nhat My:** Supervision, Project administration, Methodology, Investigation. **Bui Tien Sy:** Resources, Project administration, Methodology, Investigation, Funding acquisition. **Nguyen Linh Toan:** Supervision, Project administration, Methodology, Investigation. **Le Huu Song:** Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition. **Thirumalaisamy P. Velavan:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare there are no competing interests. The funder has no role in the study design, data collection and analysis, decision to publish or preparation of the manuscript.

Data availability

All data generated or analysed during this study are included in this article. A total of 87 successfully sequenced samples were submitted to the NCBI GenBank database, with accession numbers for ORF1 ranging from PP504786 to PP504831 and PP150468 to PP150473 (n = 52), and for ORF2 ranging from PP531178 to PP531212 (n = 35).

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Chapter 2: HEV seroprevalence and RNA detection in general and high-risk populations in Central Vietnam

Publication No.2

High Hepatitis E Virus (HEV) Seroprevalence and No Evidence of HEV Viraemia in Vietnamese Blood Donors

Cao LC, Martin V, Linh LTK, Giang TT, Chau NTM, Anh TNP, Nghia VX, The NT, My TN, Sy BT, Toan NL, Song LH, Bock CT, Velavan TP.

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viruses

MDPI

Brief Report

High Hepatitis E Virus (HEV) Seroprevalence and No Evidence of HEV Viraemia in Vietnamese Blood Donors

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Abstract: The prevalence of hepatitis E virus (HEV) in the Vietnamese population remains underestimated. The aim of the present study was to investigate the seroprevalence of HEV IgG/IgM antibodies and the presence of HEV RNA in blood donors as a part of epidemiological surveillance for transfusion-transmitted viruses. Serum samples from blood donors (n = 553) were analysed for markers of past (anti-HEV IgG) and recent/ongoing (anti-HEV IgM) HEV infections. In addition, all serum samples were subsequently tested for HEV RNA positivity. The overall prevalence of anti-HEV IgG was 26.8% (n = 148/553), while the seroprevalence of anti-HEV IgM was 0.5% (n = 3/553). Anti-HEV IgG seroprevalence in male and female donors was similar (27.1% and 25.5%, respectively). A higher risk of hepatitis E exposure was observed with increasing age. None of the blood donors were HEV RNA positive, and there was no evidence of HEV viraemia. Although the absence of HEV viraemia in blood donors from Northern Vietnam is encouraging, further epidemiological surveillance in other geographical regions is warranted to rule out transfusion-transmitted HEV.

Keywords: hepatitis E virus; blood donors; transfusion; seroprevalence

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1. Introduction

Hepatitis E virus (HEV) is a major cause of acute viral hepatitis and is mainly transmitted through contaminated water or food, especially in areas with poor sanitation. HEV infections affect about 20 million people in developing countries each year, with more than 3 million symptomatic cases and 44,000 reported deaths [1]. In most cases, hepatitis E is a self-limiting disease. However, in pregnant women, organ transplant recipients, people with underlying liver disease, and HIV-infected and immunocompromised patients, the disease can be more severe [2]. HEV is an enterically transmitted small quasi-enveloped or non-enveloped single-stranded RNA virus and belongs to the Hepeviridae family with several species in the subfamily *Orthohepevirinae*. Currently, five genotypes (HEV-1 to

HEV-4, and HEV-7) classified in the genus *Paslahepevirus* are known to infect humans [3]. HEV genotype 1 and genotype 2 (common in Asia and Africa) are mainly transmitted via the faecal–oral route and lead to acute hepatitis [2,4]. HEV genotype 3 and genotype 4 (in developed and developing countries) are transmitted mainly through the consumption of contaminated food, especially raw or undercooked pork. Additionally, other animal products such as liver pâté, sausage, or fresh shellfish are often associated with sporadic human cases [2,5]. Infection with HEV-7 occurs through the consumption of HEV-contaminated camel products, which are common in some regions of the Middle East [2]. In Vietnam, only HEV genotypes 3 and 4 are currently circulating, suggesting predominantly zoonotic transmission of HEV in this country [6,7]. Testing of blood and stool samples is valuable for understanding transmission patterns and monitoring the prevalence of HEV. HEV can be detected in both blood (at 2 to 6 weeks) and stool (in the acute phase) during the course of the infection [2]. The virus occurs as quasi-enveloped virus particles in blood and urine and as non-enveloped virions in bile and faeces [8]. Blood transfusions can be a potential route of transmission, and performing HEV screening on blood donations, especially in regions where HEV is endemic, can minimize the risk of transfusion-transmitted infection [9]. A first report involved a patient in Japan, who developed acute hepatitis E after transfusion of HEV-positive blood products in 2004 [10]. Since that time, studies have accumulated showing an increasing incidence of transfusion-transmitted HEV and associated risks in immunocompromised individuals [11]. Certain individuals, including pregnant women, immunocompromised individuals, and patients with pre-existing liver disease, may be more vulnerable to severe complications if they receive blood from an infected donor.

Many European countries have introduced HEV testing for blood donations [2]. More than 3.2 million blood donor samples have been tested for HEV RNA in the European Union, and one study reports that 1 in 3109 donations is HEV RNA positive [12]. As more cases of transfusion-transmitted HEV are reported in Asia [13–15], transfusion safety is a challenge in countries with dynamic health policies and practices. Vietnam is an HEV endemic area in Southeast Asia with high rates of faecal–oral and animal transmission [7], making hepatitis E a significant public health problem in a country with high HBV prevalence [16]. A previous study has shown that HEV superinfection can worsen the clinical course and progression of HBV-related liver disease in a population [16]. As part of an epidemiological surveillance for transfusion-transmitted viruses, the study aimed to investigate the prevalence of HEV IgG/IgM antibodies and the presence of HEV RNA in blood donors.

2. Materials and Methods

2.1. Ethics Statement

The study was approved by the ethics committee of 108 Military Central Hospital, Hanoi, Vietnam (108/RES/OBI-HEP-VGCARE-V-D1-20-09-2021). Informed written consent was obtained from all donors. All experiments were performed following GCP/GCLP guidelines.

2.2. Study Cohort

The blood donation for this cross-sectional study was obtained from the Hematology and Blood Transfusion department of the 108 Military Central Hospital, Hanoi, Vietnam, in 2021. Based on the studies conducted in the region [16] and the sample size calculation, we hypothesized that we needed to screen blood donors (n = 553) (with a power of 80% and a two-sided confidence interval of 95%) to determine the seroprevalences. Demographic data and blood samples (n = 553) were collected from healthy adult donors mostly belonging to the Kinh ethnicity. In accordance with standard hospital algorithm, all blood donor samples were serologically tested for HIV, HCV, and HBV (HBsAg) and were confirmed to be negative (using VITROS Immunodiagnostic Products HBsAg, Anti-HIV 1 + 2, and Anti-HCV from Ortho-Clinical Diagnostic, Felindre Meadows, Bridgend, UK).

2.3. Serological Assays

The blood donor serum samples were screened for anti-HEV IgG and anti-HEV IgM antibodies using specific enzyme-linked immunosorbent assay (ELISA) (WANTAI, Beijing, China). The Wantai HEV test was selected for our analysis based on our own interlaboratory tests and on the sensitivity and specificity performances of the assays used in many studies in Europe and Asia. The anti-HEV IgG (sensitivity of 99.08% and specificity of 99.9%) and anti-HEV IgM (sensitivity of 97.1% and specificity of 98.4%) ELISA assays were previously evaluated in multicentre clinical trials involving acute hepatitis E samples, samples from individuals living in hepatitis E outbreak areas, and 10,587 blood donation samples (also refer the following: https://www.ystwt.cn/wp-content/uploads/2018/04/Wantai-HEV-IgG-ELISA.pdf; accessed on 3 July 2023). The ELISA protocol was followed according to the manufacturer's instructions. The test was considered positive if the optical density was $\geq$0.4 + non-reactive control (NRCx) and $\geq$0.5 + NRCx for IgM and IgG, respectively. Absorbance was measured with a CLARIOstar microplate reader (BMG Labtech, Ortenberg, Germany).

2.4. Nucleic Acid Isolation

Nucleic acid isolation from serum samples was conducted using QIAmp Viral RNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. For the isolation, 140 µL of serum was used, and the nucleic acids were eluted in 60 µL of elution buffer. The quality and quantity of the RNA were measured using NanoDrop™ (Thermo Fisher Scientific, Waltham, MA, USA) and stored at −80 °C until use.

2.5. HEV-Specific RT-PCR

HEV RNA was reverse transcribed into cDNA using a High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific, Waltham, MA, USA). The presence of HEV RNA was examined using a nested polymerase chain reaction (PCR) assay using primers targeting the conserved regions of the overlapping HEV ORF1 as described in Hoan et al. [16]. The outer primer pairs were HEV-38 (sense) 5′-GAGGCYATGGTSGAGAARG-3′ and HEV-39 (antisense) 5′-GCCATGTTCCAGACRGTRTTCC-3′; while the inner primers were named as HEV-37 (sense) 5′-GGTTCCGYGCTATTGARAARG-3′ and HEV-27 (antisense) 5′-TCRCCAGAGTGYTTCTTCC-3′. The PCR amplification was performed in 25 µL volume containing 5 ng viral cDNA, 1 × PCR buffer, 0.4 mM dNTPs, 0.4 mM MgCl2, 0.6 µM specific primer pairs, and 1 unit of Taq polymerase (Qiagen GmbH, Hilden, Germany). The thermal cycling parameters for the outer PCR were an initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation (95 °C for 30 s), annealing (54 °C for 30 s), and extension (72 °C for 30 s), followed by a final extension at 72 °C for 10 min. The thermal cycling parameters for the inner nested PCR were an initial denaturation at 94 °C for 5 min, followed by 40 cycles of denaturation (95 °C for 30 s), annealing (56 °C for 30 s), and extension (72 °C for 30 s), followed by a final extension at 72 °C for 10 min. A plasmid containing HEV cDNA served as a positive control. Amplicons (306 bp) were visualized on 1.2% agarose gels stained with SYBR Green.

2.6. Statistical Analysis

Based on the confidence interval for a proportion of positive results and the sample size, 95% Cis was calculated. The lower bound and upper bound were determined using the Sample Size Calculator online tool for clinical research planning (https://sample-size.net; accessed on 15 August 2023).

The age of 40 years was selected according to the average value in the range of minimum and maximum value (19–59 years). There was no specific age difference between the two genders.

All analyses were performed employing the R software (version 4.0.5). When comparing differences between and among groups, we used Fisher's exact or Chi-squared tests where appropriate. The level of significance was set at a p value of <0.05.

3. Results

3.1. Baseline Characteristics

All relevant donor demographic data, including gender, age, self-reported ethnicity, and place of residence, were collected and analysed together with the serological and molecular results. Place of residence was used to categorize the communities to which the donors belong. Each donor was considered only once, i.e., only the first blood donation was included in the analysis. All blood donors were serologically tested negative for HIV, HCV, and HBV (HBsAg). None of them had a previous history of receiving blood transfusion. All donors were from the northern region and represented the urban population of Hanoi, predominantly of Kinh ethnicity (94%, n = 520). The median age of the cohort was 34 years with an interquartile range (IQR) of 19–59 years. The blood donor population comprised 451 males and 102 females. None of the female blood donors were pregnant at the time of blood donation. The other demographic parameters showed that 97% (n = 536) lived in urban areas and consumed either well or tap water.

3.2. HEV Seroprevalence

Serological tests revealed that 26.8% (148/553) were positive for anti-HEV IgG and 0.5% (3/553) were positive for anti-HEV IgM (Table 1).

Table 1. Hepatitis E virus IgG and IgM seroprevalences in investigated blood donors.

Biomarkers	*n* (%)	95% CI
anti HEV-IgG	148/553 (26.8)	23.1–30.6
anti HEV-IgM	3/553 (0.5)	0.1–1.5

Male and female donors had high anti-HEV IgG positivity (27.1%, 122/451, and 25.5%, 26/102, respectively), whereas the gender distribution was not statistically significant (p = 0.74) (Figure 1A). The age group 40–60 years had a high anti-HEV IgG seroprevalence compared to the age group 19–40 years, but the difference was not statistically significant (p = 0.23) (Figure 1B).

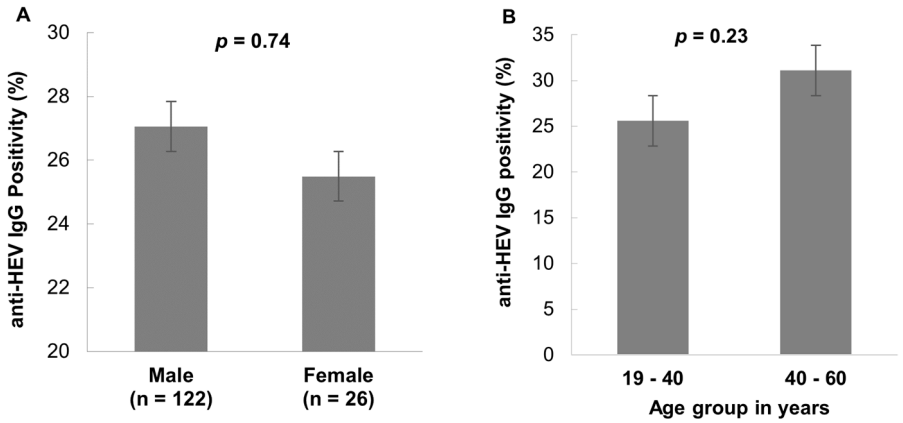

Figure 1. Distribution of seroprevalence of anti-HEV IgG and IgM based on sex (**A**) and age (**B**). Data are provided as percentages. No significant differences were observed between sex (p = 0.74) and age (p = 0.23).

Three anti-HEV IgM-positive samples were obtained from three male donors, aged 32, 39, and 42 years, respectively. Observing the age distribution, anti-HEV IgG was detected in individuals as young as 23 years old, while the oldest individual was 54 years old. The majority of anti-HEV IgG-positive cases were between 27 and 40 years old, and this fact is consistent with the characteristics of the general blood donor population (Figure 2).

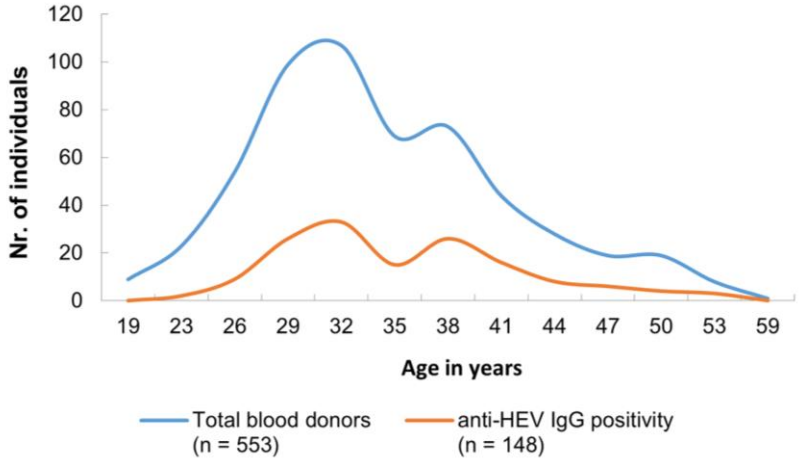

Figure 2. Age-stratified distribution of anti-HEV IgG positivity among all blood donors. The data are provided as absolute numbers. The median age of blood donors was 34 years with an age range of 19–59 years (min.–max.). The majority of anti-HEV IgG-positive cases were between 27 and 40 years old, and this result is a characteristic of the general blood donor population.

3.3. HEV Nucleic Acid Testing

Nucleic acid testing of conserved regions of the overlapping HEV ORF1 region of a 306 bp fragment revealed no HEV RNA positivity, indicating that there was no evidence of HEV viremia.

4. Discussion

Emerging viral infectious diseases are a constant threat in Southeast Asia, including Vietnam, and the safety of blood transfusions could be compromised as the region remains a hotspot for emerging infectious diseases, making epidemiological surveillance for transfusion-transmitted viruses indispensable. While HEV infection is asymptomatic in immunocompetent blood donors, HEV-infected blood transfusion products can exacerbate the clinical course of HEV infection in immunocompromised patients. In this study, we report about HEV exposure in the blood donor population. Although there was no evidence of HEV viremia, we found a high prevalence of HEV IgG seropositivity. HEV is increasingly recognized in Vietnam, and there are no practices of HEV screening, especially in one of the main hospitals where most blood donations are conducted. While European countries such as Germany, Spain, the Netherlands, Iceland, France, and the United Kingdom have introduced mandatory HEV screening of blood donors [2], HEV is not integrated in routine blood donor testing in Vietnam. In Vietnam, testing is only recommended for five transfusion-transmissible infectious agents, namely HIV, HBV, HCV, *Treponema pallidum*, and *Plasmodium* spp.

Previous studies from Vietnam reported an anti-HEV IgG seroprevalence in the general population of 31% [7], which is comparable to the anti-HEV IgG seroprevalence observed in this study (26.8%). A very low anti-HEV IgM seroprevalence (0.5%) was observed in this study compared to other studies in Vietnam in occupationally unexposed individuals (6%) [7] and in pregnant women in the third trimester (2%) [4]. When comparing HEV seroprevalence in individuals who have frequent contact with pigs, such as pork vendors, pig farmers, and slaughterhouses workers, significantly high seropositivity for anti-HEV IgG (53%) and anti-HEV IgM (11.3%) was found in a study in Northern Vietnam [7]. These exposed individuals are undoubtedly at an increased risk of zoonotic HEV transmission. Comparing HEV seroprevalence with European countries showed that the rate of seropositivity varies widely among different countries and regions. In the Netherlands, the seroprevalence for anti-HEV IgG was 27% using the same test systems as in our study [12]. Another study in South–West France found that a high proportion of blood donor were positive for anti-HEV IgG (53%) [17]. In contrast, low seroprevalence

of anti-HEV IgG was found in adults in Germany (15%) [18] and among blood donors in Italy (9%) [19]. Although the majority of seropositive cases in our study were male, no significant difference in seroprevalence rates was observed between the sexes (males 27.1% versus females 25.5%) (Figure 1A). In addition, there are no differences in seroprevalence across different age distributions in this study (Figure 1B). Given the geographic differences in seroprevalence in different study cohorts within Vietnam, our data provide valuable information about HEV exposure in a healthy adult and relatively young blood donor population. However, other studies on both white and Asian ethnic groups, and particularly in the male population, showed that hepatitis E risk increases significantly with age [20,21], and accordingly, men are much more likely to develop symptomatic hepatitis E [22].

The incubation period of HEV lasts 2–9 weeks (15–64 days) in the case of acute infection, and RNA can usually be detected in the blood 2–6 weeks after infection, while HEV-specific IgM antibodies appear in the bloodstream 3–4 weeks after infection and persist between four and six months [2]. Our results showed that <1% of blood donors were tested positive for anti-HEV IgM but did not have HEV viraemia, indicating recent exposure and that detection with molecular testing must have resulted in false negatives due to the diagnostic time window. Similar to what was observed in this study, HEV RNA viraemia and/or HEV RNA positivity was low in Europe (France: 0.045%; Denmark and Spain: 0.03%; and Germany: 0.08%) and in Asian countries (China: 0%) [12,14]. In addition, the HEV subgenotypes 3c, 3e, and 3f reported in these studies suggest a zoonotic transmission pattern among blood donors, as these genotypes have been frequently detected in domestic pigs and wild boar in Europe [23–25]. This pattern of HEV infection has been increasingly recognized in several European countries, and in Vietnam, where HEV genotype 3 is prevalent [7]. An epidemiological link has been established between hepatitis E cases and consumption of undercooked pork, clearly indicating a zoonotic transmission consistent with our HEV IgG seroprevalence results [7,16].

A limitation of the study is that the blood donors were representative of the healthy adult population living in urban areas in the northern region of Vietnam and were geographically distinct. There may be differences in local exposure risks in other rural areas and highland regions, but further studies are needed to investigate this observation in other communities. Another limitation of our study could be the sensitivity for our RT-PCRs, which are approximately 1×10^3 copies/mL (approx. 840 IU/mL). Thus, it cannot be ruled out that individuals with a lower HEV viral load might have been tested false negative although they were HEV IgM positive. However, a recent study by Lhomme et al. [26] showed that in asymptomatic blood donors, a median HEV load of 717 IU/mL was determined, and symptomatic individuals showed a median load of 2.82×10^5 IU/mL. Therefore, the PCR detection limit was acceptable for this study analysing apparently healthy individuals showing no hepatitis E-like symptoms. Overall, while the absence of HEV viraemia in blood donors from Northern Vietnam is encouraging, further surveillance studies will help unravel the burden of the disease in the sub-region.

5. Conclusions

The HEV IgG seroprevalence in the blood donor population studied in Northern Vietnam is high (26.8%), although the proportion of HEV IgM antibodies is low (<1%), and no HEV RNA was detected. Therefore, according to the results of the present study, the risk of transmission of HEV infection from Vietnamese blood donors to, for example, immunocompromised patients, is considered to be rather low. Nevertheless, from a public health policy perspective, HEV screening of blood samples, as regulated in few European countries, is recommended to minimize the risk of chronic hepatitis E associated with acute liver inflammation, especially in immunocompromised patients. Further epidemiological surveillance is warranted to confirm our data in other geographical areas of Vietnam to rule out transfusion-transmitted HEV.

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Institutional Review Board Statement: The study was approved by the ethics committee of 108 Military Central Hospital, Hanoi, Vietnam (108/RES/OBI-HEP-VGCARE-V-D1-20-09-2021). Informed written consent was obtained from all donors. All experiments were performed following GCP/GCLP guidelines.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: All data generated or analysed during this study are included in this article.

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Conflicts of Interest: The authors declare no conflict of interest.

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Publication No.3

High Seroprevalence of Hepatitis E Virus among patients with Hepatitis B and those with Hepatitis of Unknown Etiology in Central Vietnam

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MAJOR ARTICLE

High Seroprevalence of Hepatitis E Virus Among Patients With Hepatitis B and Those With Hepatitis of Unknown Etiology in Central Vietnam

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Background. Hepatitis E virus (HEV) is a significant cause of acute viral hepatitis, particularly in regions with poor sanitation. Co-infection with hepatitis B virus (HBV) is common in high-risk populations, such as individuals with chronic liver disease, but data on HEV prevalence in patients living with HBV in Southeast Asia are limited. This study investigates HEV seroprevalence among patients with HBV and those with hepatitis of unknown etiology (HUE) in central Vietnam

Method. Blood samples from 587 patients with HBV and 158 individuals with HUE were collected and analyzed for anti-HEV immunoglobulin G (IgG) and immunoglobulin M antibodies by enzyme-linked immunosorbent assay. HEV RNA was detected using reverse transcriptase-polymerase chain reaction, and HBV viral load was quantified via real-time polymerase chain reaction.

Results. The overall anti-HEV IgG seroprevalence was 26% in patients with HBV and 36% in those with HUE. Although patients with liver cirrhosis and hepatocellular carcinoma exhibited higher rates of anti-HEV IgG seropositivity, these differences were not statistically significant. Among patients with HBV, the seroprevalence was highest in those with liver cirrhosis and hepatocellular carcinoma (46%), whereas the lowest (24%) was found in patients with symptomatic chronic hepatitis B. Only 1 (0.1%) tested positive for anti-HEV immunoglobulin M, and no HEV RNA positivity was detected. Furthermore, biochemical laboratory parameters in patients with liver diseases were not found to be associated with anti-HEV seropositivity.

Conclusions. Anti-HEV IgG seroprevalence was relatively high among patients with chronic liver disease, including those with HBV. However, no association was observed between HEV exposure and liver disease progression. HEV is not a common cause of hepatitis in Central Vietnam.

Keywords. hepatitis b; hepatitis e; hepatitis of unknown etiology; liver disease; seroprevalence.

Hepatitis E virus (HEV) is a major cause of acute viral hepatitis, particularly in regions with poor sanitation and hygiene conditions, which are common in low- and middle-income countries, and in rural areas where pigs, a major reservoir of HEV, are often reared. Every year, an estimated 20.1 million people are infected with the HEV, of which around 3.4 million become symptomatic, resulting in >70,000 deaths and 3000 stillbirths annually [1]. Although HEV often resolves without medical intervention, it poses a significant risk to certain high-risk groups, including pregnant women, organ transplant recipients, people with chronic liver disease, and those with HIV [2, 3]. In addition, HEV infections can lead to severe extrahepatic complications such as neurological manifestations and glomerulonephritis [4].

HEV is a small RNA virus with a 7.2-kb genome, comprising 3 distinct open reading frames. The virus exists in 2 forms: quasi-enveloped particles that circulate in the bloodstream and nonenveloped virions excreted in feces [5]. The virus belongs to the family *Hepeviridae* and the subfamily *Orthohepevirinae*, which includes 4 genera. The genus *Paslahepevirus*, which is associated with human and zoonotic HEV, contains the species *Paslahepevirus balayani* with 8 distinct genotypes (HEV-1 to HEV-8) [6]. Genotypes 1 and 2 are human-specific pathogens, predominantly transmitted via the fecal-oral route, and are most common among regions with inadequate and/or poor sanitation. In contrast, genotypes 3, 4, and 7 are zoonotic, infecting both humans and animals and are also prevalent in high-income countries [7]. Genotypes 5, 6, and 8 have been identified exclusively in animals to date [3].

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In Southeast Asia, the burden of HEV has been investigated in several studies, with reported seroprevalence ranging from 2% among blood donors in Malaysia to 77.7% in lowland communities in Laos [8]. In particular, the co-infection and interactions between HEV, hepatitis B virus (HBV), and other chronic liver diseases have received increasing attention because of their potential to exacerbate liver pathology even in immunocompetent individuals [9, 10]. Given that SEA is also an endemic region for hepatitis B, with >61 million people chronically infected [11], continued research into HEV-HBV interactions remains essential. Vietnam, in particular, is recognized as a high endemicity region for HBV, with approximately 9% of the general population testing positive for hepatitis B surface antigen (HBsAg), making HBV a major contributor to liver cancer [12]. Additionally, prior exposure to HEV has been shown to influence the clinical course of chronic HBV infection, underscoring the important role of this HEV RNA virus in HBV treatment management and cryptogenic hepatitis [9, 13].

Vietnam is a well-recognized as a hotspot for zoonotic HEV infection [14, 15]. Epidemiological studies in the country have reported significant HEV exposure in the healthy population [9, 15], with prevalence ranging from 27% in the northern region to 31% in the southern region [16, 17]. In addition, Vietnam is among the leading countries in pig production and consumption [18], with pigs serving as a primary reservoir for HEV [15]. However, inadequate food safety measures and absence of effective control strategies may contribute to an increased risk of widespread HEV transmission [15]. Given these factors, there is a critical need for further serological surveillance of HEV, particularly among high-risk groups such as individuals with liver diseases, including those with HBV.

This study investigates the seroprevalence of HEV infection among individuals with HBV and those with hepatitis of unknown etiology (HUE) in Central Vietnam. The aim is to elucidate the burden of HEV in high-risk populations within this region and assess its potential impact on the clinical progression of HBV infection.

MATERIALS AND METHODS

Ethics Statement

The study was approved by the ethics committee of Hue University of Medicine and Pharmacy, Hue University, Vietnam (H2024/585). All experiments were performed following Good Clinical Laboratory Practice guidelines.

Study Design and Collection

Blood samples were collected from both inpatients and outpatients at the Hospital of Hue University of Medicine and Pharmacy, Central Vietnam, between June 2023 and June 2024. The blood samples were initially obtained as part of routine diagnostic procedures during standard medical care. With approval from the institutional ethics committee, a portion of each sample was later used for research purposes in this study. All patients provided written informed consent before participation. For patients who tested positive for HEV-RNA or HEV-immunoglobulin M (IgM), the results were forwarded to the responsible physicians to ensure appropriate clinical treatment. A total of 587 samples were obtained from individuals with HBV, confirmed by a positive HBsAg result, including those undergoing treatment for HBV. In addition, 158 blood samples were collected from patients with HUE (non-B and non-C hepatitis). Clinical manifestations and laboratory parameters were obtained from the medical records of all enrolled patients for comprehensive analysis.

HBV infection was classified according to World Health Organization guidelines. Acute hepatitis B (n = 3) was defined as HBsAg positivity for <6 months, with anti-HBc IgM positivity, elevated liver enzymes (typically more than 5 times the upper limit of normal), with no history of previous HBV infection. Chronic hepatitis B (CHB, n = 584) was characterized by HBsAg positivity for more than 6 months and anti-HBc immunoglobulin G (IgG) positivity [19]. To assess how HEV exposure influences the progression of HBV infection, patients with CHB were stratified into the following clinical categories: (1) symptomatic CHB (SYMP + CHB) included individuals with current or prior hepatitis symptoms and elevated liver enzyme levels, but without evidence of liver cirrhosis (LC) or hepatocellular carcinoma (HCC); (2) CHB with liver cirrhosis (LC + CHB) and (3) CHB with hepatocellular carcinoma (HCC + CHB) were defined by the presence of LC or HCC, respectively; (4) CHB with both LC and HCC (LC + HCC + CHB) included patients diagnosed with both complications; (5) asymptomatic CHB (ASYMP + CHB) comprised individuals with HBV infection lasting more than 6 months, but without clinical symptoms or elevated liver enzymes. Similarly, 158 patients with HUE were categorized into 4 subgroups: (1) symptomatic hepatitis without liver cirrhosis or hepatocellular carcinoma (SYMP + HUE); (2) HUE with liver cirrhosis (LC + HUE); (3) HUE with hepatocellular carcinoma (HCC + HUE); and (4) HUE with both liver cirrhosis and hepatocellular carcinoma (LC + HCC + HUE).

Cirrhotic patients were identified based on clinical symptoms, and biochemical abnormalities including hyperbilirubinemia, elevated aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels, prolonged prothrombin time, decreased serum albumin levels, and ultrasonographic evidence of chronic liver parenchymal damage. In addition, the diagnosis of HCC was based on clinical manifestations, imaging findings, and histopathological confirmation.

HEV Serological Assays

Serum samples were analyzed for anti-HEV IgG and anti-HEV IgM antibodies using enzyme-linked immunosorbent assay kits (WANTAI, Beijing, China). The anti-HEV IgG assay

(sensitivity: 99.08%; specificity: 99.9%) and the anti-HEV IgM assay (sensitivity: 97.1%; specificity: 98.4%) were previously validated in multicenter clinical studies as provided by the manufacturer (https://www.dbaitalia.it/newsletter/we–7296_hev). Testing was performed according to the manufacturer's protocol. Absorbance was measured using a CLARIOstar microplate reader (BMG Labtech, Rotenberg, Germany).

Nucleic Acid Isolation

Nucleic acid isolation from serum samples was performed using the QIAamp Viral RNA Mini Kit (Qiagen GmbH, Hilden, Germany) for HEV RNA extraction and the QIAamp Viral DNA Mini Kit (Qiagen GmbH) for HBV DNA extraction according to the manufacturer's instructions. A total of 140 µL of serum was used for each isolation and the nucleic acids were eluted in 80 µL of elution buffer. The extracted products were stored at −80°C until used.

Reverse Transcriptase-Polymerase Chain Reaction for HEV-RNA Detection

HEV RNA was reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific, Waltham, MA, USA). The presence of HEV RNA was detected using a nested polymerase chain reaction (PCR) assay targeting the conserved regions of HEV ORF1, as previously described by Hoan et al [15]. The outer primers were HEV-38 (5′-GAGGCYATGGTSGAGAARG-3′) and HEV-39 (5′-GCCATGTTCCAGACRGTRTTCC-3′), while the inner primers were HEV-37 (5′-GGTTCCGYGCTATTGARAA RG-3′) and HEV-27 (5′-TCRCCAGAGTGYTTCTTCC-3′). PCR amplification was performed in a 25-µL reaction containing 5 ng cDNA, PCR buffer, dNTPs, MgCl2, specific primers as indicated previously (Eurofins Genomics, Ebersberg, Germany), and Taq polymerase (Qiagen GmbH). Thermal cycling parameters for the outer and nested PCRs included initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation (94°C for 30 seconds), annealing (54°C for PCR outer or 56°C for PCR inner in 30 seconds), and extension (72°C for 30 seconds), with a final extension for 5 minutes at 72°C. A plasmid containing HEV cDNA was used as a positive control. Amplicons (306 bp) were visualized on 1.2% agarose gels stained with SYBR Green.

Quantitative Detection of HBV

Real-time PCR targeting a conserved 90-bp region of the HBV S gene (GenBank #X75657, position 182–271) was performed using the SensiFAST One-Step RT-PCR Kit (Meridian Biosciences, Memphis, TN, USA) on a LightCycler 480-II (Roche, Mannheim, Germany). Each 20-µL reaction contained 10 µL of 2× master mix, 0.8 µL of each 10 µM primer (Eurofins Genomics, Ebersberg, Germany) (forward: HBV-61 (5′-GGACCCCTGCTCGTGTTACA-3′); reverse: HBV-62 (5′-GAGAGAAGTCCACC ACGAGTCTAGA-3′), 0.3 µL of 10 µM probe HBV-TM-05 (FAM-5′-TGTTGACAARAATC CTCACAATACCRCAGA-3′-DabCyl) and 5 µL (15–20 ng) of DNA. Cycling conditions were 95°C for 5 minutes followed by 45 cycles of 95°C for 10 seconds and 60°C for 34 seconds. The detection limit of the assay was 25 IU/mL, calibrated with a 10^6 copies/µL plasmid standard and 10-fold dilutions. Cycle threshold values were used to calculate viral load, with a maximum cycle threshold value of 40 corresponding to 221 copies/mL.

Statistical Analysis

All analyses were performed using GraphPad Prism (version 9.5.1), with a P value <.05 considered statistically significant. Quantitative data were presented as median with ranges, and categorical data as counts and percentages. Categorical variables were analyzed using chi-squared or Fisher exact tests, whereas continuous variables were analyzed using t-tests or Kruskal-Wallis tests. Multivariate analyses adjusted for age and sex were performed using SPSS (version 20).

RESULTS

Demographic Data and Clinical Characteristics of Study Cohorts

The demographic characteristics of 587 patients with HBV and 158 patients with HUE are presented in Table 1. The median age of patients with HBV and patients with HUE were 50 (range, 5–97 years) and 55.5 (10–83 years) respectively. The male/female ratio of patients with HBV and patients with HUE were 331/256, and 107/51, respectively. Among the 587 patients with HBV, the majority were diagnosed with CHB (n = 584), whereas a small number had acute hepatitis B (n = 3). Of the 584 patients with CHB, most were symptomatic (SYMP + CHB; n = 426), followed by asymptomatic individuals (ASYMP + CHB; n = 88). Additional subgroups within the CHB cohort included those with liver cirrhosis (LC + CHB; n = 43), hepatocellular carcinoma (HCC + CHB; n = 14), and both liver cirrhosis and hepatocellular carcinoma (LC + HCC + CHB; n = 13) (Table 1). Among 158 patients with HUE, the majority had liver cirrhosis (LC + HUE; n = 79), followed by symptomatic hepatitis (SYMP + HUE; n = 58), hepatocellular carcinoma (HCC + HUE; n = 11), and both LC and HCC (LC + HCC + HUE; n = 10) (Table 2). Significant differences were observed in the HBV subgroups with regard to age, red blood cells, platelets, AST, ALT, prothrombin time, and alpha-fetoprotein (AFP). In the HUE subgroups, age, platelets, prothrombin time, and AFP differed significantly, whereas liver enzymes showed no statistical differences.

Anti-HEV IgG Seroprevalence and RNA Positivity in HBV and HUE

In this study population, anti-HEV IgG seroprevalence was high among both patients with HBV (26%, n = 153/587) and those with HUE (36%, n = 57/158) (Table 3). Among patients

Table 1. Demographic and Clinical Characteristics of Patients Living With Hepatitis B Infection

	Patients With Hepatitis B (n = 587)					
	SYMP + CHB (n = 426)	ASYMP + CHB (n = 88)	LC + CHB (n = 43)	HCC + CHB (n = 14)	LC + HCC + CHB (n = 13)	AHB (n = 3)
Age (y)*	50 [5–97]	44 [14–89]	56 [26–79]	70 [40–77]	58 [29–68]	34 [21–43]
Sex ratio (M/F)	232/194	42/46	30/13	12/2	12/1	3/0
WBC (10^3/μL)	6.6 [2.4–20.0]	7.0 [4.4–19.5]	6.3 [2.7–12.4]	6.8 [2.9–11.1]	7.5 [4.6–19.4]	7.8 [5.7–8.6]
RBC (10^6/μL)*	4.6 [2.8–7.2]	4.6 [2.9–6.9]	4.2 [2.6–5.3]	4.4 [3.0–5.5]	4.2 [3.3–4.6]	4.3 [3.9–4.5]
Hb (g/L)	140 [82–179]	139 [63–184]	130 [91–175]	144 [94–170]	129 [89–153]	133 [117–152]
PLT (10^3/μl)*	201 [43–597]	239 [129–546]	148 [39–415]	192 [91–508]	152 [103–278]	129 [64–188]
AST (IU/L)*	31 [14–737]	23 [12–53]	47 [18–392]	40 [24–72]	80 [29–660]	405 [344–1504]
ALT (IU/L)*	30 [6–513]	23 [7–56]	34 [14–517]	38 [10–88]	87 [21–741]	949 [716–2053]
Prothrombin time (s)*	12.6 (3.1–38.4)	12.2 [9.3–16.9]	12.9 [9.7–25.4]	13.7 [11.1–17.1]	14.0 [12.4–25.9]	15.9 [10.9–18.6]
AFP (ng/mL)*	3 [1–195]	2 [1–8]	10 [1–280]	17.2 [3–29136]	864 [3–14727]	n/a
HBV viral loads (copies/mL)	1431 [221–3.2×10^9]	2802 [221–3.1×10^8]	1142 [221–4.1×10^8]	4.7×10^4 [284–1.2×10^7]	1112 [221–6.2×10^6]	1.5×10^6 [4.1×10^5–2.8×10^6]

Values are presented as medians and ranges. HBV quantification limit: 221 copies/mL (Ct 40).

Abbreviations: AFP, alpha-feto protein; AHB, acute hepatitis B; AST and ALT, aspartate and alanine amino transferase; ASYMP, Asymptomatic; CHB, chronic hepatitis B; F, female; HCC, hepatocellular carcinoma; LC, liver cirrhosis; M, male; n/a, not applicable; PLT, platelets; RBC, red blood cells; SYMP, symptomatic; WBC, white blood cells.

*$P < .001$ for comparisons with all other groups using a 1-way analysis of variance test.

with HBV, anti-HEV IgG seropositivity was highest in those with both liver cirrhosis and hepatocellular carcinoma (46%, LC + HCC + CHB; n = 6/13) and those with hepatocellular carcinoma alone (43%, HCC + CHB; n = 6/14), followed by patients with liver cirrhosis (33%, LC + CHB; n = 14/43), asymptomatic CHB (28%, ASYMP + CHB; n = 25/88), and symptomatic CHB (24%, SYMP + CHB; n = 102/426) (Table 3). Among patients with hepatitis of unknown etiology, anti-HEV IgG seropositivity was highest in those with both liver cirrhosis and hepatocellular carcinoma (40%, LC + HCC + HUE; n = 4/10), followed by those with liver cirrhosis alone (39%, LC + HUE; n = 31/79), hepatocellular carcinoma alone (36%, HCC + HUE; n = 4/11), and symptomatic HUE (31%, SYMP + HUE; n = 18/58) (Table 3). No significant differences in anti-HEV IgG seropositivity were observed between the patients with HBV and HUE subgroups ($P = .271$), as age and sex were significantly associated with anti-HEV IgG seropositivity ($P < .001$) (Table 3). Seropositivity, which appeared to be higher in patients with liver cancer than in patients without liver cancer (38% vs 26%) and higher in patients with hepatocellular carcinoma than in patients without hepatocellular carcinoma (42% vs 27%), was also not significant after adjustment for age and gender ($P > .05$) (Figure 1). None of the patients with HUE tested positive for anti-HEV IgM, whereas 1 patient with LC + HBV was anti-HEV IgM and IgG positive. All 3 acute hepatitis B patients were negative for both anti-HEV IgG and IgM. No HEV viremia or HEV RNA was detected in the studied population.

Association of HEV Infection With Biochemical Parameters in HBV and HUE Patients

The relationship between anti-HEV IgG seropositivity and various biochemical parameters including AST, ALT, AFP, prothrombin time, platelet count, and HBV viral load was examined. Although minor differences in these clinical laboratory values were observed between individuals with and without anti-HEV IgG, none was statistically significant after adjusting for age and sex ($P > .05$), as shown in Figure 2. Significant differences were observed in AST levels and prothrombin time ($P < .05$) between patients with HBV and HUE on anti-HEV IgG positivity, whereas ALT, AFP, and platelet count showed no significant differences (Figure 3*A*). Significant differences were observed in AST, ALT levels, and prothrombin time ($P < .001$) between patients with HBV and HUE who were HEV seronegative, whereas AFP and platelet count showed no significant differences (Figure 3*B*).

DISCUSSION

HEV seroprevalence differs worldwide, reflecting diverse epidemiological patterns. A meta-analysis reported a 21% pooled anti-HEV IgG seroprevalence across Southeast Asia, with significant variation between countries. Myanmar had the highest (33.5%), followed by Vietnam (31.4%), whereas Malaysia had the lowest (5.9%) [20]. These differences are likely influenced by factors such as the pork consumption habits and the prevalence of pig farming in the region. Notably, both Vietnam and Myanmar are among the countries with high pork consumption, which is a key contributor to increased HEV exposure. In our study, IgG seropositivity ranged from 26% in patients with HBV to 36% in those with hepatitis of unknown etiology, consistent with previous reports from Northern and Southern Vietnam (27% among blood donors and 31.7% in hospital populations, respectively) [16, 17]. Northern Vietnam studies showed even higher seroprevalences (53% in occupationally

Table 2. Demographic and Clinical Characteristics of Patients With Hepatitis of Unknown Etiology (HUE)

	Hepatitis of Unknown Etiology (n = 158)			
	LC (n = 79)	SYMP (n = 58)	HCC (n = 11)	LC + HCC (n = 10)
Age (y)*	55 [33–83]	54 [10–81]	67 [55–80]	69 [37–82]
Sex ratio (M/F)	65/14	27/31	8/3	7/3
WBC (10^3/μL)	5.9 [1.7–16.1]	7.3 [2.8–17.0]	6.7 [3.7–9.2]	6.9 [4.6–16.6]
RBC (10^6/μL)*	4.0 [1.8–5.6]	4.4 [2.7–6.0]	4.6 [3.2–5.1]	4.4 [4.1–4.7]
Hb (g/L)	122 [47–195]	130 [90–160]	138 [98–161]	138 [121–150]
PLT (10^3/μL)*	128 [11–332]	234 [76–437]	252 [131–461]	136 [79–375]
AST (IU/L)	64 [11–940]	40 [14–1550]	33 [14–82]	76 [23345]
ALT (IU/L)	37 [9–1543]	38 [3–1579]	26 [6–54.3]	51 [17–584]
Prothrombin time (s)*	13.6 [10.3–41.2]	12.2 [10.1–21.2]	11.4 [9.8–17.3]	13.5 [10.7–17.4]
AFP (ng/mL)*	4.7 [2–325]	3 [1–97]	5 [2–2697]	103 [2–8849]

Values are presented as medians and ranges.

Abbreviations: AFP, alpha-feto protein; AST and ALT, aspartate and alanine amino transferase; F, female; HCC, hepatocellular carcinoma; LC, liver cirrhosis; M, male; PLT, platelets; RBC, red blood cells; SYMP, symptomatic; WBC, white blood cells.

*$P < .001$ for comparisons means of the independent groups using a 1-way analysis of variance test.

Table 3. Anti-HEV IgG Seropositivity in Patients With HBV and Hepatitis of Unknown Etiology (HUE) Stratifying to Clinical Stages, Age, and Sex

	CHB % (n = 584)	HUE % (n = 158)	*P* Value
Clinical stages			$P = .271$[a]
LC + HCC	46% (6/13)	40% (4/10)	
HCC	43% (6/14)	36% (4/11)	
LC	33% (14/43)	39% (31/79)	
SYMP	24% (102/426)	31% (18/58)	
ASYM	28% (25/88)	n/a	
Total	26% (153/584)	36% (57/158)	
Age group			$P < .001$
<25	3% (1/32)	13% (1/8)	
25–34	11% (9/79)	14% (1/7)	
35–44	30% (32/108)	29% (5/17)	
45–54	24% (31/129)	48% (20/42)	
55–64	37% (38/102)	49% (20/41)	
65–74	35% (34/96)	24% (8/33)	
≥75	35% (8/38)	20% (2/10)	
Gender			$P < .001$
Male	31% (103/328)	44% (47/107)	
Female	20% (50/256)	20% (10/51)	

The *P* value for comparing IgG seropositivity among groups was calculated using logistic regression.

Abbreviations: ASYMP, asymptomatic; CHB, chronic hepatitis B; HCC, hepatocellular carcinoma; LC, liver cirrhosis; SYMP, symptomatic.

[a]*P* value after adjusting age and sex.

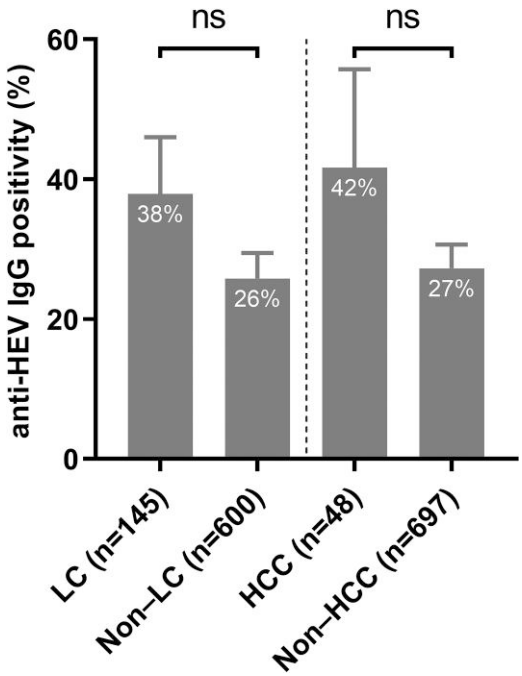

Figure 1. Anti-HEV IgG seroprevalence in patients with liver cirrhosis (LC) and hepatocellular carcinoma (HCC). *P* values were calculated using the chi-square test, adjusted for age and sex.

exposed, 45% liver disease patients) [9, 15], suggesting Vietnam remains highly endemic for HEV with significant regional variations. Although previous studies have reported higher HEV seropositivity among patients with chronic liver diseases compared to the general population [20, 21], our findings indicate no significant difference in seropositivity between patients with HBV, HUE, and the general population in Vietnam.

In our study, the low prevalence of anti-HEV IgM, with only 1 case detected among 745 patients, indicates a low occurrence. A similar trend was observed in Vietnam, where IgM seropositivity was only 0.5%, despite a high 42% IgG prevalence [22]. Other studies in Vietnam have reported higher IgM rates, ranging from 2% in pregnant women [23], 3% in acute hepatitis [24], to 11%–12% in individuals occupationally exposed to pigs and with liver disease [9, 15]. In Southeast Asia, Cambodia and Laos had low IgM prevalence (around 1%), whereas Thailand and Indonesia reported higher rates (7.3%–12.4%), with Vietnam at 4.72% [20]. The difference in

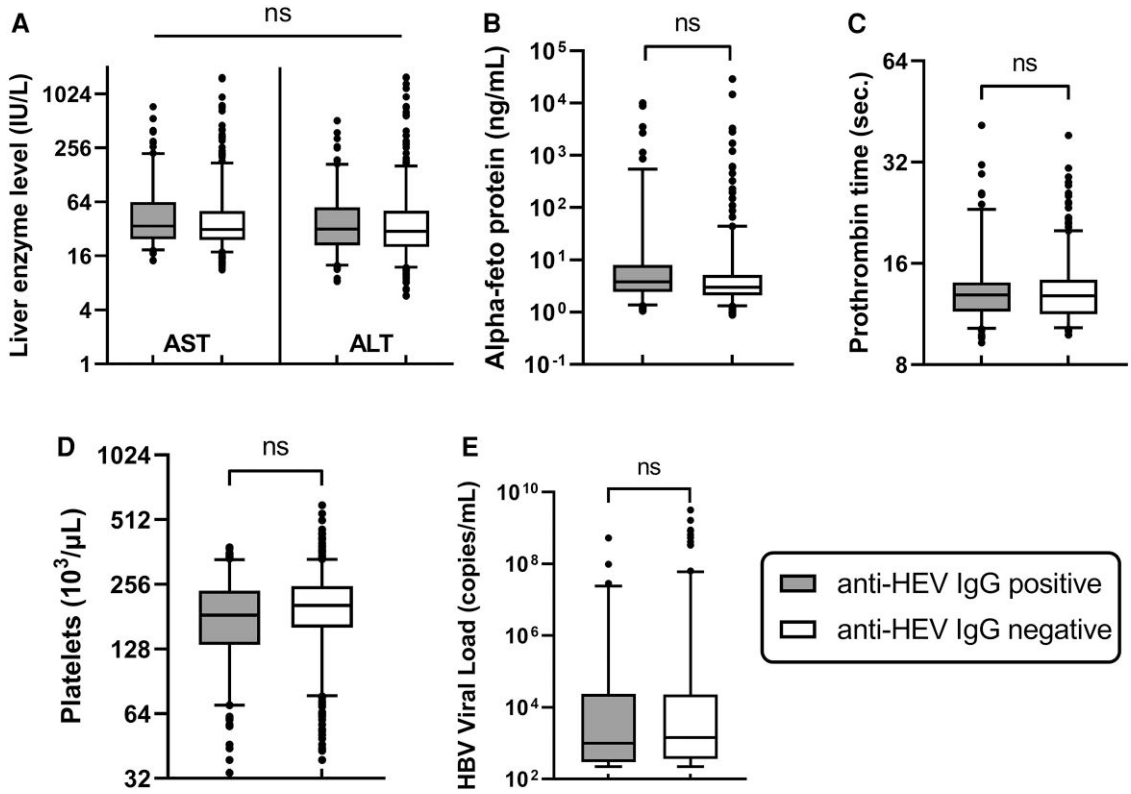

Figure 2. Comparison of biochemical parameters in anti-HEV IgG-positive and -negative patients. (*A*) Liver enzyme levels; (*B*) alpha-fetoprotein; (*C*) prothrombin time; (*D*) platelet count; (*E*) HBV viral load. ns, not significant. Box plots display medians with 25th and 75th percentiles and *P* values were calculated using multivariable logistic regression, adjusted for age and sex. HBV, hepatitis B virus; HEV, hepatitis E virus; IgG, immunoglobulin G.

seropositivity between IgG and IgM in our study suggests a high prevalence of previous HEV exposure with limited evidence of ongoing transmission. The case of a patient with CHB who tested positive for both IgM and IgG but had undetectable HEV RNA suggests that the patient is in the later stages of acute or resolving hepatitis E infection, with the absence of HEV RNA, indicating a lack of active viral replication. Furthermore, the absence of IgM positivity in the HUE group supports the notion that HEV is a rare cause of hepatitis in Central Vietnam.

The seroprevalence of HEV in patients with HBV has been widely documented. A study in the United States and Canada found an anti-HEV IgG seroprevalence of 28.5% among 600 adults with CHB, similar to our findings, with no HEV RNA positivity [25]. In Vietnam, a study on pregnant women with HBV reported 26% IgG seroprevalence, suggesting comparable exposure across high-risk populations [26]. Another study in Vietnam reported higher rates (45% IgG, 12% IgM) in people living with HBV, with even higher rates in those with cirrhosis (54% IgG, 19% IgM) [9]. These variations may be due to differences in assay sensitivity, HBV disease progression, and geographical distribution of HEV infection [27]. A Chinese study reported lower seroprevalence, (9.1% IgG, 0.1% IgM) in patients with CHB, reflecting geographical differences in HEV exposure [28]. HEV superinfection in patients with HBV has been associated with increased mortality, particularly in cirrhotic patients [29]. Although anti-HEV IgG seropositivity was highest (46%) in patients with both LC and HCC, no significant difference was observed between those with and without these conditions. The elevated anti-HEV IgG seroprevalence in the LC/HCC group may be attributed to the older age and male predominance in this group, as both older individuals and males are more likely to have been exposed to HEV.

The relationship between anti-HEV IgG seropositivity and platelet count in patients with HBV is clinically significant, as platelet count is a known prognostic marker for HEV-related acute liver failure [30]. A study by Hoan et al found lower platelet levels in patients with HBV with HEV seropositivity compared to those without HEV exposure [9]. Although we observed lower platelet counts in anti-HEV IgG-positive patients compared to IgG-negative patients, this difference was not statistically significant after adjustment for age and gender. These results suggest that platelet counts are more strongly influenced by age and gender than by HEV exposure itself. We also analyzed other biochemical parameters such as AST and ALT, AFP, and prothrombin time. No significant differences

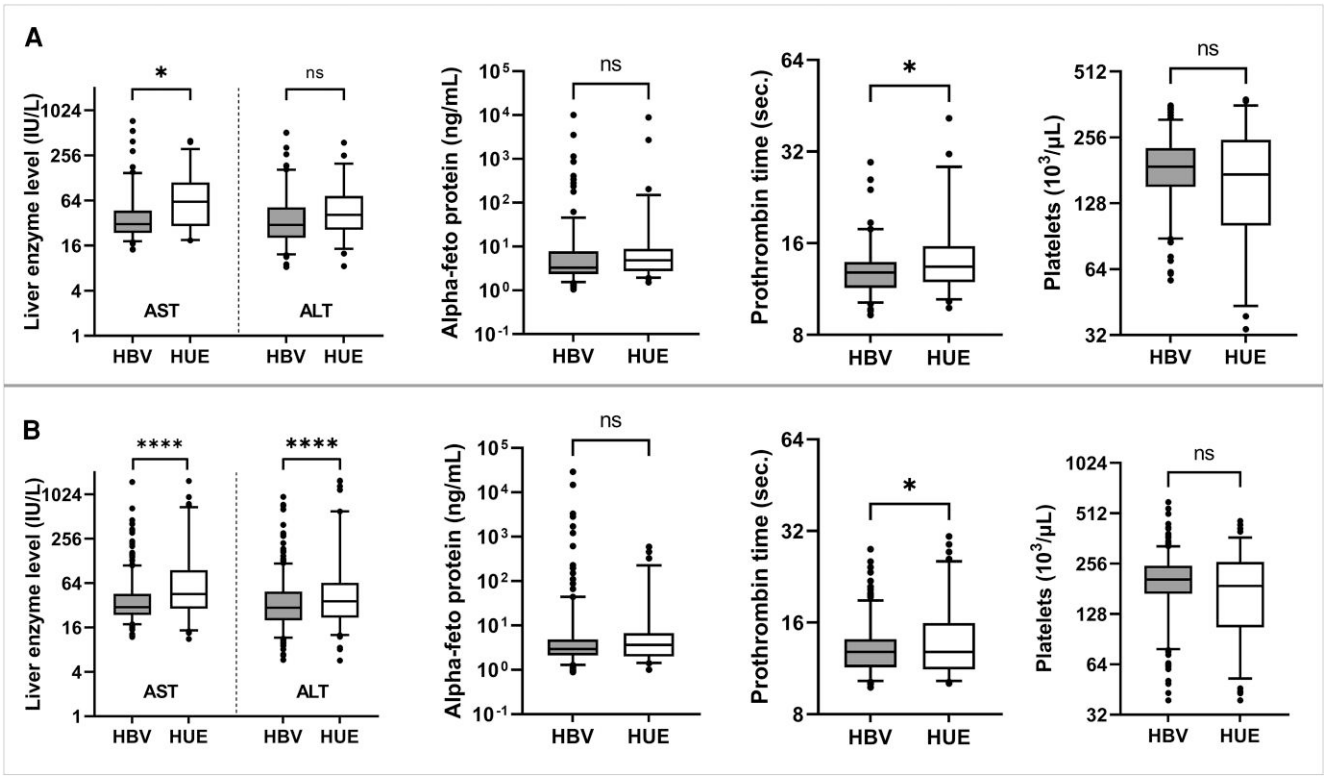

Figure 3. Biochemical parameters in patients with hepatitis B virus (HBV) and patients with hepatitis of unknown etiology (HUE). (*A*) Individuals positive for anti-HEV IgG positive; (*B*) individuals negative for anti-HEV IgG. ****$P < .001$. HEV, hepatitis E virus; IgG, immunoglobulin G.

were found between patients with anti-HEV IgG-positive and those without this antibody, which is in contrast to the results of studies in Vietnam and China [9, 31]. These discrepancies may be attributed to the high prevalence of current HEV infection in those studies and the high inclusion of patients with liver cirrhosis and hepatocellular carcinoma. We initially hypothesized that HEV exposure would influence biochemical parameters in patients with HBV. However, the comparable results observed between anti-HEV IgG-positive and -negative groups suggest that HEV exposure does not significantly impact the clinical parameters in patients with HBV. This finding indicates that, unlike other hepatitis co-infections, such as hepatitis D [32, 33], HEV may not exacerbate liver function abnormalities or disease progression in patients with HBV, underscoring the need for further studies to explore the interaction between these viruses in distinctive clinical contexts.

This study showed a high seroprevalence of anti-HEV IgG in patients with advanced liver disease, though it has several limitations. The small number of acute hepatitis B cases and the unequal size of the subgroups limit the evaluation of the role of HEV in different disease stages. In addition, the absence of anti-HEV IgG avidity results and lack of liver fibrosis data constrain the analysis of HEV exposure in relation to disease progression. In conclusion, although HEV exposure is common in patients with CHB and HUE with cirrhosis and/or HCC, disease progression appears to be more strongly related to age and gender than to HEV exposure. Furthermore, HEV does not appear to be a common cause of hepatitis in central Vietnam.

Notes

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Chapter 3: Distribution and species Diversity of *Entamoeba* and *Cryptosporidium* in domestic pigs and wild boars.

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Prevalence and Genetic Diversity of *Entamoeba* and *Cryptosporidium* in Pigs and Wild Boars in Central and Southern Vietnam: Implications for Zoonotic Risks and Surveillance

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Abstract

Background: Parasites of *Entamoeba* and *Cryptosporidium* genera, prevalent among various vertebrates such as humans and pigs, pose a zoonotic threat as common protozoan pathogens. This study investigated the prevalence and genetic diversity of *Entamoeba* and *Cryptosporidium* species in pigs and wild boars across central and southern Vietnam, to ascertain parasite transmission dynamics.

Methods: A total of 113 independent stool samples from 77 pigs and 36 wild boars were analyzed using PCR-based molecular methodologies to detect the presence of *Entamoeba* spp. and *Cryptosporidium* spp. The identified species were further characterized through Sanger sequencing, and phylogenetic relationships were analyzed.

Results: The study revealed a high prevalence of *Entamoeba* spp. (62%, $n = 70/113$) and *Cryptosporidium* spp. (31%, $n = 35/113$). *Entamoeba suis* (57%, $n = 40$) was predominant, followed by *Entamoeba polecki* (40%, $n = 40$) and *Entamoeba hartmanni* (3%, $n = 2$). Among *Cryptosporidium* species, *Cryptosporidium scrofarum* (89%, $n = 31$) was the most common, followed by *Cryptosporidium suis* (11%, $n = 4$). Wild boars exhibited a higher prevalence of *Entamoeba* infection compared with domestic pigs ($p = 0.019$).

Conclusions: The study highlights a high prevalence of *Entamoeba* and *Cryptosporidium,* suggesting a potential for zoonotic transmission in Vietnam. Further investigations are necessary to determine the extent to which these parasites in pigs and wild boars contribute to the burden in the human population.

Keywords: *Entamoeba*, *Cryptosporidium*, zoonotic transmission, wild boars, pigs, one health

Introduction

Zoonotic diseases have gained global attention, accounting for 60% of emerging infectious diseases (Jones et al., 2008). Viral and bacterial pathogens are more frequently studied, but zoonotic parasites are often neglected. Swine population are the reservoirs for several emerging zoonotic parasites that pose a potential threat to human health (VanderWaal and Deen, 2018). In addition, the close interaction between humans and pigs can result in zoonotic infections, as humans share a significant proportion of parasites with pig populations (Ledger and Mitchell, 2022). *Entamoeba* spp. and *Cryptosporidium*

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spp. are two widespread parasites that cause diarrhea in domestic and wild pigs, with several pathogenic species also occurring in humans (Wang et al., 2022a; Wang et al., 2022b). Vietnam, renowned for its significant pig production and consumption, stands as a potential hotspot for possible zoonotic parasite transmission.

The genus *Entamoeba* is an intestinal protist that includes several free-living and parasitic species from all classes of vertebrates and some invertebrates. *Entamoeba suis* and *Entamoeba polecki* have been identified as the most common causative agents of diarrhea in pigs (Wang et al., 2022b), while *Entamoeba histolytica* can cause amoebiasis and lead to severe disease in humans. Although experimental observations have shown *E. histolytica* infections in pigs, no cases have been reported in farmed animals (Girard-Misguich et al., 2011). Of note, *E. polecki* observed in pigs are occasionally found in humans, suggesting cross-infection of *Entamoeba* species between the human and animal reservoirs (Stensvold et al., 2018). In addition, a variety of *Entamoeba* species have been reported in humans (*Entamoeba bangladeshi*, *E. histolytica*, *Entamoeba dispar*, *Entamoeba coli*, *Entamoeba moshkovskii*, *Entamoeba hartmanni*, and *E. polecki*) (Dos Santos Zanetti et al., 2021), indicating the importance of studying these protozoa in nonhuman reservoirs to understand their transmission dynamics.

Cryptosporidium, on the other hand, belongs to the genus of Apicomplexan protozoa and is an opportunistic parasite that causes self-limiting diarrhea in both humans and a wide range of animals (Chen et al., 2023). *Cryptosporidium* is commonly found in the intestines of humans and animals and is transmitted by the fecal–oral route. Interestingly, considerable genetic variation in the genus of *Cryptosporidium* has been reported worldwide with 44 known species and more than 120 identified genotypes (Ryan et al., 2021). Pigs are recognized as a reservoir of *Cryptosporidium* with 13 different species/genotypes reported thus far. Of note, the majority of cases of cryptosporidiosis in pigs are caused by *Cryptosporidium scrofarum* (formerly *Cryptosporidium* pig genotype II) and *Cryptosporidium suis* (Wang et al., 2021), which occasionally infect humans, suggesting that these two species may be zoonotic and pose a potential risk to humans (Kvac et al., 2009; Moore et al., 2016).

Detection and monitoring of *Entamoeba* spp. and *Cryptosporidium* spp. in pigs have been carried out in several Asian countries as these parasites have an impact on human and animal health (Chen et al., 2023; Dos Santos Zanetti et al., 2021; Matsubayashi et al., 2015). In Vietnam, pig farming stands as a pivotal component of the livestock sector, and the increased interaction between humans and pigs amplifies the potential for zoonotic disease transmission (Coker et al., 2011). However, only limited research has delved into protozoan infections in pigs in this region, particularly on *Entamoeba* spp. Consequently, monitoring *Entamoeba* spp. in pigs is imperative not only for enhancing public health safety but also for elucidating their genetic diversity to understand genotypes/subtypes in circulation. Concurrently, there is mounting interest in understanding the distribution and genetic attributes of *Cryptosporidium* species, given the documented evidence of *Cryptosporidium* infections associated with pigs in this region (Iwashita et al., 2021). Additional surveillance studies are warranted to ascertain the presence of zoonotic species such as *Cryptosporidium hominis* or *Cryptosporidium parvum* in these animals, thus evaluating the potential risk of pathogen transmission.

This study aims to identify *Entamoeba* and *Cryptosporidium* parasites in pigs and wild boars in Vietnam, while also subjecting the specific species/genotypes to molecular analysis through sequencing of the small subunit ribosomal RNA (SSU rRNA) gene to elucidate their genetic diversity.

Materials and Methods

Ethical statement

The study was approved by the Ethics Committee of Hue University of Medicine and Pharmacy, Hue University, Vietnam (H2022/020) and the Animal Ethics Committee of the International University (IU)—Vietnam National University—Ho Chi Minh City (VNUHCM, August 2022).

Study design and sampling

Between April and June 2022, a total of 113 fecal samples were collected from 77 pigs and 36 wild boars from various farms and slaughterhouses in southern and central Vietnam. In central Vietnam, 88 samples (77 pigs and 11 wild boars) were collected from eight different farms and slaughterhouses. In southern Vietnam, 25 stool samples were collected from wild boars from two different farms. Fecal samples were collected from various cages in each farm and slaughterhouse to ensure that the samples originated from independent sources.

DNA isolation and PCR amplification

DNA isolation was performed using the QIAamp Fast DNA Stool Mini Kit (Qiagen GmbH, Hilden, Germany). The manufacturer's protocol was followed with minor modifications. For partial amplification of the SSU rRNA gene of *Entamoeba* species, conventional PCR with Taq DNA polymerase (Qiagen GmbH) at an annealing temperature of 64°C was performed. Forward and reverse primers that were utilized were sourced from a prior publication, resulting in a PCR product of 580 bp (Verweij et al., 2001). For amplification of the SSU rRNA gene of *Cryptosporidium* species, nested PCR was performed with Taq DNA polymerase (Qiagen GmbH) at an annealing temperature of 55°C. The outer and inner primer pair was described in the study by Xiao et al. (2001). The inner PCR product was 830 bp. Amplicons were visualized on 1.5% agarose gels stained with c Green. Positive samples underwent an additional round of PCR screening to ensure reproducibility.

Sanger sequencing

All samples positive for PCR, which produced clear amplicons, underwent Sanger sequencing. PCR products were purified using ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher Scientific, Carlsbad, CA, USA). Amplicons were sequenced using both forward and reverse primers using the BigDye Terminator V.1.1 Sequencing Kit (Applied Biosystems™, Foster City, CA, USA) and were subsequently purified on Sephadex G50 (Cytiva, Uppsala, Sweden). The product templates were sequenced with the Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems).

Phylogenetic analysis

The obtained SSU rRNA gene sequences were assembled using Lasergene SeqMan II software (Jin and Sun, 2018) and aligned using EditSeq software (DNASTAR, Madison, Wisconsin, USA). The sequences were analyzed together with the representative nucleotide sequences of *Entamoeba* and *Cryptosporidium* species and/or respective genotypes using MAFFT version 7 using the G-INS-i model (Katoh et al., 2019). All reference sequences were obtained using the GenBank database (www.ncbi.nlm.nih.gov/GenBank). Phylogenetic trees were reconstructed using MEGA version 11 (www.megasoftware.net) (Tamura et al., 2021), employing the Maximum Likelihood method and the general time reversible plus gamma distribution model. The statistical robustness and reliability of the branching order were confirmed *via* bootstrapping with 1000 replicateset al. The resulting phylogenetic tree was annotated and visualized using the online tool iTOL v6 (https://itol.embl.de/) (Letunic and Bork, 2019). The representative sequences of *Entamoeba* spp. and *Cryptosporidium* spp. obtained in this study have been deposited in the National Center for Biotechnology Information (NCBI) GenBank database and can be retrieved with accession numbers PP735745–PP735814 (*Entamoeba* spp., $n = 70$) and PP748483–PP748517 (*Cryptosporidium* spp., $n = 35$).

Data analysis

All analyses were conducted using GraphPad Prism (version 9.5.1). A p-value <0.05 was deemed statistically significant. When comparing differences between and among groups, Fisher's exact or chi-squared tests were used where appropriate.

Results

Demographic and sample characteristics

This study, conducted in central and southern Vietnam, involved the analysis of a total of 113 fecal samples. In central Vietnam, 88 samples were collected from various pig holdings, comprising adult pigs and wild boars sourced from five slaughterhouses ($n = 64$), one pig farm ($n = 13$), and two wild boar farms ($n = 11$). In southern Vietnam, 25 samples were collected from two wild boar farms, including wild boar piglets sampled between 24 and 160 days after birth (mean: 100 days ±54) and weighing between 4 and 35 kg (mean: 17.2 kg ±30.5).

DNA positivity of Entamoeba spp. and Cryptosporidium spp.

PCR analysis targeting the SSU rRNA gene indicated that 62% ($n = 70/113$) of the tested samples were positive for *Entamoeba* spp. Among these, 55% ($n = 42/77$) were from pigs, and 78% (28/36) were from wild boars. A statistically significant difference in the *Entamoeba* spp. prevalence was observed between pigs and wild boars ($p = 0.019$) (Table 1). Regarding *Cryptosporidium* spp., the prevalence was 31% ($n = 35/113$). The prevalence was marginally lower in wild boar at 29% ($n = 8/36$) compared with pigs at 35% ($n = 27/77$), with no statistical difference observed between the groups ($p = 0.5$) (Table 1). Coinfection with both parasites was detected in 27% of the samples ($n = 30/113$).

Species identification and phylogenetic analysis

Among the *Entamoeba*-positive samples, *E. suis* ($n = 40/70$) predominated with 57%, followed by *E. polecki* at 40% ($n = 28/70$) and *E. hartmanni* at 4% ($n = 2/70$). Subtyping of *E. polecki* isolates in this study revealed subtypes ST1 ($n = 14$, 50%) and ST3 ($n = 14$, 50%) (Table 1). In addition, two species of *Cryptosporidium* were detected, with *C. scrofarum* being the most prevalent at 89% ($n = 31/34$), followed by *C. suis* at 11% ($n = 4/35$) (Table 1). The distribution of all *Entamoeba* species was uniform across domestic pigs and wild boar, except for the two *E. hartmanni* samples, which were exclusively found in pig samples from central Vietnam (Fig. 1). This is also the first study reporting on *E. hartmanni* in the pig population. Sequences of the two *E. hartmanni* samples in this study (#PP735749 and #PP735767) share >99% homology with the human isolate (#KX618191). The *Entamoeba* spp. reliably clustered in a distinct branch with a bootstrap value exceeding 95 (Fig. 1). Similarly, the *Cryptosporidium* spp. clustered with *C. scrofarum* and *C. suis* branches with a bootstrap value exceeding 99. Of note, *C. suis* was found only in pigs from central Vietnam (Fig. 2), which are phylogenetically related to other pathogenic species (*C. parvum* and *C. hominis*), suggesting a potential risk of human infection from *C. suis*.

TABLE 1. DISTRIBUTION OF *ENTAMOEBA* SPP. AND *CRYPTOSPORIDIUM* SPP. IN DOMESTIC PIGS AND WILD BOARS

Positivity	Entamoeba *spp.* n (%) *[95% CI]*	Cryptosporidium *spp.* n (%) *[95% CI]*	*All detected* Entamoeba *and* Cryptosporidium *spp.*
Pigs (n = 77)	42 (55%) [43–65]	27 (35%) [24–46]	*E. suis* ($n = 21$; 50%) *E. polecki* ST1 ($n = 8$; 19%) *E. polecki* ST3 ($n = 11$; 26%) *E. hartmanni* ($n = 2$; 5%) *C. scrofarum* ($n = 23$; 85%) *C. suis* ($n = 4$; 15%)
Wild boar (n = 36)	28 (78%) [61–90]	08 (22%) [10–39]	*E. suis* ($n = 19$; 68%) *E. polecki* ST1 ($n = 6$; 21%) *E. polecki* ST3 ($n = 3$; 11%) *C. scrofarum* ($n = 8$; 100%)

CI, confidence interval.

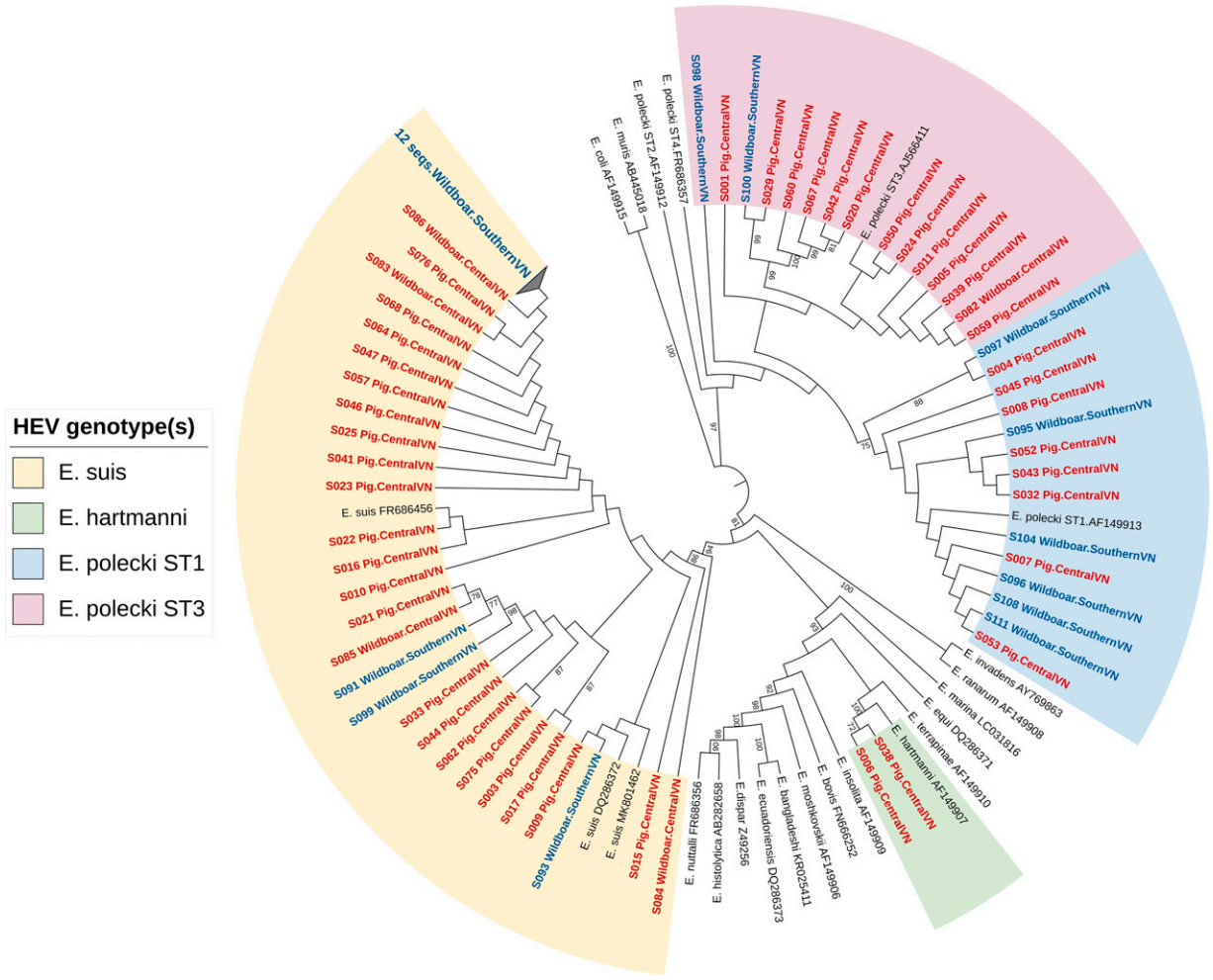

FIG. 1. Reconstructed phylogenetic tree using *Entamoeba* spp. SSU rRNA gene nucleotide sequences (n = 70, this study) along with 23 reference nucleotide sequences from 18 distinct *Entamoeba* species retrieved from GenBank. The sequences from this study are highlighted in red (central VN) and blue (southern VN). Only bootstrap values above 70 were shown. SSU rRNA, small subunit ribosomal RNA; VN, Vietnam.

Discussion

This study investigated the prevalence and genetic diversity of *Entamoeba* and *Cryptosporidium* species in pigs and wild boars across central and southern Vietnam, offering insights into potential zoonotic risks and parasite transmission dynamics. Notably, this study revealed significant differences in the prevalence of *Entamoeba* spp. between pigs and wild boars, alongside the exclusive detection of pathogenic *Cryptosporidium* species in pigs from central Vietnam.

The distribution of *Entamoeba* and *Cryptosporidium* in vertebrates varies geographically (Cui et al., 2019). However, the differences in prevalence may stem from varied detection methods. Microscopy, commonly employed, is less sensitive than molecular techniques, as it can misdiagnose parasite morphology due to stool sample artifacts (Stensvold and Nielsen, 2012). This study revealed a high prevalence of *Entamoeba* spp. at 62%, as observed in Wang's study in China, where 60% of 1254 pigs were infected (Wang et al., 2022b), with similar rates observed in Nepal at 61% (Adhikari et al., 2021). In our study, determining the age and sex of pigs is a challenge as different pigs live together in the same barn. However, the study samples from pigs were only taken from adult animals. The prevalence of porcine *Entamoeba* varies depending on the methodology utilized. For instance, microscopy-based studies reported lower prevalence in Korea (4%) (Ismail et al., 2010), Greece (8%) (Symeonidou et al., 2020), and Cambodia (27%) (Schar et al., 2014) compared with molecular screening studies in Indonesia (85%) (Wardhana et al., 2020) and Vietnam (92%) (Jacob et al., 2016), which indicated much higher prevalence rates. Numerous factors, including geographical location, climate, housing conditions, farming practices, feed and water quality, animal density, cohabitation with other livestock, and veterinary care, are likely contributors to such a varying prevalence of parasites in farmed pigs. *Entamoeba* spp. has been detected across a range of vertebrates, with prevalence rates varying from 0.8% in dogs to around 63% in nonhuman primates and 100% in long-tailed macaques (Cui et al., 2019). Despite its presence in a wide array of vertebrates, studies on the pathogenicity induced by the parasite in animals are limited or rarely documented. This study marks the first reporting of *Entamoeba* spp. prevalence in the wild boar population in Vietnam, revealing a rate of approximately 78%, notably higher than that documented in an Iranian study (52%) (Yaghoobi et al., 2016). While the prevalence of

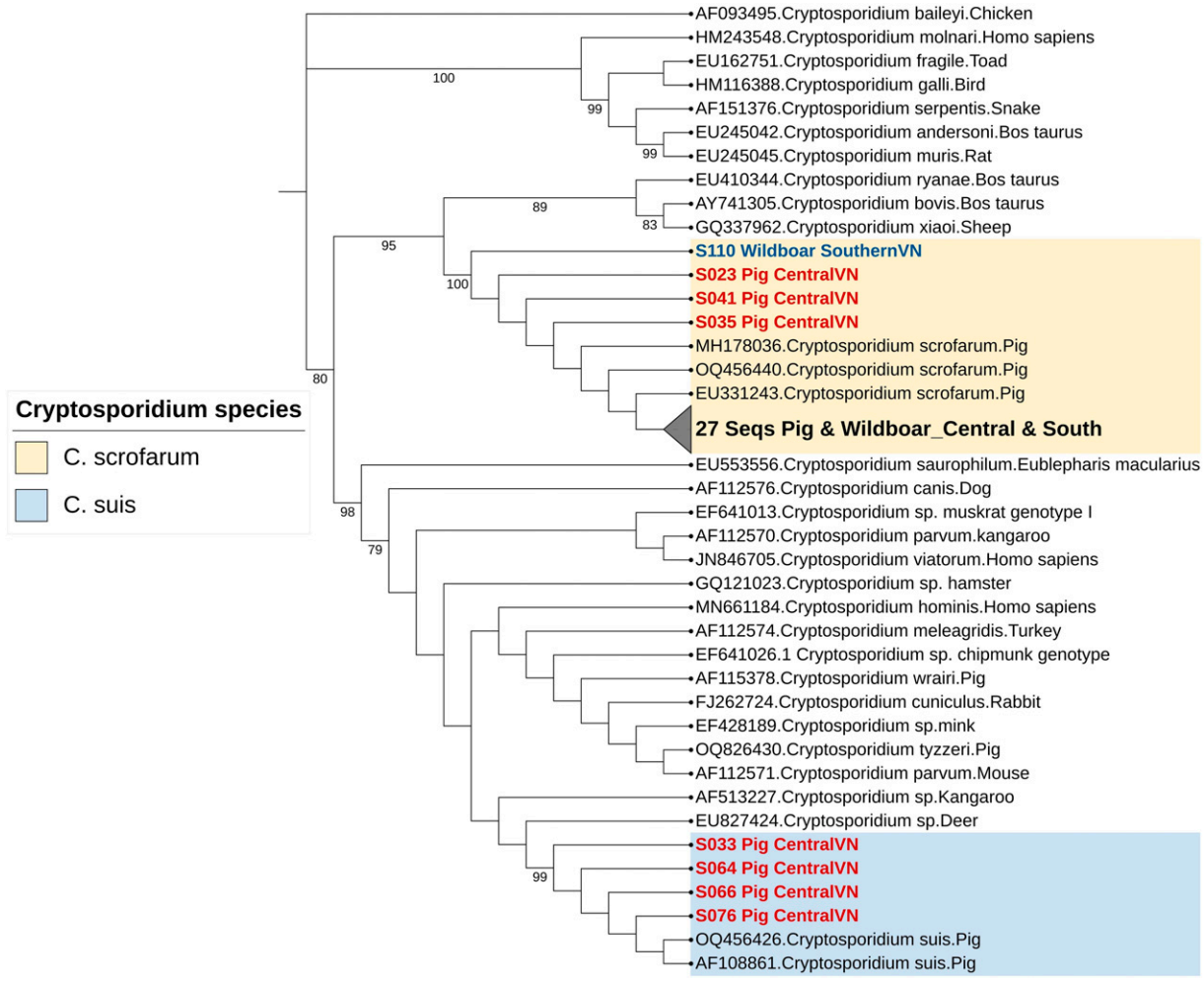

FIG. 2. Reconstructed phylogenetic tree using *Cryptosporidium* spp. SSU rRNA gene nucleotide sequences (n = 35, this study) along with 34 reference sequences from 28 distinct *Cryptosporidium* spp. retrieved from GenBank. The sequences from this study are highlighted in red (central VN) and blue (southern VN). Only bootstrap values above 70 were shown.

Entamoeba in wild boar surpasses that in domestic pigs (78% compared with 55%), it is important to note the limitation of our study, which had a small sample size of 36 wild boars.

In this study, the presence of three *Entamoeba* species was identified as follows: *E. suis*, *E. polecki*, and *E. hartmanni*. Domestic pigs are the primary hosts for *E. suis* and *E. polecki* (ST1, ST3) worldwide (Cui et al., 2019), although the distribution of these species and subtypes varies. For instance, Wang et al. showed that *E. polecki* (82%) was the predominant species, followed by *E. suis* (18%) (Wang et al., 2022b). Conversely, our findings show that the majority of *Entamoeba*-positive samples consisted of *E. suis* (57%) and *E. polecki* (40%). *E. polecki* exhibits zoonotic potential and has been detected in several hosts, including humans and pigs. There are four subtypes of *E. polecki* (ST1–ST4), all of which have been observed in humans. ST1 and ST3 have been identified in pigs, while ST2 has solely been detected in nonhuman primates. Notably, *E. suis* has not yet been detected in humans (Ji et al., 2019). Interestingly, two *E. hartmanni* detected from two distinct slaughterhouses in central Vietnam displayed significant sequence homology with human isolates (>99%), suggesting a zoonotic potential in this species. While *E. hartmanni* is widely distributed among nonhuman primates and humans [21], this study marks the first detection of this species within the pig population. Although *E. hartmanni* is generally considered nonpathogenic in humans, a recent study from Indonesia reported a significantly higher prevalence of *E. hartmanni* in loose stools (69%) compared with its prevalence in healthy schoolchildren (31%) aged 7–15 years (Matsumura et al., 2019). This finding suggests a potential role for *E. hartmanni* in mild diarrhea among children. However, further research is needed to confirm its pathogenicity, as the study by Matsumura et al. did not account for the presence of true pathogens.

Cryptosporidium infections are widespread in pigs, but data on wild boar are limited (Nemejc et al., 2013). The prevalence of *Cryptosporidium* in domestic pigs varies globally, with rates ranging from 0.4% in Germany to 80% in South Africa (Chen et al., 2023). This study found moderate prevalence in both domestic pigs (35%) and wild boar (29%), surpassing previous findings in central Vietnam (14%) (Nguyen et al., 2013) and China (4.5%) (Wang et al., 2022a). Globally, *Cryptosporidium* pig infection rates are estimated at 16.3%, with Asia and Africa being a hotspot (Chen et al., 2023). Surveillance studies conducted in Vietnam have revealed varied

infection rates, with reports of both *C. suis* and *C. scrofarum* species (Iwashita et al., 2021; Nguyen et al., 2013). The rates observed align with the findings of this study. Although no *C. suis* was observed in the wild boar population in this study, they remain susceptible hosts (Nemejc et al., 2013). Although numerous *Cryptosporidium* species have been identified in pig populations, over 90% of cases in pigs are attributed to *C. scrofarum* and *C. suis*, indicating pigs as the main hosts (Chen et al., 2023). *C. scrofarum* has been identified as a causative pathogen in patients presenting with diarrhea, including those who are HIV positive (Xiao et al., 2002).

Conclusion

Although our study has limitations regarding sample characteristics, limited sample size, and the need for further confirmation of pathogenicity among animals, the prevalence of *Entamoeba* and *Cryptosporidium* species suggests a potential for zoonotic transmission, underscoring the need for additional epidemiological surveillance efforts. In summary, the study highlights a significant prevalence of *Entamoeba* and *Cryptosporidium* infections in domestic pigs and farmed wild boars in central and southern Vietnam. The diverse species of *Entamoeba* identified, including the first detection of *E. hartmanni* in domestic pigs in Vietnam, underscore the importance of surveillance in animal populations. Further investigations in the human population of these regions are essential to assess whether the parasites found in pigs and wild boars contribute to the human disease burden.

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Authors' Contributions

T.P.V. designed and supervised the study and contributed to the study materials for all experimental investigations. L.C.C., D.M., T.T.G., V.M.T., N.T.M.C., T.N.P.A., L.N.N.H., N.T.T.H., T.N.M., and L.H.S. participated in the study design. L.C.C., T.T.G., V.M.T., and L.N.N.H. collected samples. L.C.C., D.M., N.H., and A.M. performed the experimental procedures. L.C.C. performed statistical and phylogenetic analysis. L.C.C. wrote the first draft, and T.P.V. revised the first draft. All authors have read and approved the article.

Data Availability Statement

All data generated or analyzed during this study are included in this article. A total of 105 successfully sequenced samples were submitted to the NCBI GenBank database, with accession numbers for *Entamoeba* ranging from PP735745 to PP735814 ($n = 70$) and for *Cryptosporidium* ranging from PP748483 to PP748517 ($n = 35$).

Disclosure Statement

The authors declare no conflicts of interest. The funder has no role in the study design, data collection and analysis, decision to publish, or preparation of the article.

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3. DISCUSSION

Vietnam remains a hotspot for zoonotic diseases, highlighting the importance of routine surveillance to prevent infections from escalating into outbreaks. In response, the country has launched the Vietnam Initiative on Zoonotic Infections (VIZIONS), which takes an integrated approach to monitoring pathogens circulating in both human and animal populations (Rabaa et al., 2015). The program primarily targets five major pathogens: avian influenza, rabies, *Streptococcus suis*, anthrax and leptospirosis as they are frequently reported in Vietnam (Pham-Thanh et al., 2022). However, given the high human exposure to HEV, the HEV surveillance in animal reservoirs should also be prioritized and integrated into the national surveillance framework. In addition, protozoal infections such as amoebiasis and cryptosporidiosis warrant further investigation to assess their potential impact on livestock production and to identify species that may pose a zoonotic risk.

3.1 Zoonotic HEV

3.1.1 HEV-RNA positivity in domestic pigs and wild boars

This study demonstrated a high overall rate of HEV RNA positivity (10%) among the pigs, which is consistent with findings from neighbouring countries such as Laos (11.6%) (Conlan et al., 2011), the Philippines (7.4%) (Liu et al., 2015) and exceeding the rates reported in Thailand (3%) (Hinjoy et al., 2013). Previous Vietnamese studies reported HEV RNA positivity rate ranging from 19% in southern Vietnam (Berto et al., 2018) and 12% in the north (Hoan et al., 2019) to 6.8% in a nationwide study (Lee et al., 2020), indicating regional variations. This first chapter of the thesis demonstrated the presence of HEV in wild boar population in Vietnam, and a substantial HEV RNA positivity of 25% in wild boars, indicating their potential role as an important HEV reservoir in Vietnam. In comparison, reported HEV RNA positivity rates in wild boar in China are lower with 0.8% in free-ranging wild boar and 2.5% in farmed wild boar (Wu et al., 2022). In contrast, several European countries have reported much higher prevalence rates, including 68.2% in Germany (Adlhoch et al., 2009), 43.6% in Italy (Beikpour et al., 2023) and 23.2% in Spain (Rivero-Juarez et al., 2018). These regional variations in prevalence may be due to differences in

management practices and the dynamics of HEV circulation within wild boar populations. In Vietnam, wild boar is typically kept in centralized enclosures and fed collectively, which may favour the spread of HEV and thus increase the overall positive rate. Furthermore, the higher HEV RNA positivity in farmed compared to undomesticated wild boar in China suggests that environmental and farming factors such as crowding and hygiene may contribute to high HEV transmission (Wu et al., 2022). In Europe, wild boar are usually hunted, providing more occasions for surveillance and detection of HEV in these animals (Fanelli et al., 2021).

The findings also revealed higher HEV RNA detection in rectal swabs and faecal samples compared to liver tissues, consistent with previous studies from Italy, where faecal samples were more sensitive for HEV detection (Carella et al., 2023). HEV RNA is also found in bile samples, with a prevalence of 17% (Fanelli et al., 2021). Lower detection rates in liver tissue may reflect viral replication and shedding dynamics, although HEV replicates in hepatocytes, it is shed in large quantities through bile into the intestine, leading to higher viral loads in stool and increased likelihood of detection in rectal swabs or faeces (P. Li et al., 2022; Marion et al., 2020).

3.1.2 HEV Genotypic characterization

Nested PCR targeting both the ORF1 and ORF2 regions was used for the detection and genotypic characterization of HEV. ORF1 showed a high sensitivity for HEV detection, while the ORF2 region enabled better subgenotype characterization. The study results revealed HEV subgenotype 3a as most prevalent, followed by HEV-4b and HEV-3f, consistent with previous studies in Vietnam, that reported the widespread of HEV-3 and HEV-4 in domestic pigs (Berto et al., 2018; Hoan et al., 2019; Lee et al., 2020). At subgenotype level, the genotype 3 show high variability with subgenotype 3a, 3d was observed in study of in Southern Vietnam (Berto et al., 2018), while Hoan et al. reported the occurrence of HEV-3a and HEV-3b in the northern region. This was the first study to report the occurrence of HEV-3f in domestic pigs in Vietnam, suggesting a possible introduction of this subgenotype through cross-border pig trade with neighbouring countries such as Thailand and Cambodia.

Globally, HEV-3 subgenotype distribution varies: 3e predominates in Europe, followed by 3f and 3c, whereas 3a, 3b, and 3f are more common in Asia (Nicot et al., 2020). For genotype 4, subgenotype 4a and 4c dominate in China and Japan, while 4b is most frequent in France (B. Li et al., 2022). In Vietnam, subgenotype 4a, 4b, 4c, and 4h have been detected in domestic pigs, with 4b being most common. The detection of 4b in wild boar suggests possible cross-species transmission from domestic pigs, reinforcing the hypothesis of inter-species HEV spread among different animal hosts. Increasing reports of HEV-4 in humans further highlight its emerging public health significance (Koizumi et al., 2004; Sridhar et al., 2019; Yamada et al., 2015).

Given the high prevalence of HEV in domestic and wild pigs in Vietnam, preventive measures should be implemented for individuals in close contact with these animals. The genetic similarity between animal and human HEV strains supports zoonotic and foodborne transmission as key dynamics in Vietnam (Hoan et al., 2015; Huy et al., 2021), particularly through consumption of raw or undercooked pig or wild boar liver and meat, the common dietary practices contributing to HEV infection.

3.2 Seroprevalence and HEV RNA positivity in healthy blood donors

Systematic screening for HEV is absent even in major hospitals in Vietnam, where most blood donations are collected. In contrast, several European countries, including Germany, Spain, the Netherlands, Iceland, France and the United Kingdom, have established mandatory HEV screening for blood donors (Velavan et al., 2021). In Vietnam, routine blood donor screening is limited to five transfusion-transmissible infections: HIV, HBV, HCV, *Treponema pallidum* and *Plasmodium* species. This study assessed the HEV exposure to blood donors and evaluated the potential risk of transfusion-transmitted HEV. While no cases of acute HEV infection (viremia) were detected in our study, 27% of blood donors tested positive for anti-HEV IgG. This finding aligns with Hoan et al., who reported a 31% seroprevalence also in blood donors (Hoan et al., 2019). However, our study found lower anti-HEV IgM seroprevalence (0.5%) compared to 6% in his study and 2% among pregnant women (2%), suggesting lower recent infection in blood donors (Hoan et al., 2019; Huy et al., 2021).

In Asia, Vietnam and Myanmar report the highest HEV seroprevalence (>30%), while Malaysia reports the lowest (6%), differences likely driven by dietary practices such as pork consumption (Mirzaev et al., 2024). Europe also shows regional disparities, with anti-HEV IgG prevalence in blood donors ranging from 27% in the Netherlands (Boland et al., 2019), to 53% in south-western France (Mansuy et al., 2011), and lower rates was found in Italy (9%) (Spada et al., 2018) and Germany (15%) (Faber et al., 2018). Although the use of different ELISA tests may contribute to global variability, the comparative evaluations suggest minimal differences in IgG detection sensitivity (Avellon et al., 2015).

Despite no HEV RNA-positive donors detected, the risk of transfusion-transmitted HEV cannot be excluded, as more than one million blood units are collected annually in Vietnam. European studies report low HEV RNA positivity among donors: France (0.045%), Denmark and Spain (0.03%), and Germany (0.08%) (Boland et al., 2019). Chinese studies did not find a single HEV RNA-positive case among 1,800 donors screened (Fu et al., 2021). This study also identified three cases of anti-HEV IgM positivity (<1%) without concurrent viremia, suggesting recent infection, but no risk of transmission to recipients. The absence of detectable HEV RNA could be due to either a very low viral load (below the detection limit of the assay) or a resolved infection (Velavan et al., 2021). In addition, we observed no significant seroprevalence differences by sex or age, contrasting with studies reporting higher seropositivity in older males (Feng et al., 2018; Mah et al., 2023).

3.3 HEV in patients infected with HBV and hepatitis of unknown etiology

While most HEV infections are asymptomatic or self-limiting, co-infection with HEV can aggravate liver disease in patients with chronic HBV or cirrhosis (Choi et al., 2022; Nasir & Wu, 2020). However, data on the impact of prior HEV exposure on progression of chronic HBV remain limited. Given that Vietnam is an HBV endemic country with high HEV exposure rates, investigating possible interactions between these infections is essential for informing treatment options. In this study, anti-HEV IgG seropositivity was detected in 26% of patients with chronic HBV and 36% of patients with hepatitis of unknown etiology, consistent with previous study on HEV seroprevalence in the general population (27-32%)

(Berto et al., 2018). However, these seroprevalence rates were lower than the 45% reported among HBV patients (Hoan et al., 2015) and the 53% observed among individuals with occupational pig exposure (Hoan et al., 2019) in northern Vietnam. Such discrepancies likely reflect regional differences and further confirm the endemic nature of HEV exposure in Vietnam.

Globally, the anti-HEV IgG seroprevalence among individuals with chronic HBV varies considerably, ranging from 9-38% in China to 28.5% in the USA (McGivern et al., 2019; Zhang et al., 2018). In this study, no significant difference in HEV seroprevalence was observed between patients with chronic HBV infection and the general population. Among Vietnamese HBV-infected pregnant women, anti-HEV IgG HEV seroprevalence was 26%, comparable to 22% observed in healthy pregnant women (Thi Hong Van et al., 2025). In contrast, Hoan et al. reported a higher seroprevalence of 45% in HBV patients compared to 31% in healthy controls, with particularly elevated rates of 52% in patients with cirrhosis (Hoan et al., 2015). We also found 46% seroprevalence in patients with cirrhosis or HCC. However, after adjusting for age and sex, this association was not significant, suggesting that older age, rather than HEV exposure, was the primary factor associated with poorer outcomes.

Only one case of recent infection (0.1% anti-HEV IgM positive) was detected in our study, a rate substantially lower than that reported by Hoan et al (11.6%) (Hoan et al., 2015), and among pregnant women (~2%) (Huy et al., 2021; Thi Hong Van et al., 2025). In Southeast Asia, similarly low rates of infection have been documented in Laos and Cambodia (~1%), whereas higher rates were observed in Thailand and Indonesia (7–12%) (Mirzaev et al., 2024). The one person in our study who tested positive for recent infection (anti-HEV IgM) also had signs of past infection (anti-HEV IgG) but no active viraemia in their blood, indicating a recovery. Furthermore, the absence of anti-HEV IgM or HEV RNA positivity among patients with hepatitis of unknown etiology suggest that HEV is not a common cause of hepatitis in Vietnam.

Previous studies have suggested that HEV infection may suppress HBV replication. For example, Kilonzo (Kilonzo et al., 2019) reported lower HBV viral loads among patients with HEV exposure compared to HBV monoinfection, and similar results were found by Hoan et

al. (Hoan et al., 2015). However, no significant difference in HBV replication was found between individuals with and without HEV antibodies in our investigations. This discrepancy may be due to the use of antiviral therapy in our cohort, which was not evaluated.

3.4 Enteric protozoa *Entamoeba* and *Cryptosporidium* in pigs and wild boars.

Pigs are the one of the most important livestock in Vietnam and are associated with several zoonotic diseases (Lin et al., 2022). This study aimed to investigate the prevalence and genetic diversity of the two protozoan parasites (*Entamoeba* and *Cryptosporidium)* in pigs and wild boar in Vietnam, as data remains limited. Globally, the distribution of these parasite species varies. *Entamoeba* is found in 63% of non-human primates (Cui et al., 2019), while *Cryptosporidium* is less common in livestock at around 19 % (Hatam-Nahavandi et al., 2019).

In this study, we found a high prevalence of *Entamoeba* spp. in both domestic pigs (55%) and wild boars (78%). Similar rates have been reported in pigs in China (58%) and Nepal (61%) (Adhikari et al., 2021; Wang et al., 2022), while lower rates were documented in Korea (4%), Greece (8%) and Cambodia (32%) (Ismail et al., 2010; Schar et al., 2014; Symeonidou et al., 2020). The variability of prevalence is attributed to the different techniques employed, as microscopy-based methods are less sensitive than molecular methods. High *Entamoeba* infection have also been documented in other livestock such as camels, yak, cattle, sheep and goat with up to 100% in some studies (Ai et al., 2021). *Entamoeba* can also infect dogs, but with a lower prevalence (0.8%) (Cui et al., 2019).

Although the presence of zoonotic *Entamoeba* has been documented in numerous studies, there are only a few investigations into its pathogenicity in hosts. Determining the age, sex and clinical signs of the pigs was a challenge in our study as the faecal samples were collected from farms where pigs were collectively raised. However this first study identified the prevalence of *Entamoeba* in wild boar in Vietnam (78%), which is higher than previous study in other regions such as Iran (52%) (Yaghoobi et al., 2016). Although *Entamoeba* is common in animals, human infection rates remain low (3-4%) (Cui et al., 2019), suggesting that humans are not fitting hosts for many of circulating *Entamoeba* species in livestock.

This study identified three different *Entamoeba* species: *E. suis* (55%), *E. polecki* (42%, including subtypes ST1 and ST3), and *E. hartmanni* (3%). *E. suis* and *E. polecki* are widespread in domestic pigs, but their prevalence and subtype distribution vary by region. For instance, *E. suis* predominated in Indonesia (80%), followed by *E. polecki* ST1 (67%) (Chrismanto et al., 2023), while *E. polecki* ST1 and ST3 were widely distributed in China (>82%), followed by *E. suis* (18%) (Wang et al., 2022). In Japan, only *E. polecki* ST3 was detected, and no cases of *E. suis* were documented (Ito et al., 2020). Notably, four subtypes of *E. polecki* (ST1-4) have also been detected in humans (Stensvold et al., 2011), indicating potential for zoonotic transmission. While *E. polecki* plays a certain role in the invasion of amebiasis in pigs (Matsubayashi et al., 2015), there is no evidence of its pathogenicity in humans. Also, no cases of *E. suis* infection in humans have been reported to date. *E. hartmanni* is commonly found in non-human primates and humans, but this is the first report of its presence in pigs. Two *E. hartmanni* isolates in this study showed high similarity to human sequences (>99%), suggesting potential zoonotic transmission. Higher prevalence of *E. hartmanni* in Indonesian children with loose stools (69%) compared to healthy children (31%) indicates a possible role for this species in causing mild diarrhoea (Matsumura et al., 2019).

Cryptosporidium spp. can cause diarrhoea and other gastrointestinal disorders in pigs (Wang et al., 2022). Although the prevalence and genetic diversity of this parasite has been widely studied in pigs, limited information is available on its occurrence in wild boars (Nemejc et al., 2013). Globally, infection rates of *Cryptosporidium* in pigs vary by region: 15% in Asia, 18% in Europe, 41% in Africa, 14% in North America, and 7% in South America. Notably, the highest prevalence has been reported in South Africa (80%), while the lowest prevalence was reported in Germany (0.4%) (Chen et al., 2023). In our study, *Cryptosporidium* was detected in 35% of domestic pigs and 22% of wild boars, rates are comparable to previous findings in Vietnam (18%) (Nguyen et al., 2012). Also, our results indicated an association between infection rates and factors such as pig age and sanitary conditions. By contrast, a study in China reported a low prevalence (4.5%) of *Cryptosporidium spp.* in diarrheic pigs, (Wang et al., 2022). In our investigation, assessing clinical symptoms was challenging, as

samples were collected from shared barns, making it difficult to attribute signs to individual animals.

Our investigations identified two *Cryptosporidium* species: *C. scrofarum* (89%) and *C. suis* (11%), which aligns with findings from China reporting 81% *C. scrofarum* and 19% *C. suis* (Wang et al., 2022). To-date, seven *Cryptosporidium* species have been identified in pigs, with *C. scrofarum* and *C. suis* considered pig-specific (Chen et al., 2023). Although pigs can harbour *C. parvum*, a species pathogenic to humans, our investigations did not detect this species. Interestingly, both *C. suis* and *C. scrofarum* have occasionally been detected in humans (Kvac et al., 2009; Wang et al., 2013), highlighting a potential zoonotic risk. Also, phylogenetic analysis further revealed that *C. scrofarum* clusters closely with other livestock-associated *Cryptosporidium* species, such as *C. ryanae* and *C. bovis* (cattle) and *C. xiaoi* (sheep), suggesting possible cross-species transmission among farm animals.

3.5 Conclusion

Although numerous studies on zoonotic HEV have been conducted worldwide, routine surveillance in Vietnam remains limited. Beyond HEV, pigs can also act as reservoirs for various parasitic infections.

The first chapter demonstrated the high prevalence of HEV in pigs and wild boars, underscoring their role as important reservoirs in Vietnam and providing an epidemiological map of circulating HEV subgenotypes. The elevated detection rates of HEV in faeces and rectal swabs also indicate that these sample types are well-suited for routine surveillance efforts.

The second chapter examined the screening of HEV antibodies and RNA in blood donors, offering valuable insights into the widespread exposure of the general population to HEV. Notably, no evidence of HEV transmission through blood transfusions was identified, and the level of ongoing HEV transmission appeared low. This chapter also revealed similar anti-HEV IgG seroprevalence rates among patients with chronic HBV infection, individuals with liver-related diseases, and blood donors. Interestingly, patients with cirrhosis and hepatocellular carcinoma exhibited the highest anti-HEV IgG seropositivity; however, this

increased seroprevalence was strongly correlated with age and gender, suggesting that HEV exposure is unlikely to play a direct role in the progression of liver disease.

The third chapter highlighted the significant prevalence of *Entamoeba* and *Cryptosporidium* protozoa in pigs and wild boars in Vietnam, findings consistent with previous studies and revealing notable species diversity. Notably, *Entamoeba hartmanni* was detected in domestic pigs in Vietnam for the first time, emphasizing the importance of ongoing parasite surveillance in animal populations. Given the zoonotic potential of these parasites, further investigations in human populations are warranted to evaluate the risk of cross-species transmission.

4. SUMMARY

This doctoral thesis explores the epidemiology of two underrecognized zoonotic pathogens: hepatitis E virus (HEV) and enteric protozoa (*Entamoeba* spp. and *Cryptosporidium* spp.) in Vietnam, a country with extensive pig farming and high risk of zoonotic spillovers. The research is structured in three chapters:

HEV in Pigs and Wild Boars: The study analysed over 500 samples from domestic pigs and wild boars across Vietnam, revealing a high HEV RNA positivity: 25% in wild boars and 7% in pigs. Molecular characterization identified HEV-3a as the dominant subgenotype, with HEV-4b detected only in wild boars. These findings demonstrate pigs and wild boars as important HEV reservoirs, emphasizing the risk of zoonotic transmission through handling or consumption of pork and wild boar meat.

HEV in Humans (Blood Donors and High-Risk Populations): Screening of 553 blood donors showed 27% anti-HEV IgG seropositivity but <1% anti-HEV IgM positivity and no HEV RNA detection, indicating widespread past exposure but low risk of transfusion-transmitted HEV. Among 745 patients with chronic hepatitis B or unexplained hepatitis, anti-HEV IgG rates were similar or higher, especially in patients with cirrhosis or liver cancer. However, HEV exposure correlated strongly with age and gender, suggesting no direct role in liver disease progression. The data confirm high endemicity but low current HEV transmission risk.

***Entamoeba* and *Cryptosporidium* in Pigs and Wild Boars:** The thesis found a high prevalence of *Entamoeba spp*. (62%) and *Cryptosporidium* spp. (31%) in pigs and wild boars. Species identified included *Entamoeba suis*, *E. polecki* (subtypes ST1 and ST3), *E. hartmanni*, *Cryptosporidium scrofarum*, and *C. suis*. Notably, *E. hartmanni* was detected in Vietnamese pigs for the first time. Several of these species have documented zoonotic potential, highlighting a possible risk of cross-species transmission to humans.

Taken together, the research thesis provides important data on zoonotic HEV and protozoan parasites in animal reservoirs and highlights the need for increased surveillance and One Health approaches to mitigate zoonotic risks. Despite widespread HEV exposure, there is no

evidence of transmission through transfusions or of HEV as a common cause of unexplained hepatitis in Vietnam. The considerable diversity and prevalence of *Entamoeba* and *Cryptosporidium* in pigs and wild boars requires further investigation of their zoonotic potential and public health implications.

5. ZUSAMMENFASSUNG

Diese Doktorarbeit untersucht die Epidemiologie zweier wenig beachteter zoonotischer Erreger: des Hepatitis-E-Virus (HEV) und enterischer Protozoen (*Entamoeba spp*. und *Cryptosporidium spp*.) in Vietnam, einem Land mit intensiver Schweinehaltung und hohem Risiko für zoonotische Spillover-Ereignisse. Die Arbeit ist in drei Kapitel gegliedert:

HEV bei Schweinen und Wildschweinen: In der Studie wurden über 500 Proben von Hausschweinen und Wildschweinen aus ganz Vietnam analysiert. Es zeigte sich eine hohe HEV-RNA-Positivität: 25 % bei Wildschweinen und 7 % bei Schweinen. Die molekulare Charakterisierung identifizierte HEV-3a als dominantes Subgenotyp, während HEV-4b ausschließlich bei Wildschweinen nachgewiesen wurde. Diese Ergebnisse bestätigen, dass Schweine und Wildschweine wichtige Reservoirs für HEV darstellen und unterstreichen das Risiko einer zoonotischen Übertragung durch den Umgang mit oder den Verzehr von Schweine- oder Wildschweinfleisch.

HEV beim Menschen (Blutspender und Risikogruppen): Das Screening von 553 Blutspendern ergab eine Anti-HEV-IgG-Seropositivität von 27 %, jedoch weniger als 1 % Anti-HEV-IgM-Positivität und keinen HEV-RNA-Nachweis, was auf eine weit verbreitete frühere Exposition, aber ein geringes Risiko einer transfusionsbedingten HEV-Übertragung hinweist. Unter 745 Patienten mit chronischer Hepatitis B oder ungeklärter Hepatitis waren die Anti-HEV-IgG-Raten ähnlich oder höher, insbesondere bei Patienten mit Leberzirrhose oder Leberkrebs. Allerdings korrelierte die HEV-Exposition stark mit Alter und Geschlecht, was auf keinen direkten Einfluss auf die Krankheitsprogression hinweist. Die Daten bestätigen eine hohe Endemizität, aber ein geringes aktuelles Übertragungsrisiko von HEV.

***Entamoeba* und *Cryptosporidium* bei Schweinen und Wildschweinen**: Die Arbeit zeigte eine hohe Prävalenz von *Entamoeba spp*. (62 %) und *Cryptosporidium spp*. (31 %) bei Schweinen und Wildschweinen. Identifizierte Arten waren *Entamoeba suis*, *E. polecki* (Subtypen ST1 und ST3), E. hartmanni, *Cryptosporidium scrofarum* und *C. suis*. Besonders bemerkenswert ist der erstmalige Nachweis von *E. hartmanni* bei vietnamesischen Schweinen. Mehrere dieser Arten haben bekanntes zoonotisches Potenzial, was auf ein mögliches Risiko einer Übertragung auf den Menschen hinweist.

Zusammenfassend liefert diese Dissertation wichtige Daten zu zoonotischem HEV und Protozoenparasiten in tierischen Reservoiren und hebt die Notwendigkeit verstärkter Überwachung und eines One-Health-Ansatzes hervor, um zoonotische Risiken zu minimieren. Trotz weit verbreiteter HEV-Exposition gibt es keine Hinweise auf eine Übertragung durch Bluttransfusionen oder darauf, dass HEV eine häufige Ursache für ungeklärte Hepatitis in Vietnam ist. Die beträchtliche Diversität und Prävalenz von *Entamoeba* und *Cryptosporidium* bei Schweinen und Wildschweinen erfordert weitere Untersuchungen ihres zoonotischen Potenzials und ihrer Auswirkungen auf die öffentliche Gesundheit.

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7. DECLARATION OF CONTRIBUTIONS

We hereby declare that the doctoral dissertation entitled "**Zoonotic Hepatitis E and Enteric Protozoa in Vietnam: Epidemiological Evidence from Animal Reservoirs to Human Health Risk**", submitted to the members of the PhD Board at the Faculty of Medicine, University of Tübingen, represents original work conducted by Dr. Le Chi Cao and co-authors at the Institute of Tropical Medicine, University of Tübingen, under the supervision of Prof. Dr. Thirumalaisamy P. Velavan. Four publications form the backbone of this doctoral dissertation, all authored primarily by Dr. Le Chi Cao.

Publication 1: One Health. 2024 Jul 10;19:100857. PMID: 39077329
Publication 2: Viruses. 2023 Oct 11;15(10):2075. PMID: 37896852
Publication 3: Open Forum Infectious Diseases. 2025; ofaf381. DOI: 10.1093/ofid/ofaf381.
Publication 4: Foodborne Pathogens and Disease. 2024 Oct 22. PMID: 39435712.

Dr. Le Chi Cao has made substantial contributions to all four manuscripts, including study design, sampling procedures, patient recruitment, experimental implementation, data analysis, and manuscript preparation. A detailed statement of the individual contributions of all co-authors for each publication, are as follows:

Contributions of PhD candidate and other co-authors

Publication 1:
Cao LC, Ha LNN, Giang TT, Tiep VM, Chau NTM, Phuong Anh TN, Duy PK, Nhan LP, Hoai NTT, Linh LTK, Hafza N, Bock CT, My TN, Sy BT, Toan NL, Song LH, Velavan TP. Characterization of zoonotic hepatitis E virus in domestic pigs and wild boar in Vietnam: Implications for public health. One Health. 2024 Jul 10:19:100857. PMID: 39077329. **IF: 4.5**

Le Chi Cao: Writing - Original Draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Le Nguyen Nhat Ha**: Methodology, Investigation. **Tran Thi**

Giang: Methodology, Investigation. **Vo Minh Tiep:** Methodology, Investigation. **Ngo Thi Minh Chau:** Project administration, Methodology. **Ton Nu Phuong Anh:** Project administration, Methodology. **Pham Khanh Duy**: Resources, Methodology. **Le Phuc Nhan:** Resources, Methodology. **Nguyen Thi Thu Hoai:** Supervision, Resources, Project administration. **Le Thi Kieu Linh:** Methodology, Investigation. **Nourhane Hafza:** Methodology, Investigation. **C.-Thomas Bock:** Writing - Review & Editing, Supervision, Resources, Methodology. **Truong Nhat My:** Supervision, Project administration, Methodology, Investigation. **Bui Tien Sy:** Resources, Project administration, Methodology, Investigation, Funding acquisition. **Nguyen Linh Toan:** Supervision, Project administration, Methodology, Investigation. **Le Huu Song:** Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition. **Thirumalaisamy P Velavan:** Writing - Review & Editing, Writing - Original Draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Publication 2:

Cao LC, Vanessa M, Linh LTK, Giang TT, Chau NTM, Phuong Anh TN, Nghia VX, The NT, My NT, Sy BT, Toan NL, Song LH, Bock CT, Velavan TP. High Hepatitis E Virus (HEV) Seroprevalence and No Evidence for HEV Viraemia in Vietnamese Blood Donors. Viruses. 2023 Oct 11;15(10):2075. PMID: 37896852. **IF:3.5**

Le Chi Cao: Methodology, Software, Formal analysis, Investigation, Writing - Original Draft. **Vanessa Martin:** Methodology, Investigation. **Le Thi Kieu Linh**: Methodology. **Tran Thi Giang**: Methodology. **Ngo Thi Minh Chau**: Validation. **Ton Nu Phuong Anh**: Validation. **Vu Xuan Nghia**: Resources. **Nguyen Trong The**: Validation. **Truong Nhat My**: Validation. **Bui Tien Sy:** Validation. **Nguyen Linh Toan**: Validation. **Le Huu Song**: Validation. **C.-Thomas Bock**: Conceptualization, Formal analysis, Writing - Review and Editing, Supervision. **Thirumalaisamy P. Velavan**: Conceptualization, Formal analysis, Resources, Data curation, Writing - Original draft, Writing - Review and Editing, Visualization, Supervision, Project administration, Funding acquisition.

Publication 3:

Cao LC, Tien Xinh TT, Gowdar T, Phuoc DN, Dung NT, Loan TTK, Duc PV, Huyen DT, Linh LTK, Song LH, Velavan TP. High Seroprevalence of Hepatitis E Virus among patients with Hepatitis B and those with Hepatitis of Unknown Etiology in Central Vietnam. Open Forum Infect Dis. 2025 (DOI: 10.1093/ofid/ofaf381). **IF:3.8**

Le Chi Cao: Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing - Original draft, Writing - Review and Editing, Visualization. **Tran Thi Tien Xinh:** Methodology, Investigation, Resources. **Taanvi Gowdar:** Investigation. **Dang Ngoc Phuoc:** Methodology, Investigation, Resources. **Nguyen Thi Dung:** Methodology, Investigation, Resources. **Tran Thi Kim Loan:** Methodology, Investigation, Resources. **Pham Van Duc:** Investigation. **Dao Thi Huyen:** Investigation, Data curation. **Le Thi Kieu Linh:** Methodology, Investigation. **Le Huu Song**: Supervision, Project administration. **Thirumalaisamy P. Velavan:** Conceptualization, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

Publication 4:

Cao LC, Muraleedharan D, Giang TT, Tiep VM, Chau NTM, Anh TNP, Ha LNN, Hoai NTT, My TN, Moussi AE, Hafza N, Song LH, Velavan TP. Prevalence and Genetic Diversity of Entamoeba and Cryptosporidium in Pigs and Wild Boars in Central and Southern Vietnam: Implications for Zoonotic Risks and Surveillance. Foodborne Pathog Dis. 2024 Oct 22. doi: 10.1089/fpd.2024.0095. PMID: 39435712. **IF: 1.9**

Le Chi Cao: Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing - Original draft, Writing - Review and Editing, Visualization. **Devika Muraleedharan:** Methodology, Validation. **Tran Thi Giang:** Methodology, Resources. **Vo Minh Tiep:** Methodology, Resources. **Ngo Thi Minh Chau:** Methodology. **Ton Nu Phuong Anh:** Methodology. **Le Nguyen Nhat Ha:** Methodology, Resources. **Nguyen Thi Thu Hoai:**

Methodology. **Truong Nhat My:** Methodology. **Awatef El Moussi:** Validation. **Nourhane Hafza:** Validation. **Le Huu Song:** Methodology, Project administration, Funding acquisition. **Thirumalaisamy P. Velavan**: Conceptualization, Writing - Review and Editing, Supervision, Project administration, Funding acquisition.

I also declare that I wrote the general introduction and discussion in this thesis.
Sincerely,

Tübingen, 5th August 2025

PhD Candidate	Primary Supervisor
Le Chi Cao, MD	**Prof. Dr. Thirumalaisamy P. Velavan**

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