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Hepatitis E

Molecular Virology, Pathogenesis, and Treatment, 2nd Edition

Edited by
Hiroaki Okamoto

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Hepatitis E: Molecular Virology, Pathogenesis, and Treatment, 2nd Edition

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Guest Editor

Hiroaki Okamoto

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About the Editor

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Hiroaki Okamoto is a Professor in the Division of Virology, Department of Infection and Immunity at Jichi Medical University School of Medicine in Japan. He graduated from Tohoku University School of Medicine in 1979 and obtained his PhD from Jichi Medical University in 1987. He has pursued sustained and impactful research on hepatitis viruses for over four decades, beginning with hepatitis B virus (HBV) in 1983 and hepatitis C virus (HCV) in 1989, and contributing to the discovery of torque teno virus (TTV) in 1997. Since 2000, his primary research focus has been hepatitis E virus (HEV). His work encompasses molecular virology, epidemiology, and zoonotic transmission of HEV. Notably, he has made seminal contributions to the establishment of robust cell culture systems for HEV, demonstrated the existence of quasi-enveloped HEV in serum and culture media, elucidated mechanisms of HEV egress, and advanced fundamental understanding of HEV genotypes, cross-species infection, and foodborne transmission. His research, particularly in Japan, has significantly influenced diagnostic strategies and strengthened public health awareness of HEV infection.

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Case Report

Case of Fatal Hepatitis Related to HEV-3 Infection in Central Italy

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Abstract: Hepatitis E virus (HEV) is a global health problem, causing an estimated 20 million infections annually. Thus, the management of HEV requires special consideration. In developed countries, hepatitis E is mainly recognized as a foodborne disease (mainly transmitted via undercooked meat consumption) that is generally caused by genotype 3 and 4 circulating in various animals, including pigs and wild boars. The current absence of officially recognized protocols for the analysis of HEV in foods and the lack of awareness of this disease among healthcare workers, together with the high percentage of asymptomatic cases, make HEV infection highly underestimated. Most HEV-3 infections in immunocompetent individuals are self-limited. Nevertheless, the possibility of serious forms of liver disease, especially in patients with co-morbidities, should be considered because it can lead to a fatal outcome. Here, we report a case of fatal hepatitis related to HEV-3 infection in a 67-year-old male patient with underlying chronic liver disease (CLD) and living in a region where a high prevalence and genetic heterogeneity of HEV-3 in wild boar has been recently demonstrated. Our case report describes the interdisciplinary approach used (from the diagnosis to the virus phylogenetic characterization) in order to improve epidemiologic HEV surveillance in central Italy.

Keywords: hepatitis E virus; genotype 3; viral hepatitis; phylogenetic analyses; foodborne disease

1. Introduction

Viruses are considered the most common cause of foodborne disease. The World Health Organization (WHO), in 2020, estimated that hepatitis A virus (HAV) causes approximately 1.4 million infections and approximately 7000 deaths each year, while hepatitis E virus (HEV) causes 20 million infections, 3.3 million symptomatic cases, and 44,000 fatalities annually [1].

HEV, a member of the *Hepeviridae* family, is a small non-enveloped or quasi-enveloped virus with a single-stranded positive-sense ribonucleic acid (RNA) genome. The viral genome encodes for three open reading frames (ORFs), namely, ORF1, ORF2, and ORF3, but a fourth open reading frame (ORF4), embedded within ORF1 and present in genotype

1 strains only, has also been described. Among these regions, ORF1 encodes functional non-structural proteins (e.g., RNA-dependent RNA polymerase), and ORF2 encodes the highly immunogenic viral capsid protein. The antibodies against this protein have neutralizing and protective features. ORF3 encodes a functional ion channel protein that has important roles in the release of viral particles. The recently discovered ORF4 is believed to play a role in the proper functioning of HEV RNA polymerase, but the lack of this sequence in the other genotypes suggests that its real function needs to be elucidated [2,3].

HEV has mainly fecal–oral or zoonotic transmission, according to the genotypes (see below). Additionally, although rare, the parenteral transmission of HEV is also possible, for example, after whole-blood or blood products transfusion [4,5]. Phylogenetic analysis classifies HEV into eight different genotypes, of which HEV-1, HEV-2, HEV-3, and HEV-4 are responsible for disease in humans [6]. The HEV-1 and HEV-2 genotypes exclusively infect humans and are transmitted by the fecal–oral route in low-income countries, especially through contaminated water. Although these two genotypes cause self-limiting disease and are not related to cases of chronicity and/or cirrhosis, HEV-1- and HEV-2-related infections still have a substantial burden on public health in low-income countries because they occur most often in young adults and have a high incidence and severity in pregnant women, with high maternal and perinatal mortality rates [2,7,8].

HEV-3 and HEV-4 infections are mainly associated with zoonotic transmission, occurring via close contact with infected animals or through the consumption of contaminated food products (most commonly raw or undercooked meat). HEV-3 and HEV-4 infections vary widely in severity, from clinically silent to fulminant hepatitis, and they may be also responsible for extrahepatic diseases. Most HEV-3 and HEV-4 infections in immunocompetent individuals are self-limited and result only in clinically silent seroconversion. Less than 2% of those infected are symptomatic [9].

Nevertheless, in specific populations, such as immunosuppressed patients or individuals with underlying chronic liver disease (CLD), HEV infection is a potential trigger of acute-on-chronic liver failure [10,11].

Older individuals usually display more severe liver disease, with a higher incidence of hepatic or non-hepatic complications (15%) and acute liver failure (8–11%) [12]. In European countries, chronic hepatitis E is mostly reported in immunocompromised patients with HEV-3 infection [2,13,14].

In May 2022, the 75th World Health Assembly defined a new set of integrated strategies for the global health sector, to be implemented in the period 2022–2030, regarding the management of HIV, viral hepatitis, and sexually transmitted infections.

Following what was established during this assembly, many Member States, including Italy, have begun to develop national programs and strategies aimed at achieving the intended objective, through community training interventions and prevention, diagnosis, and treatment actions.

In Italy, HEV infection is subject to notification through the infectious disease information system (PREMAL), based on reports from doctors who alert the Public Health and Hygiene Service every time a diagnosis of acute hepatitis E is made [15].

Since 2007, it has been possible to notify cases of acute viral hepatitis also through an integrated epidemiological surveillance known as Sistema Epidemiologico Integrato dell'Epatite Virale Acuta (SEIEVA).

SEIEVA describes and monitors the trends of the various forms of acute viral hepatitis, differentiated by specific type, throughout Italy. The integrated analysis of the information collected with the epidemiological questionnaires allows for the estimation of the incidence of disease and the relative contribution of the different risk factors. This also allows for the definition of preventive measures to be prioritized and for the monitoring of the effects of the various prevention programs.

Thanks to this surveillance system, between February 2021 and August 2023, 16 cases of acute hepatitis E were diagnosed and monitored in Umbria (Central Italy), and here, we

describe a case of fatal hepatitis related to HEV-3 infection in a 67-year-old male patient with underlying CLD.

2. Case Description

2.1. Clinical Presentation

A 67-year-old male, presenting with asthenia and loss of weight, accompanied by the presence of jaundice, hyperchromic urine, and hypocolic feces was admitted to the Internal Medicine Section of the Perugia Hospital (Umbria, Italy) on 3 February 2022. He denied abdominal pain, vomiting, diarrhoea, fever, and other associated symptoms. His medical history included a previous diagnosis of type II diabetes mellitus treated at home with oral hypoglycemic therapy, hypertensive cardiopathy with mitro-aortic valvulopathy, hypercholesterolemia associated with obesity (Body Mass Index = 31), mild congenital interventricular septal defect, and a history of knee prosthesis and inguinal hernioplasty.

At admission, SARS-CoV-2 and hemato-biochemical tests were performed. SARS-CoV-2 molecular and antigenic tests were negative upon admission and during the stay in the Internal Medicine Section. The results of the hemato-biochemical tests are reported in Table 1.

Table 1. Laboratory results at admission.

Laboratory Measurement	Result	Unit of Measurement	Normal Range
White Blood Cells (WBCs)	8.03	$\times 10^3$/uL	(3.60–9.60)
Red Blood Cells (RBCs)	4.60	$\times 10^6$/uL	(4.30–5.80)
Hemoglobin	14.1	g/dL	(13.0–17.0)
Hematocrit	40.6	%	(38.0–52.0)
MCV	88.3	fL	(82.0–97.0)
MCH	30.7	pg	(27.0–33.0)
MCHC	34.7	g/dL	(32.0–36.0)
RDW	* 14.9	%	(11.6–14.5)
Platelets	289	×1000/UL	(140–440)
MPV	10.1	fL	(8.0–13.0)
Lymphocytes	* 15.7	%	(20.5–51.5)
Monocytes	* 12.0	%	(1.0–10.0)
Neutrophils	69.1	%	(42.0–75.0)
Eosinophils	2.2	%	(0.0–5.0)
Basophils	1.0	%	(0.0–1.0)
I.N.R	* 1.41		(0.80–1.20)
Glycemia	* 146	mg/dL	(74–106)
Azotemia	* 138	mg/dL	(17–43)
Creatinine	* 3.03	mg/dL	(0.62–1.18)
CKD-EPI eGFR	* 20.4	mL/min/1.73 mq	(>60.0)
Total Bilirubin	* 33.71	mg/dL	(0.30–1.20)
Direct Bilirubin	* 15.55	mg/dL	(0.00–0.20)
AST/GOT	* 459	IU/L	(0–50)
ALT/GPT	* 1244	IU/L	(0–50)
Gamma GT	* 290	IU/L	(0–55)
Alkaline Phosphatase	* 202	IU/L	(30–120)
Sodium	* 135	mEq/L	(136–146)
Potassium	4.0	mEq/L	(3.5–5.1)
Amylase	45	IU/L	(28–100)

* Values out of normal range; Note: Highly jaundiced specimen.

Based on the laboratory results, gastroenterological and nephrological consultation were requested.Abdominal ultrasound revealed hepatosplenomegaly with hypertrophy of the right and caudate lobes of the liver. In addition, the presence of collateral circles (probably esophageal varices) and ectasia of the left gastric vein were identified. Pulsed

Wave Doppler (PW) examination highlighted the presence of hepatoportal flow with significantly reduced average velocity. The gallbladder, normally distended, appeared filled with biliary sludge. Ascitic effusion of moderate size was detected, estimated to be at least 2000 cc.

A diagnosis of liver cirrhosis with portal hypertension was made, with a probable dysmetabolic genesis, aggravated by a picture of acute renal failure (ARF) with tubular damage due to hyperbilirubinemia. From the anamnestic collection, the exotoxin and iatrogenic hypothesis of the liver pathology was excluded.

Major causes of hepatitis, including HAV, HBV, and HCV, *Cytomegalovirus*, and Epstein–Barr virus, were excluded. Meanwhile, from the serological analysis for major hepatotropic viruses, on 4 February 2022, positivity for HEV emerged. The IgM and IgG, detected using VIDAS® Anti-HEV IgM and VIDAS® Anti-HEV IgG kits (bioMérieux, France), were 39.4 S/Co and >10.00 U/mL, respectively (IgM cut-off = 1.0 S/Co, IgG cut-off = 0.56 U/mL).

A detailed medical history excluded recent trips abroad or contact with travellers in the 9 weeks preceding the onset of hepatitis. The patient also denied a history of transfusion while reporting frequent consumption of wild boar meat, as he was a hunter.

As required by the protocol, the anti-HEV IgM positive serum of the patient was sent to the Laboratory for Viral Hepatitis, Oncovirus, and Retrovirus Diseases (Istituto Superiore di Sanità, ISS), where further analyses were conducted to confirm the diagnosis of acute hepatitis E. The serological analyses confirmed the presence of anti-HEV IgG and IgM (Wantai HEV-IgG and Wantai HEV-IgM ELISA assays, Beijing WANTAI Biological Pharmacy Enterprise Co. Ltd., Beijing, China). In parallel, molecular investigation detected the presence of the viral RNA (10.000 IU/mL) using a Real-Time PCR assay (RealStar HEV RT-PCR Kit 2.0, Altona Diagnostics GmbH, Hamburg, Germany), according to the manufacturer instructions.

Considering the diagnosis of HEV infection, gastroenterological consultation was requested. The ultrasound examination of the abdomen highlighted an unknown full-blown liver cirrhosis with portal hypertension.

Based on the overall clinical picture of the patient, the liver failure attributable to acute HEV infection was not interpreted as acute liver failure (ALF) but as acute-on-chronic failure (ACLF) with dysmetabolic genesis, complicated by portal hypertension. His Mayo End-stage Liver Disease (MELD) score, a prognostic scoring system based on laboratory parameters, used to predict 3-month mortality due to liver disease, was 38. The MELD score ranges from 6 to 40, and the higher the score, the higher the 3-month mortality related to liver disease. Therefore, for our patient, the prognosis was poor.

During the hospital stay, a new basal-bolus insulin therapy was started, obtaining a good glycemic profile. Additionally, an antibiotic prophylaxis with Meropenem was started, and vitamin K and albumin were administered to compensate for liver dysfunction. The patient remained paucisymptomatic throughout his hospitalization. Gradually, there was a partial recovery of liver function (Figure 1).

Following repeated episodes of vomiting after meals, the patient was readmitted to the emergency room and monitored for one day on 15 March 2022. Patient was apyretic and had no other symptoms. Biological examinations notably revealed a modest neutrophilic leucocytosis (WBC = 9.87 $\times$ 10^3/uL, 84.1% of neutrophils, INR = 2.43, azotemia = 90 mg/dL, creatinine = 1.71 mg/dL, CKD-EPI eGFR = 40.8 mL/min/1.73 mq, total bilirubin = 42.63 mg/dL, direct bilirubin = 17.41 mg/dL, AST/GOT = 117 IU/L, ALT/GPT = 61 IU/L, CRP = 2 mg/dL). A SARS-CoV-2 test was negative. HEV serology was repeated, showing IgM and IgG values of 37.9 S/Co and >10.00 U/mL, respectively. After 24 h of observation, blood tests showed a spontaneous improvement in leucocytosis. Considering the clinical picture with a treatable and non-painful abdomen, apyrexia, and the positive course of the hospital stay, the patient was transferred to Ancona Transplant Centre and evaluated for the antiviral therapy and liver transplant. HEV RNA was tested in serum and stools during the stay; it was not detected in either sample. Antiviral therapy

was not initiated and transplant was denied because of the patient's age and the presence of significant comorbidities.

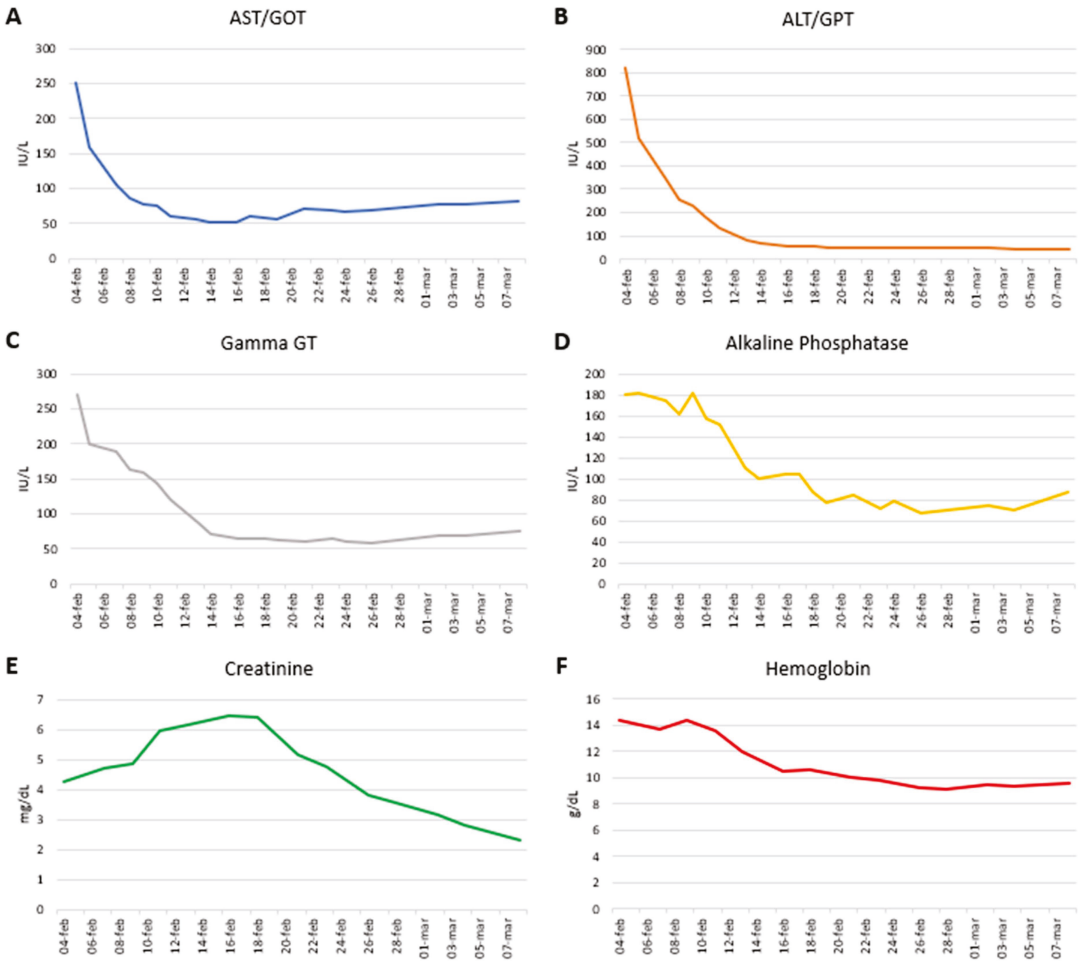

Figure 1. Graphic presentation of patient's liver enzyme levels throughout the hospitalization. Trend of liver enzymes from the day after the hospital admission (4 February 2022) to the day before the discharge (7 March 2022). (**A**) Aspartate aminotransferase/glutamic oxaloacetic transaminase (AST/GOT); (**B**) alanine aminotransferase/glutamic–pyruvic transaminase (ALT/GPT); (**C**) gamma glutamyltransferase (Gamma GT); (**D**) alkaline phosphatase; (**E**) creatinine; (**F**) hemoglobin.

Subsequently, on 28 May 2022, following a picture of liver cirrhosis in the phase of ascitic decompensation, asthenia, and new episodes of vomiting, a new hospitalization was necessary. During the hospitalization, a new serological investigation was performed, and anti-HEV IgM and anti-HEV IgG levels were still high (IgM = 14.2 S/Co and IgG > 10.00 U/mL). Urine culture resulted positive for *Escherichia coli* (>100.000 CFU/mL), and antibiotic therapy with Fosfomycin (3 gr/die per 3 days) was prescribed. Despite the treatments provided, due to the slow and irreversible deterioration of the clinical picture and following the complications that arose in relation to the HEV infection, the patient's death was declared on 19 June 2022.

2.2. HEV Genotype/Subtype and Phylogenetic Analyses

To characterize the virus, the extracted RNA was subjected to nested PCR amplification of an ORF2 fragment (nucleotide position 5948-6513 in the reference sequence, accession number M73218) of the viral genome, according to a previously described procedure [16]. The nested PCR product (size: 566 nt) was then subjected to double-strand sequencing by a Sanger Sequencing kit (Applied Biosystems by Thermo Fisher Scientific) and an

automated sequencer (SeqStudio Genetic Analyzer, Applied Biosystems by Thermo Fisher Scientific). After removal of the primer sequence ends from the raw output sequences, the final consensus sequence (size: 493 nt) was subjected to phylogenetic analysis with subtype 3 reference sequences as well as with HEV-3 sequences obtained from wild boars collected in Umbria in 2021–2022 [17].

Figure 2 shows the resulting phylogenetic tree. The patient sequence (red circle) is placed in the 3f clade, well separated from the 3e clade by a statistically supported node (bootstrap > 70); so, it can be assigned to subtype 3f.

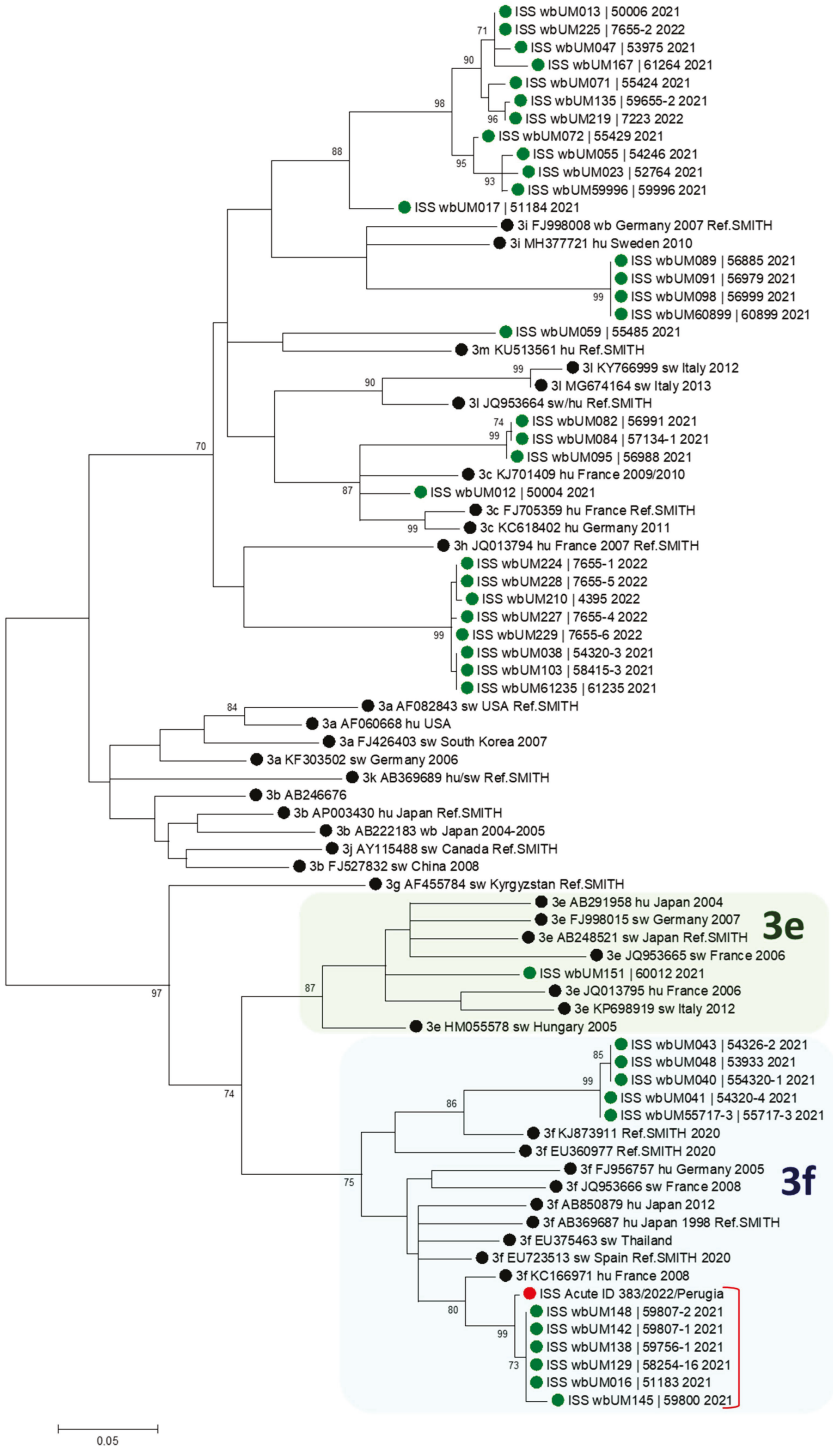

Figure 2. Phylogenetic analysis. Phylogenetic tree resulting from analysis of a 361 nt region shared by the HEV sequence from the case, sequences from wild boars sampled in 2021–2022 in Umbria, and

reference sequences representing subtypes 3a to 3m [18]. A maximum likelihood approach was applied, with a TN93+G+I evolutionary model preliminarily estimated by the Model tool in MEGA. The red circle highlights the sequence from the patient, the black circles mark reference sequences, and the green circles mark sequences from wild boars from the region of Umbria. The suffix "Ref. SMITH" at the end of some reference sequence names marks those reference sequences recommended by an international group of experts as the best representative subtype references [18]. The 3e and 3f clades are highlighted by green and light blue shading; a red square bracket delimits the cluster that includes the patient sequence and six wild boar sequences.

It is noteworthy that the sequence also forms a separate cluster with a group of six HEV sequences from wild boars sampled in November–December 2021 in places within 60 kilometers from Perugia (Città di Castello 52 Km; Ascagnano, 22 Km; Ficareto, nearby Todi, 50 Km; San Giustino, 61 Km). Significantly, the patient had his residence in Perugia, he was a wild boar hunter, and he reported frequent consumption of wild boar meat. Five wild boar sequences of this cluster are identical to each other; the patient sequence shows three nt differences vs. these five wild boar sequences and seven nt differences vs. the sixth remaining sequence.

Although the patient and wild boar sequences are not identical, sequence comparison shows that the patient HEV strain was genetically highly related to viruses circulating in wild boars in the same geographical area and in the same period in which the patient hunted wild boars.

2.3. Food Analyses

Concurrently with the investigation conducted on the patient, experts from the Prevention, Veterinary Health, and Food Safety Service of the Umbria Region, in collaboration with the Experimental Zooprophylactic Institute of Umbria and Marche "Togo-Rosati" (IZSUM), and together with the National Reference Laboratory for Foodborne Viruses, recovered from the patient's household and tested food matrices suspected to be vehicles of the infection. Eight packages of frozen wild boar meat and four packages of home-made frozen sausages, made with mixed wild boar/pig minced meat, were recovered from the patient's freezer. Analyses were performed following an in-house standardized protocol, including lysis of sample (1 g), according to Szabo et al. [19], nucleic acid extraction using a bioMérieux NucliSens MiniMag system, and HEV detection by RT-qPCR, as described in Di Pasquale et al. [20]. HEV nucleic acid was detected at a low concentration (~10 genome copies/g) in two sausage packages, confirming the patient's hunting activity as a plausible source of exposure. Subsequent attempts to amplify a viral genome fragment by nested PCR for sequencing and comparison with the HEV sequence from the patient were unsuccessful, probably due to the low viral load in the samples.

3. Discussion

Many studies, mostly outside Europe, report HEV infection as one of the most important causes of acute-on-chronic liver failure (ACLF) in patients with CLD, which is associated with high mortality rates [21,22]. Indeed, HEV infection is potentially fatal in patients with underlying liver disease [23].

Data collected through SEIEVA confirmed what is already known from the literature [24]: the category most at risk of contracting the infection is that made up of males aged >50 years. For instance, in our region, of the 16 positive subjects with HEV infection between February 2021 and August 2023, 13 (81.25%) were male and had an average age of 54 years (range 39–83), including the patient reported in this case report.

Our patient had a regular lifestyle before the infection, despite having cirrhosis of metabolic origin. Thus, HEV infection had a significant negative impact on his quality of life and on the management of the numerous preexisting pathological conditions. Moreover, as recently reported by Abravanel et al., cardiovascular comorbidities represent an important risk factor for severe HEV infection and are associated with higher hospitalization [25].

Considering the rapidly changing epidemiological situation and based on the European Food Safety Authority (EFSA) statement that the number of HEV infection cases in Europe shows a steadily increasing trend [4], an active surveillance plan was activated in the Umbria region. HEV has thus become a special watchdog.

Following reports from hospital clinicians, thanks to the SEIEVA, it was possible to quickly activate a series of epidemiological investigations that allowed the Unit of Viral Hepatitis and Oncovirus and Retrovirus diseases (at ISS) to promptly isolate viral RNA from an early sample of patient serum. This should be emphasized because, as is well known, the diagnosis of acute hepatitis E in humans is based on the specific detection, in the patient's serum, of IgM antibodies directed against HEV. In case of IgM positivity of the serologic test, the laboratory also proceeds with molecular tests for direct detection of the virus in the patient's blood.

Of note is the fact that the detection of viral RNA in serum may be difficult because very often the diagnosis of HEV infection is made at a late stage and the viremia is under the detectable level [26].

This case of infection must be considered autochthonous because the patient had reported no travel in HEV hyperendemic areas before the onset of hepatitis. Moreover, he referred to habitual consumption of wild boar meat, which is considered an important risk factor especially in our region, where Beikpour et al. [17] recently demonstrated a high prevalence and genetic heterogeneity of genotype 3 HEV in wild boar. The laboratory results of the present study (sequence comparison, phylogenetic analysis, detection of HEV in home-made sausages in the patient freezer), together with previous data collected in the same region [27], overall suggest, although do not definitively prove, HEV transmission associated with the hunting activity of the patient, either through slaughtering wild boars and handling their meat or through consumption of food originating from them.

Based on the analysis carried out and the real risk of an increase in cases, hepatitis E should be considered as an emerging public health issue in our region too.

Underestimating HEV infection in European and American patients is the most common mistake that clinicians make, due to the predominantly asymptomatic course of the disease. Nevertheless, the possibility of sudden deterioration of liver function, especially in patients with CLD, should be considered regardless of their travel history or their conscious exposure to known potential risk factors. The European Association for the Study of the Liver (EASL) suggests that patients with unexplained CLD flares should be tested for hepatitis E [28].

As reported by the ISS, in the last few years, the number of cases of hepatitis E has slightly exceeded cases of acute hepatitis C. In 2023, hepatitis E was the third most frequent cause of viral hepatitis in Italy [29].

To date, neither vaccines nor specific antiviral treatments against HEV are available. The control of HEV at a territorial level can be guaranteed exclusively through the implementation of constant prevention and monitoring programs, which must involve all those operating in the sector of public health, veterinary health, food safety and environmental protection with a One-Health approach.

In developed countries, HEV is principally recognized as a foodborne disease, and while an ISO standard method for the detection of HEV in meat products is under development, the current absence of officially recognized protocols for the analysis of HEV in foods hinder the implementation of targeted control plans in the EU.

Furthermore, the lack of awareness of this disease among healthcare workers and the high percentage of asymptomatic cases make HEV infection highly underestimated.

HEV can cause very serious forms of liver disease and be associated with the onset of severe forms of chronic hepatitis in immunosuppressed subjects or with previous diagnoses of liver cirrhosis. The present case report documents that the occurrence of HEV infection in a patient with CLD and significant comorbidities may lead to an exacerbation of the clinical conditions, leading to a fatal outcome. Taking into account that antiviral drugs,

such as ribavirin, in cases of anemia and/or renal failure should be dosed with caution [28], prevention of infection remains the only protection tool.

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Data Availability Statement: The data are available upon request from the corresponding author. The data are not publicly available because of privacy or ethical restrictions.

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

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Production and Characterization of Self-Assembled Virus-like Particles Comprising Capsid Proteins from Genotypes 3 and 4 Hepatitis E Virus (HEV) and Rabbit HEV Expressed in *Escherichia coli*

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Abstract: The zoonotic transmission of hepatitis E virus (HEV) genotypes 3 (HEV-3) and 4 (HEV-4), and rabbit HEV (HEV-3ra) has been documented. Vaccination against HEV infection depends on the capsid (open reading frame 2, ORF2) protein, which is highly immunogenic and elicits effective virus-neutralizing antibodies. *Escherichia coli* (*E. coli*) is utilized as an effective system for producing HEV-like particles (VLPs). However, research on the production of ORF2 proteins from these HEV genotypes in E. coli to form VLPs has been modest. In this study, we constructed 21 recombinant plasmids expressing various N-terminally and C-terminally truncated HEV ORF2 proteins for HEV-3, HEV-3ra, and HEV-4 in *E. coli*. We successfully obtained nine HEV-3, two HEV-3ra, and ten HEV-4 ORF2 proteins, which were primarily localized in inclusion bodies. These proteins were solubilized in 4 M urea, filtered, and subjected to gel filtration. Results revealed that six HEV-3, one HEV-3ra, and two HEV-4 truncated proteins could assemble into VLPs. The purified VLPs displayed molecular weights ranging from 27.1 to 63.4 kDa and demonstrated high purity (74.7–95.3%), as assessed by bioanalyzer, with yields of 13.9–89.6 mg per 100 mL of TB medium. Immunoelectron microscopy confirmed the origin of these VLPs from HEV ORF2. Antigenicity testing indicated that these VLPs possess characteristic HEV antigenicity. Evaluation of immunogenicity in Balb/cAJcl mice revealed robust anti-HEV IgG responses, highlighting the potential of these VLPs as immunogens. These findings suggest that the generated HEV VLPs of different genotypes could serve as valuable tools for HEV research and vaccine development.

Keywords: hepatitis E virus; capsid protein; virus-like particle; genotype; *Escherichia coli*; assembly in vitro

1. Introduction

Hepatitis E virus (HEV) is the leading cause of acute viral hepatitis worldwide, accounting for over 3.3 million symptomatic cases of hepatitis annually and resulting in approximately 44,000 hepatitis E-related deaths [1,2]. Globally, seroprevalence studies have indicated that one in eight individuals has been infected with HEV, as evidenced by the presence of anti-HEV IgG antibodies [3]. Typically, HEV infection results in asymptomatic or self-limited disease; however, in immunocompromised patients, it can become chronic or be associated with extrahepatic manifestations, including neurological and renal disorders [4,5]. The mortality rate in the general population ranges from 0.5 to 4.0%, while in pregnant women, it can reach up to 30% [6,7]. The primary cause of the increasing number

of autochthonous human infections in industrialized countries is zoonotic transmission of HEV [8].

HEV is a quasi-enveloped virus with an icosahedral capsid that encloses its viral genome [9]. The HEV genome consists of a single-stranded, positive-sense RNA, ranging from 6.6 to 7.3 kilobases (kb) in length, flanked by 5′ and 3′ untranslated regions (UTRs) [10,11]. It features a 7-methylguanine cap at the 5′ end and a poly(A) tail at the 3′ end, resembling host mRNA [12]. The HEV genome contains three open reading frames (ORFs: *ORF1–3*). *ORF1* encodes a large non-structural polyprotein with multiple functional domains involved in viral replication, such as methyltransferase, helicase, and RNA-dependent RNA polymerase [13]. *ORF2* and *ORF3* proteins are translated from a 2.2 kb bicistronic subgenomic RNA [14]. *ORF2* encodes a structural capsid protein (660 amino acids [aa]) that interacts with putative host receptors and is a major target for neutralizing antibodies; a secreted form of ORF2 does not prevent virus entry but interferes with antibody-mediated neutralization [15,16]. *ORF3* encodes a small multifunctional phosphoprotein (112–114 aa) that functions as an ion channel (viroporin) and is essential for virion morphogenesis and egress [17,18].

HEV is classified within the family *Hepeviridae*, which comprises two subfamilies: *Orthohepevirinae* and *Parahepevirinae*. The *Orthohepevirinae* encompasses four genera: *Paslahepevirus*, *Rocahepevirus*, *Chirohepevirus*, and *Avihepevirus* [10]. The genus *Paslahepevirus* comprises two species, *P. alci* and *P. balayani*. The species *P. balayani* includes eight viral genotypes, HEV-1 to HEV-8 [19]. HEV-1 and HEV-2 strains are exclusively found in humans and are responsible for large outbreaks of hepatitis E in developing countries. In contrast, HEV-3 and HEV-4 strains have been isolated from domestic pigs and wild boars, causing sporadic hepatitis E cases in both developing and industrialized countries [20,21]; HEV-5 and HEV-6 strains have been detected solely in wild boar populations in Japan [22–25]; HEV-7 infects dromedary camels [26], while HEV-8 infects Bactrian camels [27,28].

HEV-3 is further divided into at least fourteen subtypes (3a–3m and 3ra), and HEV-4 is divided into at least nine subtypes (4a–4i) [19]. Notably, HEV subtypes 3a, 3b, 3e, 3k, 4c, 4g, and 4i are actively circulating in Japan, with subtypes 3b and 4c being the most prevalent for each genotype. These subtypes are associated with zoonotic food-borne transmission in Japan [24,29–32]. Notably, HEV-3ra (rabbit HEV) strains have been isolated from both farmed and wild-caught rabbits, forming a distinct clade closely related to HEV-3 [33,34]. Recent studies have demonstrated that HEV-3ra is capable of infecting humans and causing hepatitis [35,36].

Recently, apart from HEV-3, HEV-3ra, HEV-4, and HEV-7, zoonotic infections of humans with rat HEV (*Rocahepevirus ratti* species, HEV-C1) have been documented. These infections have caused chronic infections in immunosuppressed individuals and acute hepatitis even in children [37–40]. Rat HEV has been isolated from wild rats globally [41–43], suggesting that rat HEV might be an underestimated source of human infection. The protein encoded by *ORF2* of rat HEV is 644 aa long, and 16 aa shorter than that of human HEV.

Vaccination-based prevention of HEV infection hinges on the highly immunogenic capsid protein, which elicits potent virus-neutralizing antibodies [44]. To date, various systems have been utilized to express the HEV capsid protein, including *Escherichia coli* (*E. coli*), insect cells, mammalian cells, and plants [45,46]. Among these, *E. coli* and the baculovirus-insect cell system are considered the most effective for producing virus-like particles (VLPs) [47–52]. In our previous study, we successfully expressed rat HEV ORF2 proteins in *E. coli* and characterized the self-assembled VLPs [53]. Despite advances in VLP production for HEV vaccination, exemplified by the recombinant VLP-based vaccine Hecolin® (HEV-1), available in China and Pakistan [51,54], research on the production of ORF2 proteins from various HEV genotypes in *E. coli* forming VLPs remains limited [45,46,53]. Notably, the construction of VLPs from HEV-3, including HEV-3ra (rabbit HEV), in *E. coli* has not been reported. In the present study, we expressed HEV ORF2 proteins from HEV-3 (subtype 3b), rabbit HEV (HEV-3ra), and HEV-4 (subtype 4c) in *E. coli* and characterized the self-assembled VLPs towards studies on the antigenicity, immunogenicity, pathogenicity,

and epidemiology of zoonotic HEV and the future development of VLP-based vaccines for HEV prevention.

2. Materials and Methods

2.1. Plasmid Construction and Truncated HEV ORF2 Protein Expression

To construct plasmids expressing truncated HEV-3 ORF2 proteins of the JE03-1760F strain (subtype 3b) [55], nine expression vectors were developed. These vectors contained truncated N- and C-termini sequences (aa 12–606, 14–606, 61–606, 89–606, 107–606, 112–606, 117–606, 122–606, and 368–606 of *ORF2*) (Figure 1A). The nucleotide sequences corresponding to aa 12–606, 14–606, 61–606, 89–606, 107–606, 112–606, 117–606, 122–606, and 368–606 of *ORF2* were amplified via polymerase chain reaction (PCR) using the full-length JE03-1760F cDNA clone (AB437316) [56] as a template. The primer set comprised a sense primer with an introduced Nde I site (hHEV_G3 Nde-ORF2 Fw, corresponding to aa 12, 14, 61, 89, 107, 112, 117, 122, or 368 of *ORF2*) and an antisense primer with a BamH I site (hHEV_G3 ORF2-Bam Rev, corresponding to aa 606 of *ORF2*) (Table 1). KOD Plus DNA polymerase (TOYOBO, Osaka, Japan) was used for PCR amplification.

Table 1. Primers used in this study.

Primer Name	Sequence (5′–3′)	Note
hHEV_G3 Nde-ORF2 aa12 Fw	CATATG GTGCTTCTGCCTATGCTGCC	Nde I site (underlined)
hHEV_G3 Nde-ORF2 aa14 Fw	CATATG CTGCCTATGCTGCCCGCGCC	Nde I site (underlined)
hHEV_G3 Nde-ORF2 aa61 Fw	CATATG CCCTTTGCCGCCGATGTCG	Nde I site (underlined)
hHEV_G3 Nde-ORF2 aa89 Fw	CATATG GACCAGTCCCAGCGCCCCTC	Nde I site (underlined)
hHEV_G3 Nde-ORF2 aa107 Fw	CATATG GCTGCGCCGTTGACTGCTATC	Nde I site (underlined)
hHEV_G3 Nde-ORF2 aa112 Fw	CATATG GCTATCTCACCAGCCCCTGAC	Nde I site (underlined)
hHEV_G3 Nde-ORF2 aa117 Fw	CATATG CCTGACACAGCCCCTGTACC	Nde I site (underlined)
hHEV_G3 Nde-ORF2 aa122 Fw	CATATG GTACCTGATGTTGATTCACG	Nde I site (underlined)
hHEV_G3 Nde-ORF2 aa368 Fw	CATATG ATTGCTCTGACACTGTTCAAC	Nde I site (underlined)
rbHEV_G3 Nde-ORF2 aa112 Fw	CATATG GCTGTTTCACCTGCACCTG	Nde I site (underlined)
rbHEV_G3 Nde-ORF2 aa368 Fw	CATATG ATAGCCCTGACGCTGTTTAAC	Nde I site (underlined)
hHEV_G4 Nde-ORF2 aa112 Fw	CATATG GCTGTGGCCCCGGCCCCCGA	Nde I site (underlined)
hHEV_G4 Nde-ORF2 aa114 Fw	CATATG GCCCCGGCCCCCGATACTGC	Nde I site (underlined)
hHEV_G4 Nde-ORF2 aa117 Fw	CATATG CCCGATACTGCTCCTGTTCC	Nde I site (underlined)
hHEV_G4 Nde-ORF2 aa366 Fw	CATATG GTCGGTCGTGGTATAGCGC	Nde I site (underlined)
hHEV_G4 Nde-ORF2 aa368 Fw	CATATG CGTGGTATAGCGCTAACTCTG	Nde I site (underlined)
hHEV_G4 Nde-ORF2 aa370 Fw	CATATG ATAGCGCTAACTCTGTTCAATC	Nde I site (underlined)
hHEV_G3 ORF2-Bam aa606 Rev	GGATCC CTA AGCCGAGTGCGGGGCTAGTAC	BamH I site (underlined)
rbHEV_G3 ORF2-Bam aa606 Rev	GGATCC CTA AACTGAGTGCGGAGCAAGC	BamH I site (underlined)
hHEV_G4 ORF2-Bam aa606 Rev	GGATCC CTA CGCAGAGTGAGGTGCGAGGAC	BamH I site (underlined)
hHEV_G4 ORF2-Bam aa609 Rev	GGATCC CTA AGCGGCCAGCGCAGAGTGAG	BamH I site (underlined)
hHEV_G4 ORF2-Bam aa612 Rev	GGATCC CTA GTCCTCTAAAGCGGCCAGCG	BamH I site (underlined)
rbHEV_G3 T6686C Fw	TACGGCTCGTCAACTAACCC	nt 6684-6703 [a] (mutated nt underlined)
rbHEV_G3 T6686C Rev	TGTAGTTTGATCATACTCTG	nt 6664-6683 [a]

[a] Nucleotide positions are numbered in accordance with the rbIM223L strain (LC775585).

In addition, two expression plasmids for HEV-3ra ORF2 proteins of the rbIM223L strain [34] were constructed with truncated N- and C-termini (aa 112–606 and 368–606 of ORF2) (Figure 1A). The nucleotide sequences corresponding to aa 112–606 and 368–606 of *ORF2* were amplified by PCR utilizing an *ORF2* cDNA clone derived from the culture medium of the rbIM223L strain (LC775585) as a template. Prior to the amplification, T at nt 6686 within the template had been converted to C through inverted RT-PCR utilizing the rbHEV_G3 T6686C Fw and rbHEV_G3 T6686C Rev primers (Table 1) to eliminate the internal Nhe I sequence (CATATG), without altering the amino acid sequence. The primer set included a sense primer with an introduced Nde I site (rbHEV_G3 Nde-ORF2 Fw, corresponding to aa 122 or 368 of *ORF2*) and an antisense primer with a BamH I site (rbHEV_G3 *ORF2*-Bam Rev, corresponding to aa 606 of *ORF2*) (Table 1). KOD Plus DNA polymerase was used for amplification.

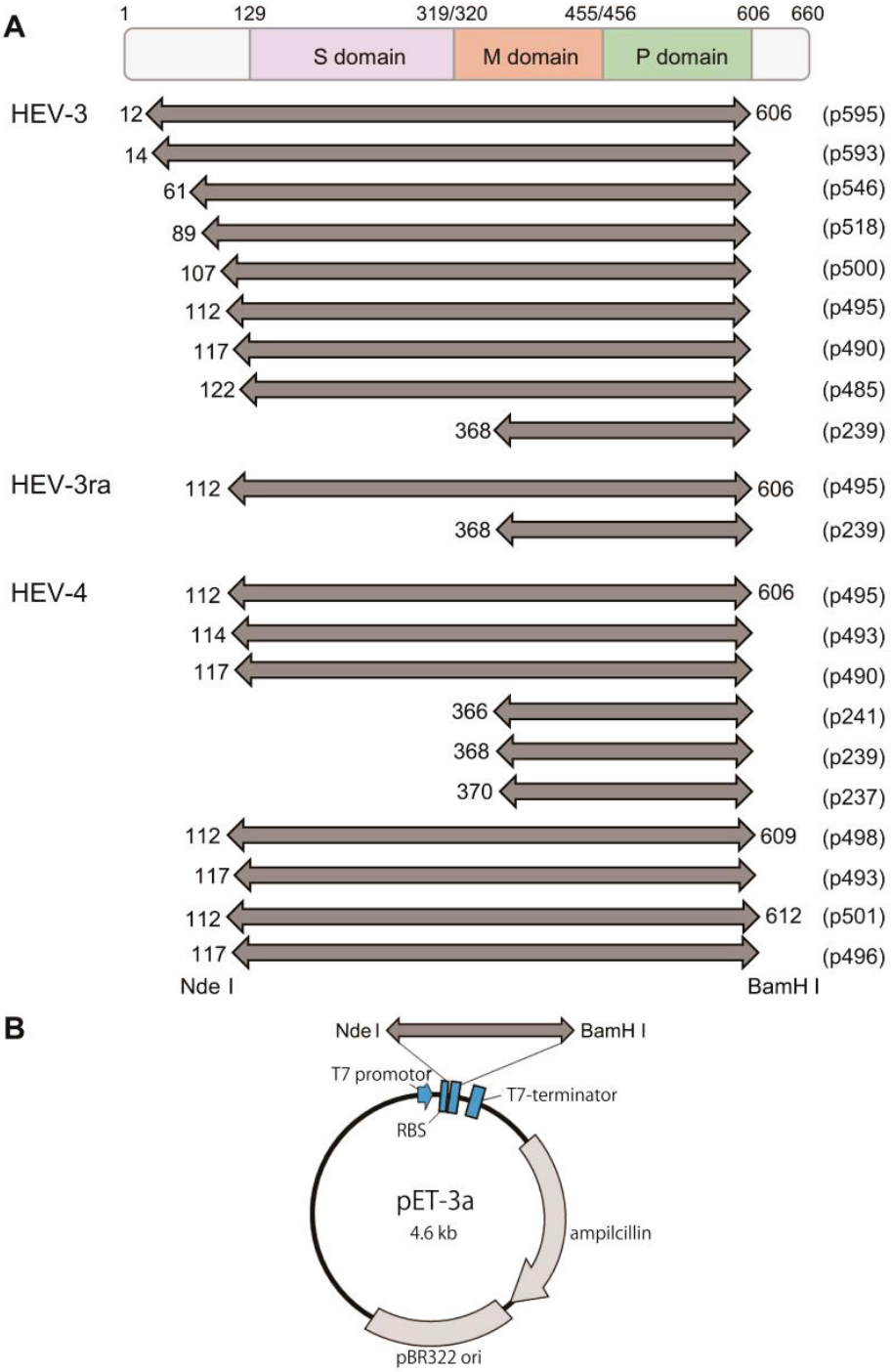

Figure 1. Construction of expression plasmids for truncated HEV ORF2 proteins. (**A**) A schematic diagram illustrating the three distinct domains within the ORF2 (capsid) protein: S (shell: aa 129–319), M (middle: aa 320–455), and P (protruding: aa 456–606) [57]. Nine truncated HEV-3 ORF2 proteins with varying lengths (aa 12–606, 14–606, 61–606, 89–606, 107–606, 112–606, 117–606, 122–606, and 368–606), two truncated HEV-3ra ORF2 proteins with different lengths (aa 112–606 and 368–606), and ten truncated HEV-4 ORF2 proteins with different lengths (aa 112–606, 114–606, 117–606, 366–606, 368–606, 370–606, 112–609, 117–609, 112–612, and 117–612) are depicted. Each protein's nucleotide sequence includes Nde I and BamH I sites at the 5′- and 3′-ends, respectively. (**B**) A map of the recombinant plasmid pET-3a vector, showing the insertion of the partial HEV *ORF2* sequence at the Nde I and BamH I sites.

Furthermore, ten expression plasmids for HEV-4 ORF2 proteins of the HE-JF5/15F strain (subtype 4c) [58] were constructed with truncated N- and C-termini (aa 112–606, 114–606, 117–606, 366–606, 368–606, 370–606, 112–609, 117–609, 112–612, and 117–612 of *ORF2*) (Figure 1A). The nucleotide sequences corresponding to aa 112–606, 114–606, 117–606, 366–606, 368–606, 370–606, 112–609, 117–609, 112–612, and 117–612 of *ORF2* were amplified by PCR using the full-length HE-JF5/15F cDNA clone (LC775584) as a template. The primer set consisted of a sense primer with an introduced Nde I site (hHEV_G4 Nde-*ORF2* Fw, corresponding to aa 112, 114, 117, 366, 368, or 370 of *ORF2*) and an antisense primer with a BamH I site (hHEV_G4 ORF2-Bam Rev, corresponding to aa 606, 609, or 612 of *ORF2*) (Table 1). KOD Plus DNA polymerase was employed for PCR amplification.

The resulting amplicons were excised and ligated into the Nde I and BamH I sites of the pET-3a expression vector (Merck, Darmstadt, Germany) (Figure 1B). A total of 21 plasmids with an insert of desired length (Figure 1A) were confirmed through nucleotide sequencing. Sequencing was performed using the Applied Biosystems 3130xl Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA) in conjunction with the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific). A sequence analysis was performed using the Genetyx software program (version 13; Genetyx Corp., Tokyo, Japan).

Protein expression was carried out in *E. coli* strain BL21 (DE3) (New England BioLabs, Ipswich, MA, USA) using 100 mL of TB medium containing 12 g/L tryptone, 24 g/L yeast extract, 8 mL/L glycerol, 9.4 g/L K_2HPO_4, 2.2 g/L KH_2PO_4, and 0.1 mg/L ampicillin. The bacterial cultures were grown at 37 °C until reaching an optical density (OD600) of 1.0. Protein expression was then induced by the addition of 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubation at 25 °C overnight. The cultures were centrifuged at 2150× *g* for 20 min at 4 °C, and the resulting pellets were either used immediately for the formation of VLPs or stored at −80 °C until further use.

2.2. Purification of Recombinant HEV ORF2 Proteins

Bacterial cell pellets obtained from a 100 mL culture in TB medium were resuspended in a sonication buffer at pH 7.5, containing 10 mM NaH_2PO_4-$2H_2O$, 10 mM Na_2HPO_4-$12H_2O$, 100 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol, 1 μg/mL RNase A, and 1 μg/mL DNase I. The suspension was then subjected to sonication on ice using an ultrasonic homogenizer (NR-50M; MICROTEC Co., LTD, Chiba, Japan) at 65% amplitude for 12 cycles of 1 min pulses. Following sonication, the lysate was incubated with 2% (*v*/*v*) Triton X-100 at 37 °C for 30 min. To separate the lysate into a supernatant fraction and a pellet fraction containing inclusion bodies, the mixture was centrifuged at 2150× *g* at 4 °C for 15 min. The resulting pellet was washed twice with the sonication buffer. The inclusion bodies were subsequently solubilized in 10–30 mL of 4 M urea buffer at pH 7.5, containing 10 mM NaH_2PO_4-$2H_2O$, 10 mM Na_2HPO_4-$12H_2O$, 100 mM NaCl, and 4 M urea, followed by sonication at 70% amplitude for 5–20 cycles of 40 s pulses. The solubilized proteins were clarified by centrifugation at 20,400× *g* for 5 min at 20 °C, and the resulting supernatant was collected. This supernatant was subsequently passed through 0.45 μm pore-sized microfilters (Sterivex-HV; EMD Millipore Corp., Billerica, MA, USA) to ensure further clarification. Eleven milliliters of the filtered supernatant were subsequently then subjected to gel filtration chromatography using fast protein liquid chromatography (FPLC) on a HiPrep 16/60 Sephacryl S-400 HR column (GE Healthcare Japan, Tokyo, Japan) at 4 °C, with a flow rate ranging from 1.0 to 1.4 mL/min. This process aimed to facilitate protein renaturation, assembly, and purification, with phosphate-buffered saline (PBS) employed as the running buffer. Fractions containing VLPs, each with a volume of 4 mL, were pooled, typically yielding three to four fractions.

2.3. SDS-PAGE and Western Blot Analysis

Various protein samples obtained in this study—including the supernatant fraction, the pellet fraction containing inclusion bodies (obtained after centrifugation of Triton X-100-treated lysates), the supernatant containing solubilized proteins (prior to gel filtration

FPLC), and purified VLPs—were suspended in a 2× sodium dodecyl sulfate (SDS) buffer composed of 125 mM Tris-HCl (pH 6.8), 2.0% (*w*/*v*) SDS, 0.01% bromophenol blue, 20% (*v*/*v*) glycerol, and 10% (*v*/*v*) 2-mercaptoethanol. These samples were subsequently incubated at 95 °C for 5 min. The denatured samples were then subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12.5% polyacrylamide gel, followed by staining with Coomassie Brilliant Blue (CBB) or subjected to Western blot analysis. CBB staining of the gel was conducted using EzStain AQua (ATTO, Tokyo, Japan).

For Western blot analysis, proteins were separated by SDS-PAGE using either a 7.5 or 12.5% acrylamide gel after heat denaturation (either at 95 °C for 5 min or at 70 °C for 10 min) in the presence of 2-mercaptoethanol or without denaturation. The separated proteins were then transferred onto a polyvinylidene difluoride (PVDF) membrane (Immobilon 0.45 μm; Merck Millipore, Tokyo, Japan). The membrane was subsequently blocked by immersion in PBS containing 0.1% (*v*/*v*) Tween-20 (PBS-T) and 5% skim milk (BD Sciences, San Jose, CA, USA), followed by thorough washing with PBS-T. The membrane was then incubated at room temperature for 1 h with an anti-HEV ORF2 mouse monoclonal antibody (MAb) (H6225 [IgG1]: 1 μg/mL) [59], which recognizes a conformational epitope (aa 551–608) of the HEV ORF2 protein and can also bind to a partially denatured epitope following heat treatment at 70 °C for 10 min [60]. After washing steps, the membrane was further incubated with horseradish peroxidase-conjugated Affinipure goat anti-mouse IgG (ProteinTech, Rosemont, IL, USA), and then visualized by chemiluminescence assay using SuperSignalTM West Atto Ultimate Sensitivity Substrate (Thermo Fisher Scientific). Protein bands were visualized using the ImageQuant LAS500 system (GE Healthcare Japan). Precision Plus Protein Dual Color Standards (Bio-Rad Laboratories, Hercules, CA, USA) served as molecular weight markers.

2.4. Analysis of Purified HEV VLPs with the Bioanalyzer

The analysis of purified VLPs was conducted using the Agilent 2100 bioanalyzer coupled with the Protein 230 kit (Agilent Technologies, Palo Alto, CA, USA). Following a 1 mg/mL dilution with PBS, 4 μL of each sample was combined with 2 μL of sample buffer containing dithiothreitol. The resulting solutions were heated to 95 °C for 5 min and subsequently diluted with 84 μL of water. A total of 6 μL of each sample was then applied to the on-chip system for analysis. Molecular weight resolution across the size range of the assays was assessed utilizing protein sizing standards provided with the kits. Run control and data analysis were performed using the Agilent 2100 Expert software program (version B.02.09).

2.5. Transmission Electron Microscopy (TEM)

The purified VLPs were diluted to a concentration of 0.5 mg/mL and deposited on a Formvar-coated EM grid (300 mesh) for 1 min. Subsequently, the samples underwent negative staining using a 2% uranyl acetate solution for 1 min and were analyzed utilizing a transmission electron microscope (model H-7600; Hitachi, Tokyo, Japan) operating at 80 kV.

2.6. Immunoelectron Microscopy (IEM)

The purified VLPs were diluted to a concentration of 0.1 mg/mL and applied onto a Formvar-coated nickel grid (300 mesh) for 20 min. Following this, PBS containing 0.2% (*w*/*v*) bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA) (PBS-0.2% BSA) was incubated on the grid for 20 min. The fixed VLPs were then exposed to an anti-HEV ORF2 MAb (H6225) (10 μg/mL in PBS-0.2% BSA) at room temperature for 1 h. After washing with PBS-0.2% BSA, the grids were treated with anti-mouse IgG conjugated with 12 nm colloidal gold (50 μg/mL in PBS-0.2% BSA; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) at room temperature for 1 h. Subsequent to washing with PBS-0.2% BSA and PBS five times each, the grids were stained with a 2% uranyl acetate solution for 1 min before examination via TEM operating at 80 kV.

2.7. Enzyme-Linked Immunosorbent Assays (ELISAs) for the Determination of Binding with Monoclonal Antibodies (MAbs) against ORF2 Protein of Human HEV (HEV-3 and HEV-4), Rabbit HEV (HEV-3ra), or Rat HEV (HEV-C1)

Enzyme-linked immunosorbent assays (ELISAs) were conducted using 96-well microplates (Greiner Bio-One GmbH, Frickenhausen, Germany) coated with purified HEV VLPs. Briefly, 50 μL of HEV-3-VLP_112–606, HEV-3ra-VLP_112–606, and HEV-4-VLP_117–606 obtained in the present study (0.01–30 μg/mL), or rat HEV VLP (HEV-C1-VLP_357–614) (0.01–30 μg/mL) [53] diluted with PBS was added to each well. Subsequently, 100 μL of PBS containing 0.1% (*w*/*v*) BSA (PBS-0.1% BSA) was added, followed by incubation at room temperature for 1 h with shaking. After discarding the blocking buffer, the wells were washed with a washing solution containing 0.05% Tween 20 in saline. Then, 50 μL of PBS-0.1% BSA containing 0.2% Tween 20 and 1 μg/mL of anti-HEV ORF2 MAbs (H6225 or H6249 [IgG1] [59], or TA7014 [IgG2b] [61]) were added to each well, followed by another incubation at room temperature for 1 h with gentle agitation, and subsequent washing. Next, 50 μL of PBS containing 25% (*v*/*v*) fecal calf serum (FCS) (Sigma-Aldrich) and peroxidase-conjugated goat IgG fraction to mouse IgG (whole molecule) (MP Biomedicals, LLC-Cappel, Santa Ana, CA, USA) was added to each well, followed by another incubation at room temperature for 1 h with gentle agitation, and subsequent washing. Then, 50 μL of tetramethylbenzidine (TMB) soluble reagent (BioFX Laboratories, Inc., Owings Mills, MD, USA) was added to each well as a substrate. The plate was incubated at room temperature for 30 min in the dark, and then 50 μL of TMB stop buffer (BioFX Laboratories, Inc.) was added to each well. Finally, the optical density (OD) of each sample was measured at 450 nm.

2.8. Immunization of HEV VLPs in Mice

To evaluate the immunogenicity of HEV VLPs, three BALB/cAJcl mice each received intraperitoneal injections of 50 μg of HEV-3-VLP_112–606, HEV-3ra-VLP_112–606, or HEV-4-VLP_117–606 in complete Freund's adjuvant (DIFCO Laboratories, Detroit, MI, USA) on day 0 and incomplete adjuvant (DIFCO Laboratories) on day 14, and serum samples were collected on day 28 at the Institute of Immunology Co., Ltd. (Tokyo, Japan). All procedures involving mice were approved by the Animal Ethics Committee of the Institute of Immunology, Co., Ltd. A pool of three non-immunized BALB/cAJcl mice served as a negative control. In addition, sera from two BALB/cAJcl mice immunized with rat HEV (HEV-C1) VLP [53] were included for comparison.

Serum samples were diluted from 3000 to 3,000,000 times with PBS-0.1% BSA and subjected to ELISA using three VLPs of HEV-3-VLP_112–606, HEV-3ra-VLP_112–606, or HEV-4-VLP_117–606 obtained in this study, rat HEV (HEV-C1) VLP_357–614 [53], or four recombinant ORF2 proteins of HEV-1, HEV-3, HEV-4 (aa 112–660) [59,62], or HEV-C1 (aa 101–644) [61] immobilized on wells of an immunoplate (Greiner Bio-One GmbH). Amino acid sequences of the immobilized proteins are depicted in Figure 2. Bound antibodies were detected using peroxidase-conjugated goat IgG fraction to mouse IgG (whole molecule) (MP Biomedicals), as described above.

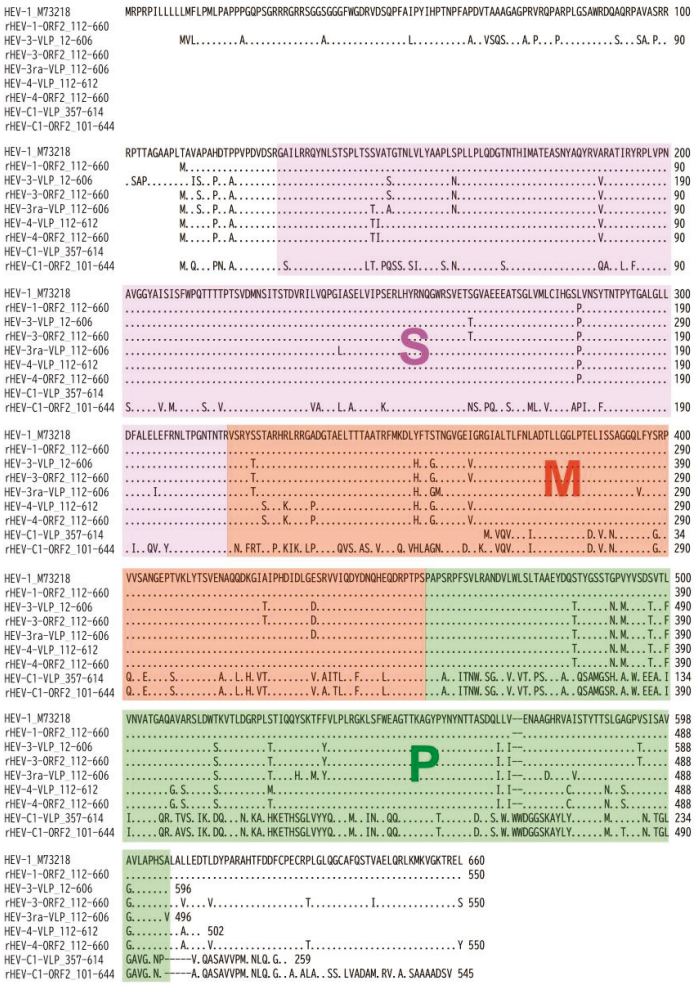

Figure 2. Comparison of amino acid sequences of various HEV ORF2 proteins used in this study. The 660-amino acid ORF2 sequence of the prototype HEV-1 strain (M73218) is displayed at the top. Below, the amino acid sequences of the longest HEV-3, HEV-3ra, and HEV-4 ORF2 proteins, expressed in *E. coli* in this study, are aligned. In addition, the sequences of three recombinant ORF2 proteins (aa 112–660) of HEV-1 (AB360347), HEV-3 (AB360348), and HEV-4 (AB082545) expressed in the silkworm pupae in our previous studies [58,61] are also aligned. The amino acid sequences of the rat HEV (HEV-C1) ORF2 protein, expressed in *E. coli* in our previous study [53] and in the silkworm pupae in our previous study [62], are included as well. Identical amino acids compared to the top sequence are represented by dots. The ORF2 protein's three distinct domains—S (shell: aa 129–319), M (middle: aa 320–455), and P (protruding: aa 456–606) [57]—are highlighted in purple, red, and green, respectively.

3. Results

3.1. The Expression and Purification of Truncated HEV ORF2 Proteins

For HEV-3, nine types of recombinant plasmid DNAs were constructed by inserting the HEV-3 *ORF2* sequence lacking the 5′-end sequence (corresponding to aa 1–11 to 1–367 (11–367 aa)) and 3′-end sequence (corresponding to aa 607–660 (54 aa)) into the pET-3a vector (Figure 1A), and eight of nine truncated ORF2 proteins with the expected length (26–56 kDa) were found to be efficiently expressed in *E. coli* BL21 (DE3) that had been transformed with the expression plasmid DNAs and induced with IPTG; aa 12–606 proteins were expressed less efficiently (Figure 3). For HEV-3ra, two types of recombinant plasmid DNAs were constructed by inserting the HEV-3ra *ORF2* sequence lacking the 5′-end sequence (corresponding to aa 1–111 or 1–367 (111 or 367 aa)) and 3′-end sequence (corresponding to aa 607–660 (54 aa)) into the pET-3a vector (Figure 1A), and both truncated ORF2 proteins with the expected length (53 or 26 kDa) were found to be efficiently

expressed in *E. coli* (Figure 3). In addition, for HEV-4, ten types of recombinant plasmid DNAs were constructed by inserting the HEV-4 *ORF2* sequence lacking the 5′-end sequence (corresponding to aa 1–111 to 1–369 (111–369 aa)) and 3′-end sequence (corresponding to aa 607–660 to 613–660 (48–54 aa)) into the pET-3a vector (Figure 1A), and all ten truncated ORF2 proteins with the expected length (26–53 kDa) were found to be efficiently expressed in *E. coli* (Figure 3).

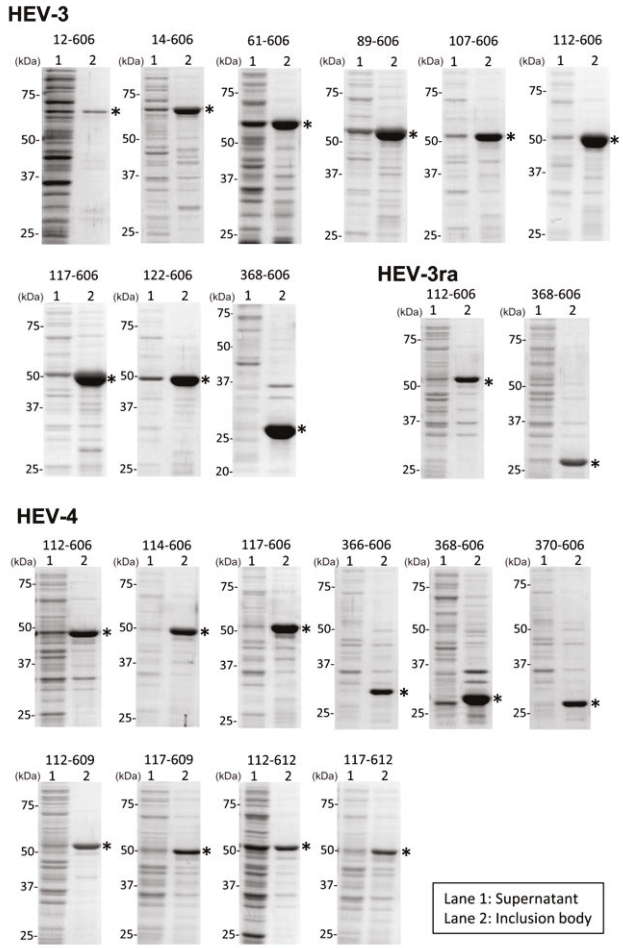

Figure 3. SDS-PAGE analysis of truncated HEV ORF2 proteins expressed in *E. coli*. The supernatant fraction (lane 1) and pellet fraction containing inclusion bodies (lane 2)—obtained after centrifugation of Triton X-100-treated lysates from cells expressing nine HEV-3 ORF2 proteins, two HEV-3ra ORF2 proteins, or ten HEV-4 ORF2 proteins with the indicated amino acid range—were subjected to SDS-PAGE on a 12.5% polyacrylamide gel and subsequently stained with Coomassie Brilliant Blue (CBB). One microliter of each fraction, corresponding to 5 μL of TB medium, were applied. Asterisks denote the expressed proteins of the desired length.

Because the ORF2 proteins were mainly expressed in inclusion bodies in all constructs tested (Figure 3), the inclusion bodies were collected after the sonication of bacterial cells and solubilized in 4 M urea. The supernatants harvested by centrifugation of the cell lysate samples containing the target proteins were visualized by SDS-PAGE as proteins with an expected length of 26–56 kDa (Figure 4, left panels), and then subjected to gel filtration. With regard to HEV-3 ORF2 proteins of aa 12–606, 14–606, and 61–606, HEV-3ra ORF2 proteins of aa 368–606, and HEV-4 ORF2 proteins of aa 112–606, 366–606, 368–606, 370–606, 112–609, 117–609, 112–612, and 117–612, only UV peaks corresponding to hexamer and dimer fractions were discernible. In contrast, regarding HEV-3 ORF2 proteins of aa 89–606, 107–606, 112–606, 117–606, 122–606, and 368–606, HEV-3ra ORF2 proteins of aa 112–606, and HEV-4 ORF2 proteins of aa 114–606 and 117–606, a UV peak of VLP

fraction was mainly found (Figure 4, right panels). These results indicated that among the expressed truncated HEV ORF2 proteins, six out of nine HEV-3 proteins, one out of two HEV-3ra proteins, and two out of ten HEV-4 proteins are capable of self-assembling into VLPs. Consequently, a total of nine HEV VLPs were successfully obtained. For subsequent analyses, solutions pooled from three to four VLP-containing fractions, as indicated by the red horizontal bar, were utilized.

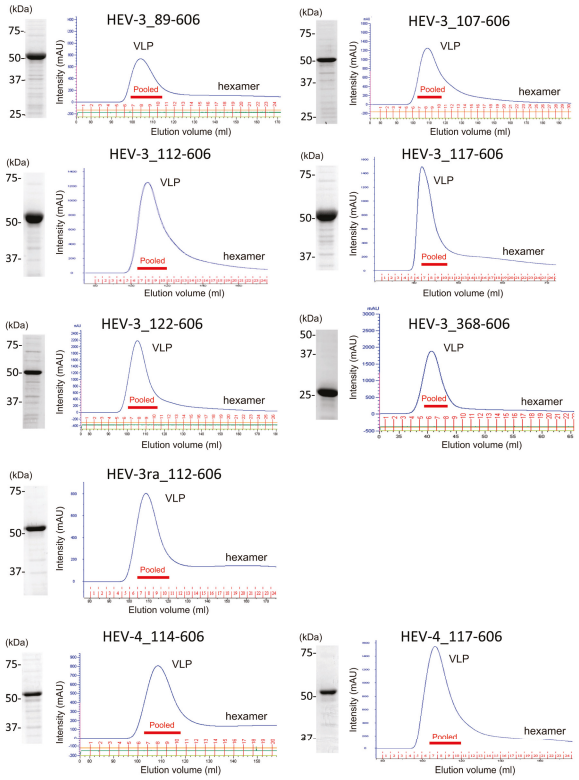

Figure 4. SDS-PAGE and gel filtration chromatography of HEV ORF2 proteins from sonicated and filtered solutions. The **left panel** shows the results of SDS-PAGE, while the **right panel** represents gel filtration chromatography (FPLC) data for HEV ORF2 proteins derived from sonicated and filtered solutions. These solutions were prepared in 4 M urea buffer and included six HEV-3 ORF2 proteins, one HEV-3ra ORF2 protein, and two HEV-4 ORF2 proteins. For SDS-PAGE, 1 µL of each solution was loaded on a 12.5% polyacrylamide gel and stained with Coomassie Brilliant Blue (CBB). In the gel filtration chromatography, 10 mL of solution containing the HEV ORF2 proteins, which were expressed from 33.3–100 mL of TB medium, was applied. The red horizontal bars indicate pooled fractions containing virus-like particles (VLPs) collected from three to four adjacent fractions.

3.2. Characterization of the Purified VLPs of HEV-3, HEV-3ra, and HEV-4

In the solutions containing purified VLPs, SDS-PAGE analysis confirmed the presence of proteins with the expected molecular weight ranges of 26 kDa and 52–56 kDa, corresponding to the monomeric forms of nine VLPs (Figure 5A). Western blot analysis of non-denatured VLPs, utilizing anti-HEV ORF2 MAb (H6225)—which specifically recognizes a conformational epitope (aa 551–608) of the HEV ORF2 protein [59,60]—revealed that three randomly selected HEV-3 VLPs (aa 89–606, 112–606, and 117–606) were detectable by MAb H6225 at molecular weights higher than those of their denatured monomeric forms. This finding confirmed that the VLPs possess the conformational epitope of the HEV ORF2 protein. Supporting this observation, the corresponding bands were undetectable by MAb H6225 when the HEV-3 VLPs (aa 89–606, 112–606, and 117–606) were heat-denatured at 95 °C for 5 min (Figure 5B). However, when the VLPs were subjected to heat treatment at 70 °C for 10 min (Figure 5C), they were detectable by MAb H6225 at molecular weights

consistent with those observed in the SDS-PAGE analysis (Figure 5A), suggesting that the purified VLPs originated from the HEV ORF2 protein.

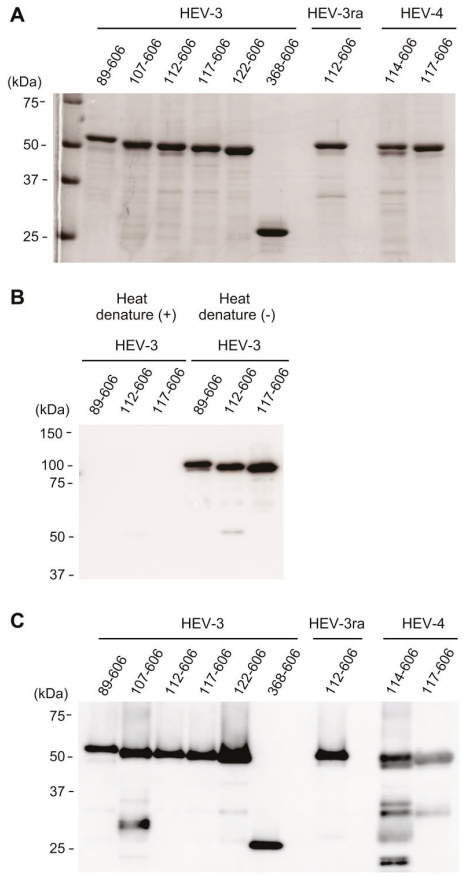

Figure 5. SDS-PAGE and Western blot analyses of purified virus-like particles (VLPs). (**A**) SDS-PAGE analysis of the purified virus-like particles (VLPs). A total of 4 μg of purified VLPs was heat-denatured at 95 °C for 5 min, loaded onto a 12.5% polyacrylamide gel, and stained by Coomassie Brilliant Blue (CBB). (**B**) A Western blot analysis of three representative purified VLPs using the anti-HEV ORF2 monoclonal antibody (MAb) (H6225) [59]. The samples contained 0.2 μg of purified HEV-3 VLPs (aa 89–606, 112–606, and 117–606), with or without heat denaturation at 95 °C for 5 min, and were loaded onto a 7.5% polyacrylamide gel. (**C**) A Western blot analysis of all nine purified VLPs using the anti-HEV ORF2 MAb (H6225). The samples contained 0.2 μg of purified VLPs, subjected to a partial denaturation at 70 °C for 10 min, and were loaded onto a 12.5% polyacrylamide gel. Notably, in repeated experiments, shorter additional bands, distinct from the expected band in the VLPs of HEV-3_107–606, HEV-4_114–606, and HEV-4_117–606, as observed in this figure, were likely due to protein degradation. These bands were not observed in other sample lots.

Bioanalyzer assessment revealed that the purified VLPs exhibited molecular weights ranging from 27.1 to 63.4 kDa with a purity of 74.7–95.3%. The yields of the VLPs were estimated to range from 13.9 to 89.6 mg per 100 mL of TB medium (Table 2). The reproducibility of the yields was confirmed from three independent experiments, demonstrating consistent yield and purity.

When observed with a TEM, VLPs of 20–50 (average 30) nm in diameter were visible (Figure 6, upper panels). Next, in order to confirm whether all of the observed particles of different sizes were derived from HEV ORF2, anti-HEV ORF2 MAb (TA6225), which recognizes the three-dimensional structure of HEV particles [59,60], and a mouse-IgG antibody, to which colloidal gold was conjugated, were used. IEM showed that colloidal gold was attached to VLPs of variable sizes (Figure 6, lower panels), indicating that the observed particles were derived from HEV ORF2.

Table 2. The yields of purified virus-like particles (VLPs) expressed in 100 mL of TB medium (*E. coli*).

Purified VLPs	Amount (mL) Recovered after Gel Filtration	Concentration (Mean ± SD, mg/mL) [a]	Molecular Weight (Mean ± SD, kDa) [b]	Yield (Mean ± SD, mg)	Purity (Mean ± SD, %) [b]
HEV-3-VLP_89-606 (p518)	48	0.5 ± 0.1	63.4 ± 0.7	24.0 ± 6.8	89.5 ± 2.8
HEV-3-VLP_107-606 (p500)	48	1.4 ± 0.1	61.1 ± 0.4	66.0 ± 6.0	90.7 ± 4.1
HEV-3-VLP_112-606 (p495)	48	1.9 ± 0.3	61.0 ± 0.5	89.6 ± 15.4	95.3 ± 1.4
HEV-3-VLP_117-606 (p490)	48	1.3 ± 0.1	60.3 ± 0.6	64.0 ± 5.5	91.9 ± 2.3
HEV-3-VLP_122-606 (p485)	48	1.6 ± 0.4	58.4 ± 1.1	76.8 ± 17.3	95.0 ± 1.6
HEV-3-VLP_368-606 (p239)	12	1.4 ± 0.6	27.1 ± 0.5	16.2 ± 7.6	93.1 ± 0.1
HEV-3ra-VLP_112-606 (p495)	16	0.9 ± 0.2	62.7 ± 0.6	13.9 ± 3.7	94.9 ± 1.9
HEV-4-VLP_114-606 (p493)	48	0.8 ± 0.1	61.9 ± 0.7	39.2 ± 5.0	74.7 ± 5.8
HEV-4-VLP_117-606 (p490)	48	1.3 ± 0.4	60.7 ± 0.3	62.4 ± 20.4	90.1 ± 8.5

[a] Mean ± standard deviation (SD) protein concentration values, quantified using the NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) from three independent experiments, are indicated. [b] Mean ± SD molecular weight or purity values, determined using the Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) from three independent experiments, are indicated.

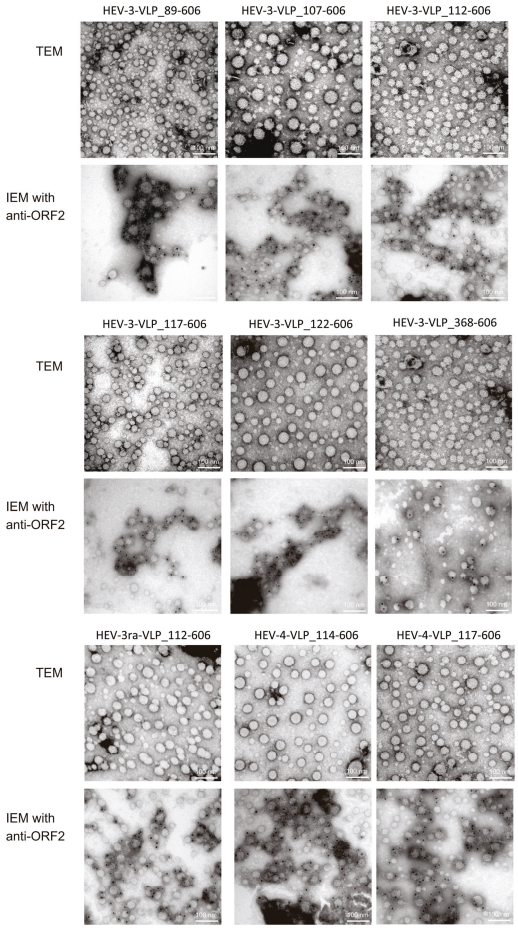

Figure 6. Electron micrographs of purified virus-like particles (VLPs). Electron micrographs of nine purified virus-like particles (VLPs) with the indicated genotype and amino acid range were analyzed using transmission electron microscopy (TEM, **upper panels**). The size of VLPs varied depending on the field of the electron microscope grid, with each image displaying representative VLPs averaging ~30 nm in diameter. Immunoelectron microscopy (IEM) was performed on the purified VLPs using anti-HEV ORF2 monoclonal antibody (H6225) [59] and immunogold-conjugated anti-mouse IgG (**lower panels**). The IEM images reveal heterogeneous VLPs ranging from 20 nm to 50 nm in diameter, coated with gold-labeled antibodies. Scale bar represents 100 nm.

3.3. The Antigenicity of the Purified VLPs of HEV-3, HEV-3ra, and HEV-4

The antigenicity of purified HEV VLPs was assessed using ELISAs using two anti-HEV ORF2 MAbs (H6225 and H6249) and one anti-rat HEV ORF2 MAb (TA7014). VLPs of HEV-3, HEV-3ra, and HEV-4, as well as HEV-C1 for comparison, were serially diluted from

30 μg/mL to 0.01 μg/mL, immobilized on a 96-well microplate, and detected by MAbs capable of recognizing the conformational epitope of HEV ORF2 (H6225) or rat HEV ORF2 (TA7014). These MAbs bound to HEV-3/3ra/4-VLPs or rat HEV VLPs, respectively, and detection occurred in a concentration-dependent manner (Figure 7). In contrast, VLPs of HEV-3, HEV-3ra, and HEV-4 were not detectable by an MAb against the linear epitope of HEV ORF2 protein (H6249) or an MAb against the conformational epitope of the rat HEV ORF2 protein (TA7014). These results indicate that the purified VLPs of HEV-3, HEV-3ra, and HEV-4 possess the antigenicity characteristic of HEV particles.

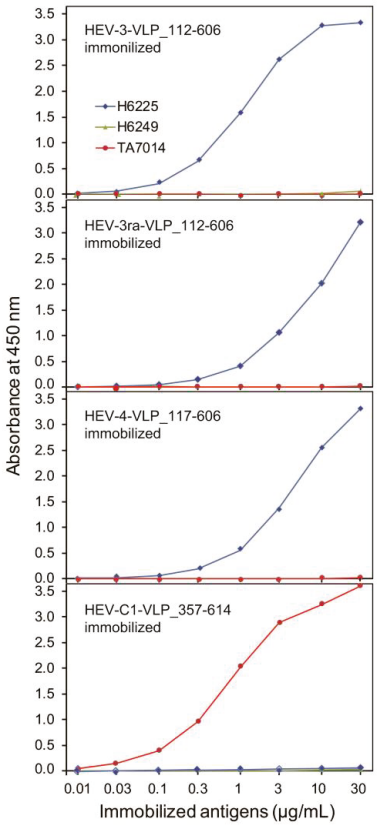

Figure 7. Assessment of the antigenicity of purified virus-like particles (VLPs) by enzyme-linked immunosorbent assay (ELISA). The antigenicity of HEV-3, HEV-3ra, and HEV-4 virus-like particles (VLPs) with the specified amino acid lengths was evaluated using an enzyme-linked immunosorbent assay (ELISA). Three monoclonal antibodies (MAbs) were employed in this analysis: H6225, which targets a conformational epitope of the human HEV ORF2 protein [59]; H6249, which targets a linear epitope of the human ORF2 protein [59]; and TA7014, which targets a conformational epitope of the rat HEV ORF2 protein [61]. The HEV-3, HEV-3ra, and HEV-4 VLPs were captured by MAb H6225 in a concentration-dependent manner, whereas the rat HEV (HEV-C1) VLP was captured by MAb TA7014 in a concentration-dependent manner. As a negative control, no HEV-3, HEV-3ra, or HEV4 VLPs were captured by MAb H6249.

3.4. Immune Response of the Balb/cAJcl Mice to the Purified VLPs of HEV-3, HEV-3ra, and HEV-4

To evaluate the immunogenicity of the VLPs of HEV-3, HEV-3ra, and HEV-4, three Balb/cAJcl mice per group were immunized twice, on days 0 and 14, with serum samples collected on day 28. The presence of anti-HEV IgG was determined using ELISA. Four different VLPs (HEV-3, HEV-3ra, HEV-4, and HEV-C1) and four recombinant non-particulate HEV ORF2 proteins (HEV-1, HEV-3, HEV-4, and HEV-C1) were employed as the immobilized antigens. In mice immunized with the VLPs of HEV-3, HEV-3ra, or HEV-4, anti-HEV IgG was detectable using six distinct ELISAs, which utilized VLPs of HEV-3,

HEV-3ra, or HEV-4, or non-particulate HEV ORF2 proteins of HEV-1, HEV-3, or HEV-4 as immobilized antigens, even after a 300,000-fold dilution (Figure 8). This suggests that HEV VLPs produced in this study exhibit high immunogenicity and can induce antibodies against both conformational and linear epitopes of the HEV ORF2 protein. In contrast, mice immunized with the VLP of HEV-C1 displayed a weak anti-HEV IgG response. However, strong anti-rat HEV IgG responses were detectable using ELISAs with HEV-C1-VLP or non-particulate HEV-C1 protein, even after a 1,000,000-fold dilution (Figure 8).

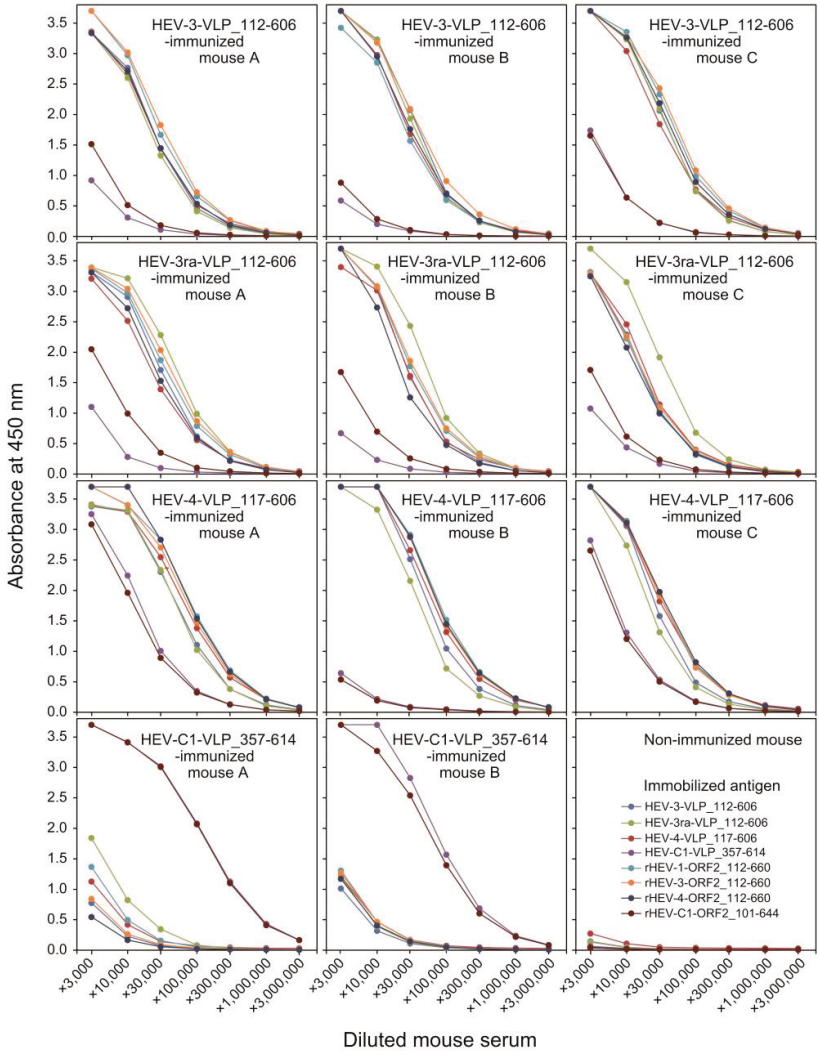

Figure 8. Immunogenicity assessment of antibodies against virus-like particles (VLPs) and recombinant HEV ORF2 proteins in mice immunized with VLPs. Anti-HEV IgG was detected in sera of Balb/cAJcl mice (n = 3 per group, designated as mouse A to C) immunized with virus-like particles (VLPs) of HEV-3, HEV-3ra, or HEV-4. Sera from mice immunized with HEV-C1 VLPs in our previous study [53] were used as a reference. Enzyme-linked immunosorbent assays (ELISAs) were conducted using VLPs (HEV-3, HEV-3ra, HEV-4, and HEV-C1) and recombinant HEV ORF2 proteins (HEV-1, HEV-3, HEV-4, and HEV-C1) as immobilized antigens. Mice immunized with HEV-3, HEV-3ra, or HEV-4 VLPs exhibited detectable anti-HEV IgG responses at a serum dilution of up to 1:300,000 across six different ELISAs. In contrast, mice immunized with HEV-C1 VLPs showed a weaker anti-HEV IgG response against the same antigen panel but exhibited a strong anti-rat HEV IgG response in ELISAs using HEV-C1 VLPs or recombinant HEV-C1 protein, detectable even at a 1,000,000-fold dilution.

4. Discussion

The present study revealed that various N- and C-terminally truncated HEV ORF2 proteins (with the shortest spanning aa 370–606 and the longest spanning aa 12–606) from HEV-3 (eight out of nine proteins), HEV-3ra (both proteins), and HEV-4 (all ten proteins) were successfully expressed in *E. coli*. Among these, six HEV-3, one HEV-3ra, and two HEV-4 proteins efficiently self-assembled into VLPs. Seven of the nine purified VLPs (27.1–63.4 kDa) exhibited high purity (> 90%), with high yields of 14–90 mg per 100 mL of TB medium. The VLPs exhibited variable sizes, with diameters of 20–50 nm (average 30 nm). Western blot analysis and IEM, utilizing an anti-HEV ORF2 MAb (H6225) that recognizes the conformational epitope of the HEV ORF2 protein and neutralizes HEV infection in vitro [59,60], confirmed that the particles were derived from the HEV ORF2 protein. Their antigenicity was verified by ELISA, demonstrating that these VLPs possess characteristic HEV ORF2 antigenicity. When tested for immunogenicity in Balb/cAJcl mice, the VLPs elicited strong anti-HEV IgG responses, detectable even at high dilutions, indicating that the HEV VLPs are highly immunogenic and capable of eliciting robust immune responses against both conformational and linear epitopes of the HEV ORF2 protein. In contrast, VLPs from rat HEV (HEV-C1), obtained in our previous study [53], induced weak anti-HEV IgG but strong anti-rat HEV IgG responses (Figure 8). Overall, this study highlights the potential of these VLPs as effective immunogens for future HEV vaccine development. It also suggests the necessity of utilizing VLPs from both HEV (HEV-3, HEV-3ra, or HEV-4) and rat HEV (HEV-C1) to prevent infection from zoonotic HEV in humans.

In the initial stages of our current study, inclusion bodies in bacterial cell pellets (Section 2.2) were solubilized in 4 M urea buffer and subjected to dialysis to allow the resulting proteins to be renatured and assembled, following the method used for preparing rat HEV-VLPs in our previous study [53]. However, during dialysis, nearly all expressed proteins, including those with longer amino acid sequences, formed white turbidity, likely due to protein aggregation, making it challenging to consistently construct stable VLPs. Considering the successful development of VLPs for HEV-1_368-606 [51] and HEV-1_112-606 [52], we hypothesized that the expressed HEV ORF2 proteins of HEV-3, HEV-3ra, and HEV-4, sharing identical N- and C-terminal ends, could potentially be refolded under optimized conditions. In light of this, we explored various refolding conditions. These included stepwise dialysis of the 4 M urea-solubilized solution in buffers containing 2 M, 1 M, and 0 M urea, the addition of 0.5 M ammonium sulfate to the 4 M urea-solubilized solution prior to dialysis, and adjustments of the dialysis buffer pH from 7.5 to 8.0 or 9.0. Despite these extensive trials, stable and reproducible formation of VLPs was not achieved following dialysis and subsequent FPLC. Therefore, we omitted the dialysis step and used gel filtration to remove the urea, allowing the solubilized proteins to be renatured and assembled. This approach eliminated the need for two time-consuming operations: dialysis and ultrafiltration concentration, reducing the process time by one day. In addition, we were able to stably and reproducibly obtain at least nine VLPs of different genotypes and varying lengths (Table 2). If the refolding process occurred at a variable rate during FPLC, the resulting VLP peaks may have broadened. Although the exact mechanism responsible for the formation of the sharp peaks observed in FPLC is not yet fully understood, it is noteworthy that this phenomenon was consistently observed across all nine HEV ORF2 proteins shown in Figure 4.

The formation of VLPs is generally considered crucial for immune recognition and response [45]. Various HEV ORF2 proteins with different lengths have been expressed in *E. coli* and evaluated for their particle-forming properties. These include E2s (aa 459–606), E2 (aa 394–606), p239 (aa 368–606), and p495 (aa 112–606) of HEV-1, and p179 (aa 439–617) of HEV-4 [45,47,51,52,63–65]. Among them, p239 (HEV-1), p495 (HEV-1), and p179 (HEV-4) exhibited particulate characteristics. Notably, p239 (HEV-1) has been licensed as the Hecolin® recombinant VLP-based vaccine against HEV in China and Pakistan [47,51,52,54,66]. In our previous study [53], among nine rat HEV ORF2 proteins expressed in *E. coli*, five proteins of aa 357–594 (corresponding to p239 of HEV-1), 357–599, 357–604, 357–609, and 357–614 exhib-

ited a particulate nature. Unexpectedly, the protein with the longest C-terminal extension (aa 357–614) exhibited the highest efficiency in VLP self-assembly. However, three rat HEV ORF2 proteins with longer N-termini (aa 109–614, 114–614, and 119–614) that covered all three domains (S + M + P) did not efficiently form VLPs [53], indicating the need for further investigation to determine the optimal N- and C-termini as well as specific amino acid sequences necessary for efficient VLP formation. This was particularly evident for the larger HEV VLPs corresponding to p495 (aa 112–606 of HEV-1), which were successfully expressed in *E. coli* and self-assembled [52]. In the current study, despite having identical N- and C-termini as p495 of HEV-1, both HEV-3_112–606 and HEV-3ra_112–606 formed particles, whereas HEV-4_112–606 did not. Notably, HEV-3_112–606 and HEV-4_112–606 differ by 4.2% (21/495) in the corresponding amino acid sequence. In addition, despite sharing identical N- and C-termini with p239 (Hecolin®, HEV-1), the smaller HEV VLPs showed differential particle formation; HEV-3_368–606 successfully formed particles, while both HEV-3ra_368–606 and HEV-4_368–606 did not consistently produce VLPs. Notably, HEV-3_368–606 differs from HEV-3ra_368–606 and HEV-4_368–606 by 3.3% (8/239) and 4.2% (10/239), respectively, in the amino acid sequence. These results suggest that the amino acid sequence plays a role in VLP formation in addition to the termini. For HEV-4, it was found that aa 606 was more conducive to VLP formation than aa 609 or aa 612 at the C-terminus, but it appears that the N-terminal amino acid requirements may vary by HEV strain.

As illustrated in Figure 1A, we have constructed expression plasmids for a range of N- and C-terminally truncated ORF2 proteins, using previous studies as references and their modifications for designing the truncations. For future investigations, we plan to incorporate a theoretical prediction model to assess VLP solubility by screening for deletions. This model will be aimed at identifying key hydrophobicity-related parameters that differentiate between aggregation conducive to VLP formation and aggregation that results in insoluble viral protein clusters [67]. In the present study, our focus was not directed towards the expression of soluble proteins, as most of the proteins expressed were predominantly found in inclusion bodies (Figure 3). In future research, we plan to enhance the solubility of target proteins expressed in *E. coli* by utilizing another pET-series vector, such as pET-15b (Merck, Darmstadt, Germany), with a solubility-enhancing tag [68].

Given the highly immunogenic nature of the capsid protein, which elicits effective virus-neutralizing antibodies, and the attractive features of VLPs composed of capsid subunits—such as safety, immunogenicity, and ease of production [44,45]—HEV VLPs are promising vaccine candidates against HEV infection, as exemplified by p239 (Hecolin®) [51]. To date, HEV VLPs have been successfully produced using *E. coli* and baculovirus–insect cell systems [45]. Notably, the baculovirus–insect cell system has yielded homogeneous VLPs with diameters of ~24 nm (T = 1 symmetry) or ~35 nm (T = 3 symmetry) for HEV-1, HEV-3, HEV-4, rabbit HEV (HEV-3ra), wild boar HEV (HEV-5 and HEV-6), and camel HEV (HEV-7), as well as rat HEV and ferret HEV [50,69–73]. In contrast, p239-based VLPs generated using the *E. coli* system have been reported to exhibit diameters of 20–30 nm, with a degree of irregularity and heterogeneity [51]. The HEV VLPs of HEV3, HEV-3ra, and HEV-4 produced in this study using the *E. coli* expression also displayed variable sizes, ranging from 20 to 50 nm, with a mean diameter of 30 nm, resembling the rat HEV VLPs in our previous study [53], despite the differences in the lengths of the expressed proteins. The formation of uneven particle sizes after expression in *E. coli* may result from the renaturation and assembly of the ORF2 protein, which had been denatured in 4 M urea under cell-free conditions, differing from the baculovirus–insect cell system. However, despite the variability in particle size, HEV-3, HEV-3ra, and HEV-4 VLPs derived from the *E. coli* expression system in this study showed a particle profile capable of eliciting strong immunity against HEV (Figure 8). In addition, the *E. coli* expression system offers significant advantages, including rapid production within two days, compared to the baculovirus–insect cell system, which requires a minimum of two weeks [52]. Furthermore, the *E. coli* system achieves a markedly higher yield, as demonstrated in Table 2.

HEVs (HEV-1 to HEV-7) belonging to the *Paslahepevirus balayani* species have been demonstrated to represent a single serotype [72,74–76]. Rat HEV (HEV-C1) differs from human HEVs (HEV-1 to HEV-4) by 60–61% over the entire genome and by 55–57% in the amino acid sequence of the ORF2 protein [77]. Reflecting the differences in the amino acid sequence between rat HEV and human HEVs, rat HEV VLPs generated in the baculovirus–insect cell system showed lower cross-reactivity with sera from HEV-1-, HEV-3-, and HEV-4-infected hepatitis E patients, despite rat HEV possessing antigenic epitope(s) in common with those of HEV-1, HEV-3, and HEV-4 HEVs [69]. In addition, prior exposure to human HEV (via infection or vaccination) does not confer protection against rat HEV infection [78], suggesting the necessity of an immunogenic rat HEV vaccine to achieve effective protection against rat HEV infection in humans. The high immunogenicity of rat HEV VLPs in mice was demonstrated in our previous study [53] and confirmed in the present study (Figure 8). Further studies are warranted on the immunogenicity of a bivalent vaccine combining HEV-VLP and rat HEV-VLP in various animal species, including Mongolian gerbil, which has been reported to support infection of both human HEV and rat HEV [79,80].

In conclusion, we successfully generated self-assembled VLPs derived from HEV-3 (n = 6), HEV-3ra (n = 1), and HEV-4 (n = 2) strains, incorporating various N- and C-terminal modifications, through an *E. coli* expression system. These findings are expected to significantly contribute to the understanding of the antigenicity and immunogenicity of HEV, and hold promise for the development of VLP-based vaccines targeting HEV infections in humans. Future research efforts should focus on elucidating the efficient and reproducible formation of VLPs using N- and C-terminally truncated HEV ORF2 proteins of diverse genotypes, particularly those with extended amino acid sequence expressed in *E. coli*. Moreover, investigations into a bivalent vaccine strategy combining two VLPs with different immunogenic profiles are warranted.

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Strain- and Subtype-Specific Replication of Genotype 3 Hepatitis E Viruses in Mongolian Gerbils

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Abstract: Since Mongolian gerbils are broadly susceptible to hepatitis E virus (HEV), including genotypes 1, 4, 5, and 8 (HEV-1, HEV-5, HEV-5, and HEV-8) and rat HEV, they are a useful small animal model for HEV. However, we have observed that the subtypes HEV-3k and HEV-3ra in genotype 3 HEV (HEV-3) were not infected efficiently in the gerbils. A small-animal model for HEV-3 is also needed since HEV-3 is responsible for major zoonotic HEV infections. To investigate whether gerbils can be used as animal models for other subtypes of HEV-3, we injected gerbils with five HEV-3 subtypes (HEV-3b, -3e, -3f, -3k, and -3ra) and compared the infectivity of the subtypes. We detected viral RNA in the gerbils' feces. High titers of anti-HEV IgG antibodies in serum were induced in all HEV-3b/ch-, HEV-3f-, and HEV-3e-injected gerbils. Especially, the HEV-3e-injected animals released high levels of viruses into their feces for an extended period. The virus replication was limited in the HEV-3b/wb-injected and HEV-3k-injected groups. Although viral RNA was detected in HEV-3ra-injected gerbils, the copy numbers in fecal specimens were low; no antibodies were detected in the sera. These results indicate that although HEV-3's infectivity in gerbils depends on the subtype and strain, Mongolian gerbils have potential as a small-animal model for HEV-3. A further comparison of HEV-3e with different genotype strains (HEV-4i and HEV-5) and different genera (rat HEV) revealed different ALT elevations among the strains, and liver damage occurred in HEV-4i- and HEV-5-infected but not HEV-3e- or rat HEV-infected gerbils, demonstrating variable pathogenicity across HEVs from different genera and genotypes in Mongolian gerbils. HEV-4i- and HEV-5-infected Mongolian gerbils might be candidate animal models to examine HEV's pathogenicity.

Keywords: genotype 3 hepatitis E virus; HEV-3; subtype; Mongolia gerbil; small-animal model

1. Introduction

Hepatitis E virus (HEV), classified in the family *Hepeviridae*, possesses a single-stranded, positive-sense RNA genome, and HEV causes type E hepatitis in humans [1,2]. The *Hepeviridae* family includes two subfamilies: *Orthohepevirinae* and *Parahepevirinae* [2,3]. *Parahepevirinae* consists exclusively of the genus *Piscihepevirus*, and the viruses in this genus infect only fish as a non-pathogenic fish virus [3,4]. *Orthohepevirinae* includes at least four genera: *Paslahepevirus*; *Rocahepevirus*; *Chirohepevirus*; and *Avihepevirus* (ictv.global/taxonomy) [3]. The genus *Paslahepevirus* is comprised of two species, *alci* and *balayani*. The species *balayani* includes at least eight genotypes, from HEV-1 to HEV-8, and it forms major HEV strains that are pathogenic to humans [3]. HEV-1 and HEV-2 are found exclusively in humans. HEV-3 and HEV-4 have been isolated from both humans and animals, including monkeys, domestic pigs, wild boars, wild deer, rabbits, and mongooses. HEV-5 and HEV-6 are detected in wild boars. HEV-7 and HEV-8 are detected in camels [2–4]. HEV-1, -2, -3, -4, and -7 infect humans and cause hepatitis E, whereas HEV-5 and HEV-8 infect monkeys

and have the potential for zoonotic infection [5–7]. Rat HEV (genus *Rocahepevirus*) is also known to transmit to monkeys and humans and cause hepatitis E [8–10].

Cell culture systems have been established to grow HEVs that belong to *Paslahepevirus* (HEV-1, HEV-3 to HEV-8) and *Rocahepevirus* (rat HEV and ferret HEV) in PLC/PRF/5 cells [6,7,11–15]. Most HEVs experimentally cross-infect cynomolgus and rhesus monkeys, and these monkeys are useful animal models for HEV infection and vaccine development [10,16–18]. However, most imported cynomolgus monkeys and farmed rhesus have been exposed to HEV [19,20], and the supply of these monkeys for infection experiments is, thus, limited. Animal models are needed for HEV research, and small-animal models are desired.

Rabbits and rats have been used as the respective animal models of HEV-3ra, HEV-4, or rat HEV; however, most HEV strains, such as HEV-1, HEV-5, and HEV-7, do not infect these animals. Our research group's earlier investigations demonstrated that Mongolian gerbils (*Meriones unguiculatus*) are broadly susceptible to HEV [21]. Although the sensitivity depended on the genotype, we observed that HEV-1, -4, -5, and -8, and rat HEV replicate in *M. unguiculatus*, thus providing a useful small-animal model for these HEVs. For example, using Mongolian gerbils as animal models, we demonstrated that open reading frame 4 (ORF 4) is not essential in the replication and infection of HEV-1 [22]. A gerbil-adapted strain that induces a robust, acute HEV infection in gerbils has also been reported [23].

HEV-3 is also responsible for major zoonotic HEV infections, and a small-animal model for HEV-3 is also needed. Mongolian gerbils have shown poor susceptibility to HEV-3 [21], and more information about HEV-3 and characterization of the strains included in this genotype are desired. HEV-3 is genetically diverse and contains at least 15 subtypes (HEV-3a to -3o) and the subtype HEV-3ra [24,25]. Recently, the HEV-3a Kernow C1 strain (HQ389544) has been confirmed to infect Mongolian gerbils [26]. We speculated that it would be informative to determine whether the subtypes of HEV-3 show differing susceptibility in Mongolian gerbils. In the present study, we used HEV-3b, -3e, -3f, -3k, and -3ra to inject Mongolian gerbils, and we then compared the strains' infectivity to determine whether the gerbils are useful as a small-animal model for these HEV-3 strains.

2. Materials and Methods

2.1. HEV Strains

Six HEV-3 strains from five subtypes were used in this study (Table 1): HEV-3b/wb (WB0567c1, LC774371); HEV3b/ch (HEV-3b-Chiba, LC786331); HEV-3e (WA1543, JQ026407); HEV-3f (JAO-SpaTok12, LC055972); HEV-3k (HEV83-2-27, AB740232); and HEV-3ra (JP-59, LC535077).

Strain HEV-3b/wb was obtained from a wild boar in Japan, and strain HEV-3k was derived from a pig fecal specimen collected from a piggery in Japan. Both strains were isolated by cell culture with a human hepatocarcinoma cell line, PLC/PRF/5 (JCRB0406) [21,27]. Strain HEV-3b/ch and strain HEV-3f were produced by a reverse genetics system using PLC/PRF/5 cells. Strains HEV-3b/ch, HEV-3b/wb, HEV-3f, and HEV-3k were grown in PLC/PRF/5 cells, and the cell culture supernatants were used for injection. HEV-3e was first detected in a Japanese monkey with persistent infection [28], and HEV-3ra was isolated from a feral rabbit by transmitting it to a Japanese white rabbit [29]. Fecal specimens collected from the above-mentioned Japanese monkey or Japanese white rabbit were used for injection in the present study. The culture supernatants or 10% fecal suspensions were centrifuged at 10,000× *g* for 30 min and passed through a 0.45-μm membrane filter (Millipore, Bedford, MA, USA). All samples were adjusted to contain viral RNA copy numbers of 10^7 copies/mL and were stored at −80 °C.

We also used three strains derived from other genotypes: HEV-4i (HEV121-12, LC657084); HEV-5 (JBOAR135-Shiz09, AB573435); and rat HEV (V-105, JX120573). Mongolian gerbils were experimentally infected with these viruses through an intraperitoneal injection, and then, fecal specimens were collected, and the 10% suspensions were clarified as described above [21].

Table 1. The hepatitis E virus (HEV) strains used in the present study.

Family	Subfamily	Genus	Species	Genotype	Subtype	Strain and Accession No.
Hepeviridae	*Orthohepevirinae*	*Paslahepevirus*	*balayani*	HEV-3	3b	HEV-3b/wb (WB0567c1. LC774371)
						HEV-3b/ch (HEV-3b-Chiba, LC786331)
					3e	HEV-3e (WA1543, JQ026407)
					3f	HEV-3f (JAO-SpaTok12, LC055972)
					3k	HEV-3k (G3HEV83-2-27, AB740232)
					3ra	HEV-3ra (JP-59, LC535077)
				HEV-4	4i	HEV-4i (G4HEV121-12, LC657084)
				HEV-5		HEV-5 (JBORA135-Shiz09, AB573435)
		Rocahepevirus	*ratti*	HEV-C1		Rat HEV (V-105, JX120573)

2.2. Injection of Mongolian Gerbils and the Sample Collection

Six-week-old female Mongolian gerbils (MON/Jms/GbsSlc, SLC, Hamamatsu, Japan) were randomly separated into six groups (n = 5 per group). Based on the results of our previous studies, each group was intraperitoneally injected with 1 mL (10^7 copies/mL) of the virus solution. The fecal specimens were collected on days 4, 7, 11, 14, 18, 21, 25, and 28 post-injection (p.i.), and the 10% fecal suspensions were used to detect the viral RNA. At the end of each experiment, the gerbils were euthanized by exsanguination from the heart under anesthesia, and the liver, spleen, bile, and serum were collected. Tissues of the liver and spleen were washed three times with phosphate-buffered saline (PBS) and homogenized with the use of a MagNA Lyser (Roche, Mannheim, Germany) according to the manufacturer's recommendations to prepare the 10% (w/v) tissue suspensions.

The animal experiments were reviewed and approved by the institutional ethics committee of Japan's National Institute of Infectious Diseases (NIID) and performed according to the Guides for Animal Experiments issued by the NIID under code 123023 (21 May 2023). All of the gerbils were negative for the serum anti-HEV IgG antibodies in an enzyme-linked immunosorbent assay (ELISA) and negative for HEV RNA as confirmed by real-time reverse transcription–quantitative polymerase chain reaction (RT-qPCR) using the fecal specimens before the injection experiments. The ELISA and RT-qPCR are described below. The gerbils were individually housed in a Biosafety Level-2 facility.

2.3. Extraction and Detection of HEV RNA

Extraction of viral RNA was carried out by a MagNA Pure 96 System (Roche Applied Science, Mannheim, Germany) with a MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche Applied Science) from 200 µL of the samples.

The copy numbers of Viral RNA were examined by a one-step RT-qPCR using TaqMan Fast Virus 1-step Master Mix (Applied Biosystems, Foster City, CA, USA) and a QuantStu-

dio 3 Real-Time PCR System (Applied Biosystems). The RT-qPCR was carried out under the condition of 5 min at 50 °C, 20 s incubation at 95 °C, followed by 40 cycles of 3 s at 95 °C and 30 s at 60 °C. A forward primer JVHEVF (5′-GGTGGTTTCTGGGGTGAC-3′ nt 5346–5363), a reverse primer JVHEVR (5′-AGGGGTTGGTTGGATGAA-3′ nt 5393–5415), and a probe JVHEVP (5′-FAM-TGATTCTCAGCCCTTCGC-TAMRA-3′ nt 5369–5386) were used to determine the RNA copy numbers of HEV-3, HEV-4, and HEV-5 [30]. A forward primer 5′-CCACGGGGGTTAATACTGC-3′ (nt 36–54), a reverse primer 5′-CGGATGCGACCAAGAAACAG-3′ (nt 189–208), and a probe 5′-FAM-CGGCTACCGCCTTT GCTAATGC-TAMRA-3′ (nt 81–102) were used to detect rat HEV RNA [14]. A 10-fold serial dilution of HEV-3 or rat HEV RNA (10^1–10^7 copies) was used as the standard to quantify the copy numbers [31]. Amplification data were collected and analyzed with Sequence Detector software ver. 1.3 (Applied Biosystems).

2.4. Detection of Anti-HEV IgG Antibodies

Serum anti-HEV IgG antibodies were detected by an ELISA using virus-like particles (VLPs) as the antigen as described with slight modification [32,33]. Flat-bottomed 96-well polystyrene microplates (Immulon 2, Dynex Technologies, Chantilly, VA, USA) were coated with the VLPs (100 ng/well): VLPs of HEV-1 were used to detect anti-HEV-3, HEV-4i, and HEV-5 IgG antibodies, and VLPs of rat HEV were used to detect the anti-rat HEV IgG antibodies. Duplicates of the serum samples (1:200) were examined.

Rabbit anti-Mongolian gerbil IgG antibody (H + L) conjugated with horseradish peroxidase (HRP) (1:1000) (Bioss, Boston, MA, USA) was used as the secondary antibody. The cut-off value of the anti-HEV IgG antibodies was 0.15, as described [21]. For the measurement of the anti-HEV IgG antibody titers, the serum samples were subjected to two-fold dilution starting from 1:200, and the highest dilution that showed a positive result was considered the antibody titer.

2.5. Determination of ALT Levels

The activities of the liver enzyme alanine transaminase (ALT) in the gerbil sera were measured using a Fuji Dri-Chem Slide GPT/ALT-PIII kit (Fujifilm, Saitama, Japan). We considered the gerbils' pre-injection mean ALT value the normal value, and a two-fold or greater increase was considered a sign of ALT elevation [21].

2.6. Histopathology

Liver tissues from HEV-infected Mongolian gerbils collected on day 28 p.i. were fixed in 10% phosphate-buffered formalin and routinely embedded in paraffin. Tissue sections were cut into 2-μm serial sections, stained with hematoxylin and eosin (H&E), and used for histopathological examination.

2.7. Statistical Analysis

Statistical analyses of viral RNA copy numbers ($\log_{10}$) and anti-HEV IgG antibody titers ($\log_2$) were performed using GraphPad Prism software ver. 10.1.2 (GraphPad, La Jolla, CA, USA). Data normality was confirmed using the Kolmogorov–Smirnov test, and the significance of differences was then examined by a one-way analysis of variance (ANOVA), followed by the Tukey–Kramer test. Probability (p)-values ≤ 0.05 were considered significant.

3. Results

3.1. The Gerbils Showed Different Susceptibilities That Were Dependent on the HEV-3 Strains and Subtypes

We randomly separated Mongolian gerbils into six groups (n = 5 per group) and intraperitoneally injected each gerbil with the virus solution containing 1.0×10^7 copies/mL of the viral RNA derived from strain HEV-3b/wb, HEV-3b/ch, HEV-3e, HEV-3f, HEV-3k, or HEV-3ra. Fecal specimens were then collected 2×/week, and 10% of the specimens were used to detect the viral RNA by RT-qPCR. As shown in Figure 1a, the viral RNAs

were detected in all of the HEV-3b/ch-, HEV-3e-, HEV-3f-, and HEV-3ra-injected gerbils. The viral RNA titers in the peaks were as follows: 1.3×10^6 to 6.6×10^6 copies/g in the HEV-3e-injected gerbils; 1.0×10^5 to 3.0×10^6 copies/g in the HEV-3b/ch-injected gerbils; 5.8×10^5 to 3.1×10^6 copies/g in the HEV-3f-injected gerbils; and 3.4×10^3 to 8.9×10^4 copies/g in the HEV-3ra-injected gerbils. Although the peak copy numbers detected in the HEV-3ra-injected gerbils were lower than those in the HEV-3b/ch-, HEV-3e-, and HEV-3f-injected gerbils, there was no significant difference, as confirmed by the statistical analysis ($p > 0.10$) (Figure 1b).

In all five of the HEV-3e-injected gerbils, the viral RNA was first detected on day 4 p.i. and remained detectable for over 18 days (from day 4 to day 21 p.i. for two gerbils, from day 4 to day 28 p.i. for the other gerbils). In contrast, in the HEV-3b/ch-injected gerbils, the viral RNA-positive period was <15 days (day 4 to day 18 p.i. for two gerbils, day 4 to day 18 p.i. for one gerbil, day 7 to day 18 p.i. for one gerbil, and day 7 to day 11 p.i. for one gerbil). In the HEV-3f-injected gerbils, the viral RNA-positive period was also <15 days (days 4–18 p.i. for four gerbils and days 4–14 p.i. for one gerbil) (Figure 1a). The viral RNA appeared later (after day 11, 14, or 18 p.i.) in the HEV-3ra-injected gerbils; however, the exact period of virus release was unknown since the experiments ended on day 28 p.i.

In contrast, the viral RNAs were detected from four of the five HEV-3b/wb-injected gerbils, and the copy numbers in the peaks were 4.7×10^3 to 1.1×10^5 copies/g, which is significantly lower than those in HEV-3b/ch-injected gerbils ($p < 0.05$), the HEV-3e-injected gerbils ($p < 0.01$), and the HEV-3f-injected gerbils ($p < 0.01$) (Figure 1a,b). Similarly, the viral RNAs were detected from three of the five HEV-3k-infected gerbils, and the copy numbers in the peaks were 3.1×10^3 to 7.6×10^4 copies/g, which is significantly lower than those in the HEV-3b/ch-injected gerbils ($p < 0.01$), HEV-3e-injected gerbils ($p < 0.001$), and HEV-3f-injected gerbils ($p < 0.001$) (Figure 1a,b). The detectable viral RNA in fecal specimens from HEV-3b/wb- and HEV-3k-injected gerbils was identified within 10 days (Figure 1a). These results suggest that HEV-3e, HEV-3f, and HEV-3b/ch were more efficiently replicated in gerbils compared to HEV-3e/wb and HEV-3k.

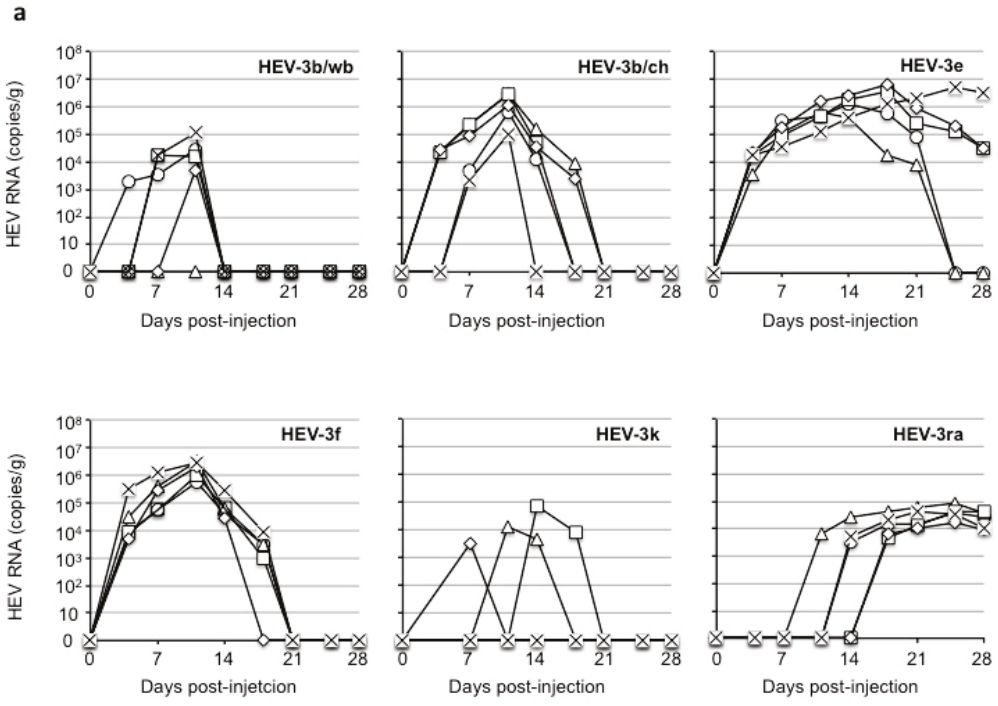

Figure 1. *Cont*.

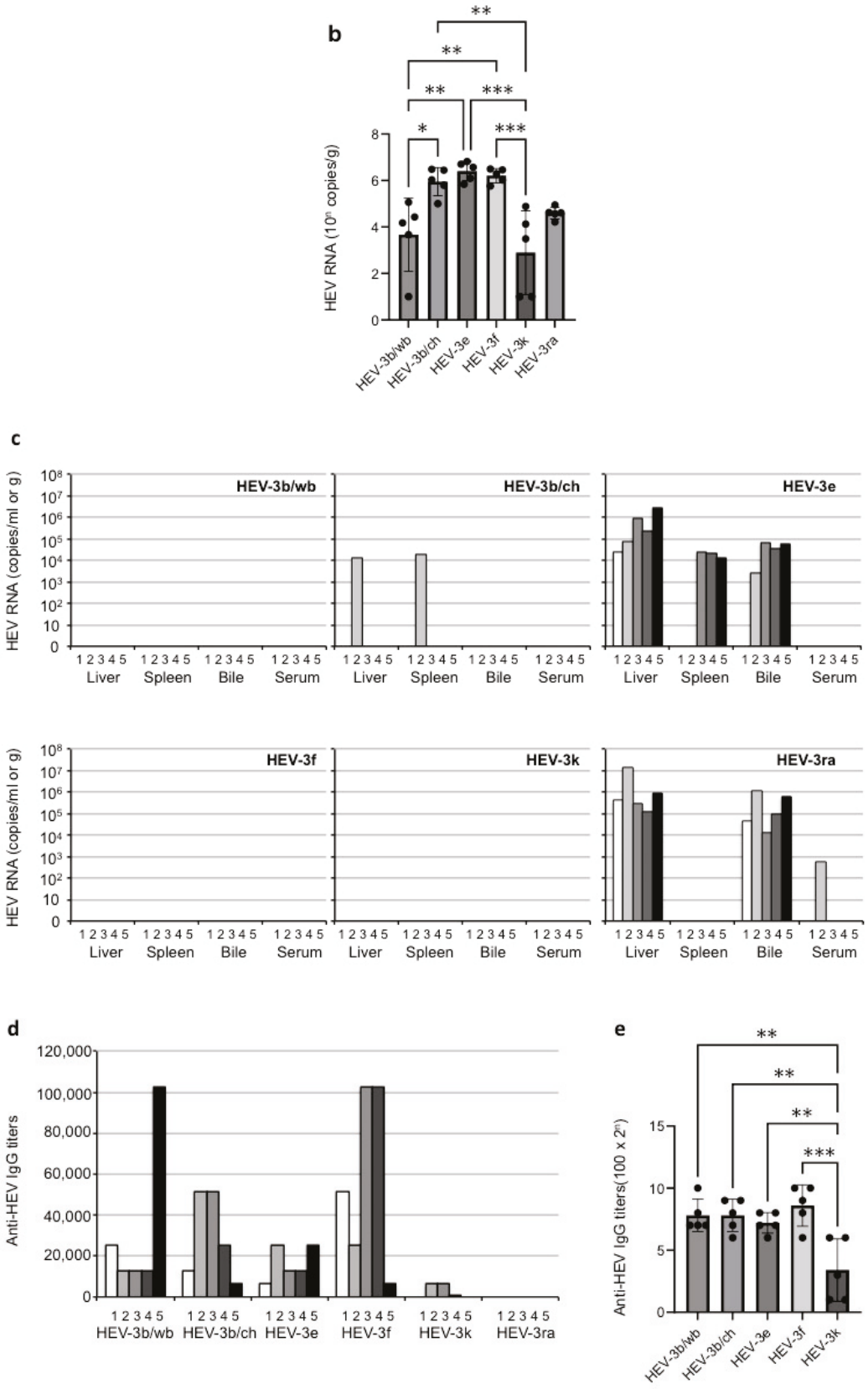

Figure 1. Replication of subtypes of HEV-3 in Mongolian gerbils. Thirty Mongolian gerbils were randomly separated into six groups (n = 5 per group). In each group, individual gerbils are numbered 1 to 5 and indicated by ○, △, □, ◇ and × (**a**) or white, gray, or black bars as shown by 1 to 5 (**c**,**d**).

Each group received 1 mL of the virus solution containing 10^7 copies of the viral RNA derived from HEV-3b/wb, HEV-3b/ch, HEV-3e, HEV-3f, HEV-3k, or HEV-3ra via intraperitoneal injection. The fecal specimens were collected 2×/week, and serum, bile, liver, and spleen samples were collected at the end of the experiment (day 28 p.i.). The kinetics of the viral RNA in the fecal samples were measured by RT-qPCR (**a**). The statistical significance of the viral RNA titer in the peaks is shown (**b**). Bars: the average titers of viral RNA. Dots: individual RNA titers. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. The viral RNA in the liver, spleen, bile, and serum samples collected on day 28 p.i. was measured by RT-qPCR (**c**). The anti-HEV-IgG antibody titers were determined by an ELISA with the virus-like particles (VLPs) of HEV-1 as the antigens (**d**). The significance of the anti-HEV IgG antibody titers is also shown (**e**). Bars: the average titers of anti-HEV IgG antibody titers. Dots: individual antibody titers. ** $p < 0.01$; *** $p < 0.001$.

These results indicate that Mongolian gerbils are susceptible to HEV-3 strains, although their susceptibility does not seem to be the same among the HEV-3 subtypes, as reflected by the varying viral RNA titers and RNA-positive periods. In addition, the gerbils showed different susceptibility to two strains, HEV-3b/wb and HEV-3b/ch, although these strains belong to the same HEV-3b subtype.

All serum, bile, liver, and spleen samples were collected on day 28 p.i. The viral RNA was detected in all of the samples from HEV-3e-injected gerbils: liver (n = 5 samples); spleen (n = 3); and bile (n = 4) (Figure 1c). These results are similar to those obtained in our previous study in which the viral RNA was detected in the liver and spleen derived from HEV4i-, HEV-5-, and rat HEV-infected gerbils [21]. Although the viral RNA was detected in all five liver and bile samples and one serum sample from HEV-3ra-injected gerbils, it was undetectable in the spleens.

In contrast, viral RNA was not detected on day 28 p.i. in any of the samples collected from the gerbils injected with the HEV-3b/wb, HEV-3f, or HEV-3k strains, and it was detected in only one liver and one spleen sample from HEV-3b/ch-injected gerbils. These results suggest that transient infection occurred in these Mongolian gerbils with a short virus RNA-positive period. Here, too, the gerbils exhibited different sensitivities dependent on the HEV-3 subtype strains.

We next measured the anti-HEV IgG antibodies in the sera collected on day 28 p.i. by an ELISA as described in the Materials and Methods section. The antibodies were detected in all of the gerbils injected with HEV-3b/wb, HEV-3b/ch, HEV-3e, or HEV-3f (Figure 1d), and the titers were 1:6400–1:102,400 in the HEV-3b/wb group, 1:6400–1:51,200 in the HEV-3b/ch group, 1:6400–1:25,600 in the HEV-3e group, and 1:6400–1:102,400 in the HEV-3f group, with no significant differences among these groups (Figure 1e). In contrast, the antibodies were detected in only three HEV-3k-inoculated gerbils, and the titers were 1:800–1:6400, significantly lower than those in HEV-3b/wb-, HEV-3b/ch-, HEV-3e-, and HEV-3f-injected gerbils ($p < 0.01$) (Figure 1d,e).

Unexpectedly, although the viral RNA was detected in the feces (Figure 1a), liver, bile, and serum (Figure 1c) of these gerbils, all of the HEV-3ra-injected gerbils were negative for the anti-HEV IgG antibody (Figure 1d), suggesting that the replication of HEV-3ra in the gerbils induced a distinct immune response compared to that of the other HEV-3 subtypes.

Together, these findings indicate that Mongolian gerbils have broad but distinguishable susceptibility to HEV-3, depending on the subtype and strain. The viral RNA was detected in the feces (Figure 1a), and high titers of the serum anti-HEV IgG antibodies were induced in all of the HEV-3b/ch- and HEV-3e-injected gerbils and nearly all of the HEV-3b/wb and HEV-3f-injected gerbils (Figure 1d), suggesting that Mongolian gerbils had potential as small-animal models for HEV-3 research.

3.2. Replication of HEV-3e, -4i, -5, and Rat HEV in Mongolian Gerbils

To further examine the susceptibilities of the gerbils to HEV-3, we compared HEV-3e with three replication-competent HEV strains: HEV-4i; HEV-5; and rat HEV (a genotype HEV-C1 classified in the genus *Rokahepevirus*) (Table 1). A total of 20 gerbils were randomly

separated into four groups (n = 5 per group). Each gerbil was intraperitoneally injected with 1.0 mL of the 10% fecal suspension containing 1.0×10^5 copies/mL of the viral RNA. The fecal specimens were collected 2×/week, and 10% fecal suspensions were used to detect the viral RNA by RT-qPCR.

Virus replication was observed in all 20 gerbils (Figure 2a). The RNA copy numbers in the peaks ranged from 2.2×10^6 to 4.0×10^7 copies/g in the HEV-3e-injected gerbils, 3.1×10^6 to 2.2×10^8 copies/g in the HEV-4i-injected gerbils, 8.2×10^7 to 4.8×10^8 copies/g in the HEV-5-injected gerbils, and 2.9×10^7 to 3.5×10^8 copies/g in the rat HEV-injected gerbils.

We used the serum samples collected on day 28 p.i. for the detection of anti-HEV IgG antibodies. The antibodies were detected in all of the HEV-infected gerbils with the exception of one HEV-3e-infected gerbil (Figure 2b). The IgG titers were 1:6400 to 1:102,400 in the HEV-3e-infected group, 1:12,800 to 1:51,200 in the HEV-4i-infected group, 1:25,600 to 1:51,200 in the HEV-5-infected group, and 1:25,600 to 1:204,800 in the rat HEV-infected group. These results demonstrate that although the viral RNA copy numbers in the HEV-3e-injected gerbils were slightly lower than those in the HEV-4i-, HEV-5-, and rat HEV-infected gerbils, the HEV-3e recovered from the gerbils' fecal specimens was infectious, again indicating that Mongolian gerbils are susceptible to HEV-3e.

To determine whether the virus infection induced liver damage in the gerbils, we collected serum samples on days 0, 14, 21, and 28 p.i. and measured the ALT levels in the sera (Figure 2c). The ALT values in the 20 gerbils before injection ranged from 54 to 88 IU/L, with a mean value of 59 IU/L considered the normal ALT value. ALT values > 118 IU/L (i.e., a two-fold increase) were considered a sign of ALT elevation. The values in all specimens from the HEV-3e-infected and rat HEV-infected gerbils were <118 IU/L. In contrast, ALT elevation was observed in three of the five HEV-5-infected gerbils on day 14 p.i., ranging from 119 to 248 IU/L; in all five gerbils on day 21 p.i., ranging from 205 to 452 IU/L; and in four of the five gerbils on day 28 p.i., ranging from 197 to 221 IU/L.

Similarly, ALT values ranging from 149 to 492 IU/L were observed on day 28 p.i. in four of the five HEV-4i-infected gerbils. These results indicated that liver damage occurred in gerbils infected with strain HEV-4i or strain HEV-5 but not in those infected with HEV-3e or rat HEV, demonstrating that HEVs belonging to different genera or genotypes showed different pathogenicity in Mongolian gerbils.

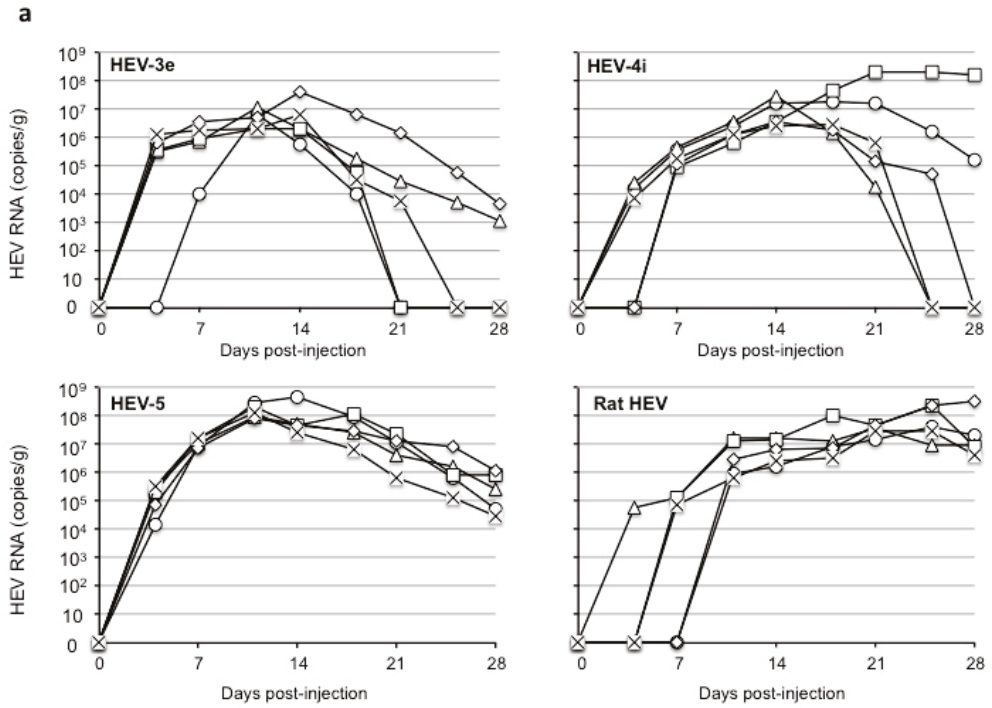

Figure 2. *Cont*.

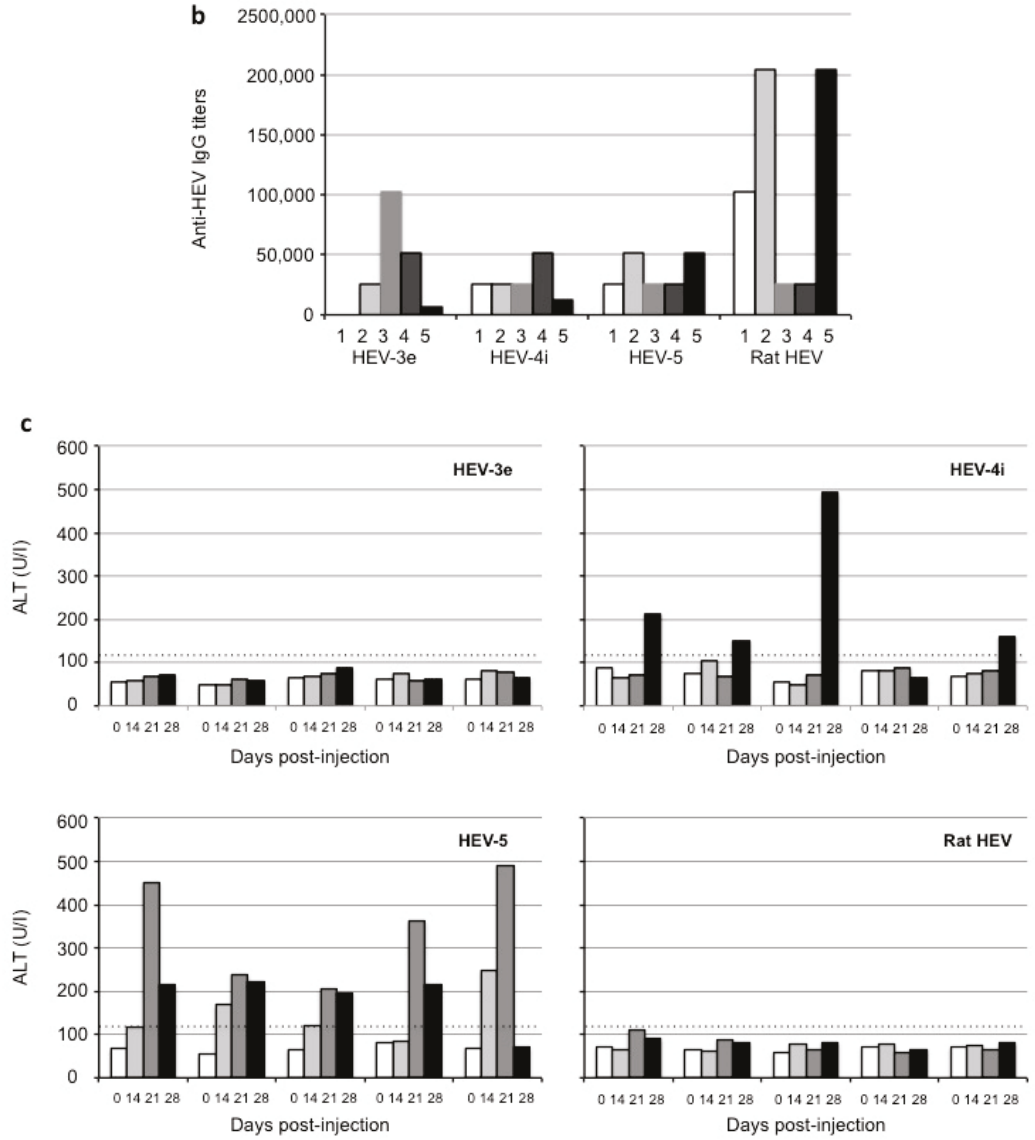

Figure 2. Replication of HEV-3e, HEV-4i, HEV-5, and rat HEV in Mongolian gerbils. Four groups of Mongolian gerbils (n = 5 per group) were injected with the 10% stool suspension prepared from HEV-3e-infected. Individual gerbils are numbered 1 to 5 and indicated by ○, △, □, ◇ and × (**a**), or white, gray, and black bars (**b**,**c**). The fecal specimens were collected 2×/week, and the kinetics of the viral RNA were determined by RT-qPCR (**a**). The anti-HEV IgG titer in serum samples collected on day 28 p.i. was determined by an ELISA (**b**). The ALT values detected in the serum are shown; the numbers 0, 14, 21, and 28 indicate the serum collected on days 0, 14, 21, and 28 p.i., respectively (**c**). Dotted lines: Two-fold the normal ALT value (118 IU/L) (**c**).

3.3. Histopathological Lesions in Liver Samples from HEV-Infected Gerbils

The histopathological examination of liver tissues from HEV-infected Mongolian gerbils collected on day 28 p.i. revealed infiltrations of lymphocytic inflammatory cells into portal areas in the gerbils infected with HEV-4i or HEV-5 (Figure 3c,e). Foci of single-cell necrosis were frequently observed in the HEV-4i-infected gerbils and occasionally noted in the HEV-5-infected gerbils (Figure 3d,f). In contrast, in the HEV-3e-infected gerbils, no clear infiltration of lymphocytes was observed, and only a few foci of single-cell necrosis appeared in the liver (Figure 3a,b). In the liver samples from rat HEV-infected gerbils, only mild lymphocyte infiltration and a few single-cell necrosis foci were observed (Figure 3g,h). Although the liver tissues were collected only on day 28 p.i., these findings

suggested that histopathological lesions occurred at least in HEV-4i- and HEV-5-infected Mongolian gerbils.

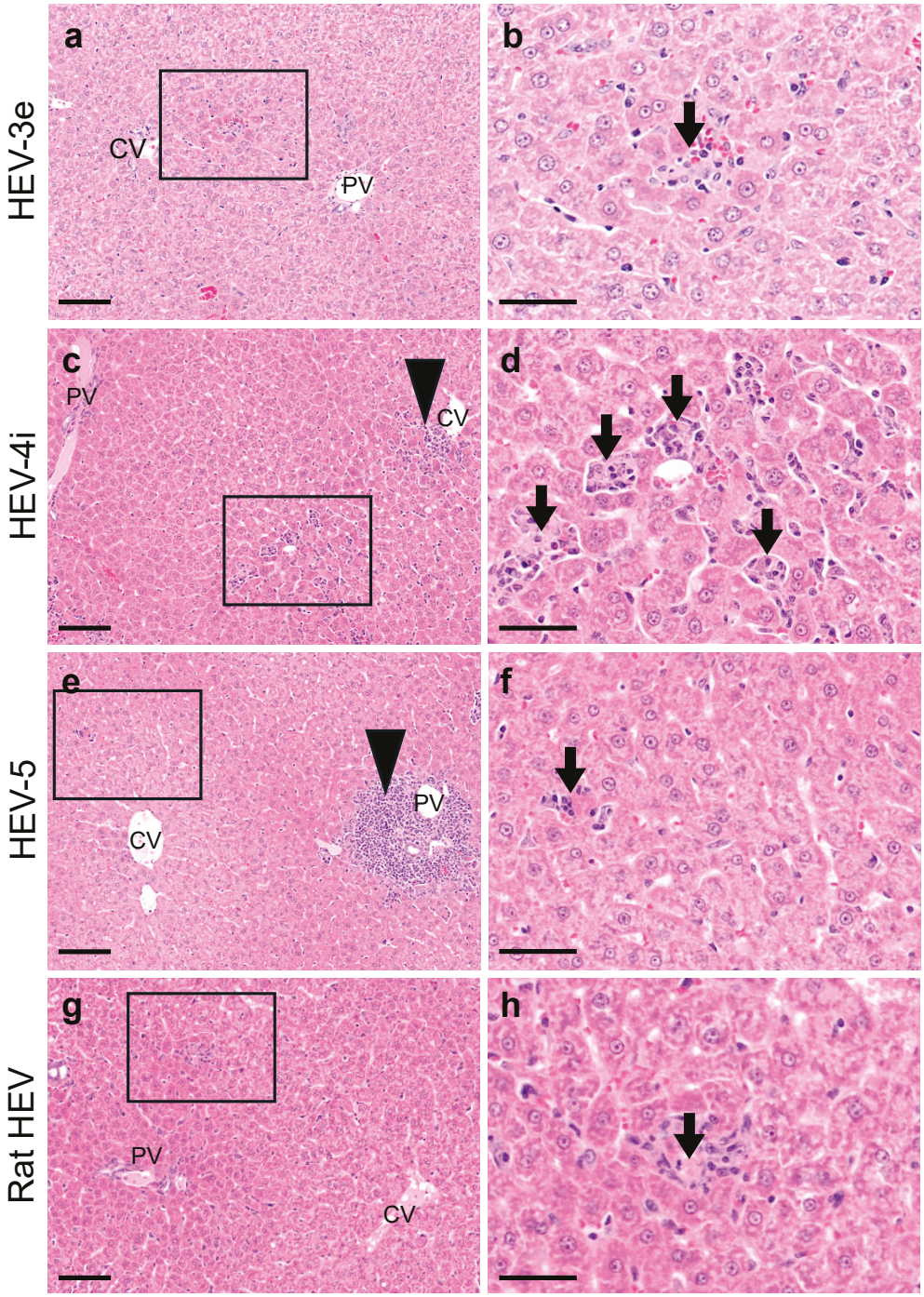

Figure 3. Histopathology of liver tissues from HEV-infected Mongolian gerbils. Representative H&E-stained liver sections from Mongolian gerbils infected with HEV-3e (**a**,**b**), HEV-4i (**c**,**d**), HEV-5 (**e**,**f**), or rat HEV (**g**,**h**) collected on day 28 p.i. are shown. Left column: photos of low-power fields (**a**,**c**,**e**,**g**); scale bars = 100 μm. Right column: photos of high-power fields (**b**,**d**,**f**,**h**); scale bars = 50 μm. Arrowheads: infiltration of inflammatory leukocytes. Arrows: foci of single-cell necrosis followed by phagocytosis by macrophages.

4. Discussion

The infectivity of six strains from five HEV-3 subtypes in Mongolian gerbils was assessed in this study, and the results of our experiments confirmed that Mongolian gerbils were broadly susceptible to these strains. The infectivity was considerably different among the subtypes and even between strains in the same subtype, HEV-3b. The subtypes HEV-3, HEV-3b, HEV-3f, and especially HEV-3e replicated extensively in Mongolian gerbils, and they produced the viruses at high titers for an extended period. Together, these results demonstrate that Mongolian gerbils have potential as an animal model of HEV-3.

Our observations also confirmed that Mongolian gerbils are poorly susceptible to HEV-3k. Although the JP-59 strain of HEV-3ra was able to infect gerbils, the copy numbers of the viral RNA were low, and no anti-IgG antibody responses were induced by this strain, suggesting that the replication of HEV-3ra in the gerbils was unusual. The reasons for the poor replication of HEV-3ra in Mongolian gerbils are still unclear. Further studies need to focus on the genetic difference in the host–virus interaction. Since the genotype HEV-3 includes at least 16 subtypes, it is worth exploring whether other subtypes would be more efficiently replicated in Mongolian gerbils.

Our group's earlier investigation confirmed that the rbIM223LR strain of HEV-3ra does not infect Mongolian gerbils after experimental inoculation [21]. However, the results of the present study confirmed that the JP-59 strain (which belongs to the same HEV-3ra subtype) infected Mongolian gerbils, again indicating that different strains in the same subtype, i.e., HEV-3ra, have different infectivity in Mongolian gerbils. The rbIM223LR strain was initially isolated from a farmed rabbit in Inner Mongolia, whereas the JP-59 strain was isolated from a feral rabbit in Japan; these strains share 87.4% of the nucleotide sequence identity [29,34]. The genetic basis underlying virus infectivity in gerbils is intriguing, and its clarification awaits further studies.

We also observed that two HEV-3b strains, HEV-3b/wb and HEV-3b/ch, exhibited a similar strain-specific difference. HEV-3b is a major subtype of HEV-3 that commonly circulates in pigs and wild boars and has caused hepatitis E in humans in Japan [27,35]. A comparison of the two strains' nucleotide sequences revealed that the strains share 87.8% identity, suggesting that genetic diversity is common in the subtype HEV-3b. Taking our present results and the past findings together, we conclude that the infectivity of HEV in Mongolian gerbils depends not only on the subtypes but also on the strains.

Our above-cited investigation confirmed that Mongolian gerbils are a suitable small-animal model for evaluating the infectivity of HEV [21], but it was not clear whether HEV-infected gerbils exhibited liver damage. In the present study, we analyzed serum samples collected from HEV-3e-, HEV-4i, HEV-5-, and rat HEV-infected gerbils, and we observed that ALT elevation occurred only in HEV-4i-infected and HEV-5- infected gerbils (Figure 2c). Histopathological lesions were also observed in the livers of HEV-4i- and HEV-5-infected Mongolian gerbils (Figure 3). These results demonstrated that (i) the pathogenicity of HEVs in the gerbils also depended on the HEV genotype, and (ii) the HEV-4i-infected Mongolian gerbils and HEV-5-infected Mongolian gerbils might be a candidate animal model to examine the pathogenicity of HEV. Together, these findings suggest that Mongolian gerbils could be a convenient small-animal model that would be useful not only to elucidate the mechanisms of HEV replication but also to evaluate the efficacy of vaccines against HEV.

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Institutional Review Board Statement: The animal experiments were reviewed and approved by the Institutional Ethics Committee of Japan's National Institute of Infectious Diseases and were performed according to the Guides for Animal Experiments at the National Institute of Infectious Diseases, Tokyo, Japan, under code 123023 (11 May 2023).

Informed Consent Statement: Not applicable.

Data Availability Statement: The sequences of HEV used in this study have been assigned (GenBank accession no. LC774371, LC786331, JQ026407, LC055972, AB740232, LC535077, LC657084, AB573435, and JX120573).

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Genetic Diversity and Molecular Evolution of Hepatitis E Virus Within the Genus *Chirohepevirus* in Bats

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Abstract: Hepatitis E virus (HEV) is a major zoonotic pathogen causing hepatitis E, with strains identified in various animal species, including pigs, wild boar, rabbits, deer, camels, and rats. These variants are capable of crossing species barriers and infecting humans. HEV belongs to the family *Hepeviridae*, which has recently divided into two subfamilies: *Orthohepevirinae* and *Parahepevirinae*, and five genera: *Paslahepevirus*, *Avihepevirus*, *Rocahepevirus*, *Chirohepevirus*, and *Piscihepevirus*. Recent advances in high-throughput sequencing, particularly of bat viromes, have revealed numerous HEV-related viruses, raising concerns about their zoonotic potential. Bat-derived HEVs have been classified into the genus *Chirohepevirus*, which includes three distinct species. In this study, we analyzed 64 chirohepevirus sequences from 22 bat species across six bat families collected from nine countries. Twelve sequences represent complete or nearly complete viral genomes (>6410 nucleotides) containing the characteristic three HEV open reading frames (ORFs). These strains exhibited high sequence divergence (>25%) within their respective host genera or species. Phylogenetic analyses with maximum likelihood methods identified at least seven distinct subclades within *Chirohepevirus*, each potentially representing an independent species. Additionally, the close phylogenetic relationship between chirohepevirus strains and their bat hosts indicates a pattern of virus–host co-speciation. Our findings expand the known diversity within the family *Hepeviridae* and provide new insights into the evolution of bat-associated HEV. Continued surveillance of chirohepevirus will be essential for understanding its potential for zoonotic transmission and public health risks.

Keywords: hepatitis E virus (HEV); chirohepevirus; bats; genetic diversity; molecular evolution; zoonotic potential

1. Introduction

Hepatitis E virus (HEV) is the causative agent of hepatitis E, a viral liver disease that remains one of the leading causes of acute viral hepatitis globally, with at least 20 million HEV infections, an estimated 3.3 million symptomatic cases, and 70,000 deaths occurring annually [1,2]. There are four major HEV genotypes that infect humans: genotypes 1 (HEV-1) and 2 (HEV-2), which are primarily transmitted through the consumption of contaminated water in areas with inadequate sanitation, and genotypes 3 (HEV-3) and 4 (HEV-4), which are mainly zoonotic, transmitted from animals, particularly pigs, and are more prevalent in industrialized countries [3]. In healthy human individuals, HEV infection is typically self-limiting and resolves within a few weeks. However, HEV-1 can

lead to severe complications in pregnant women, particularly in the third trimester, with case fatality rates as high as 30% [4]. In contrast, HEV-3 has been associated with chronic infection in immunocompromised individuals, such as organ transplant recipients and patients undergoing chemotherapy, which can result in cirrhosis or even liver failure [5]. However, treatment options for chronic hepatitis E are limited, with ribavirin being the primary antiviral drug [6]. Furthermore, HEV has been implicated in a variety of extrahepatic manifestations, including neurological and renal complications, adding to its clinical complexity [7]. Despite the growing burden of HEV-associated diseases, no specific antiviral treatment for HEV is currently available [8]. As such, HEV continues to pose a significant global health challenge.

HEV is a quasi-enveloped, positive-sense, single-stranded RNA virus with a genome approximately 7.2 to 7.5 kb in length. The viral genome comprises three major open reading frames (ORFs): ORF1, which encodes a non-structural polyprotein involved in virus replication; ORF2, which encodes the viral capsid protein necessary for virus particle formation; and ORF3, which encodes a small phosphoprotein implicated in viral particle release [9]. HEV belongs to the family *Hepeviridae*, which is genetically diverse and currently expanding in taxonomy as increasing new numbers of viruses have been discovered and identified in animal reservoirs [10]. According to the International Committee on the Taxonomy of Viruses (ICTV) in 2022, *Hepeviridae* is divided into two subfamilies: *Orthohepevirinae* and *Parahepevirinae*. The subfamily *Orthohepevirinae* includes four genera (*Paslahepevirus*, *Avihepevirus*, *Rocahepevirus*, and *Chirohepevirus*), which are based on phylogenic analysis and host tropism, while the subfamily *Parahepevirinae* comprises only one genus *Piscihepevirus*, which infects fish such as trout and salmon [11]. The genus *Paslahepevirus* can infect a range of mammalian hosts, including humans, pigs, deer, rabbits, and camels; the genera *Avihepevirus* and *Rocahepevirus* primarily infect birds and rodents, respectively; the genus *Chirohepevirus* was recently identified in bats worldwide [11]. In addition to these well-characterized viruses, there are still many HEV-related strains that remain unclassified due to incomplete genomes or unresolved phylogenetic relationships [10]. Of particular concern is the zoonotic potential of HEV, as variants originating from pigs, wild boar, rabbits, deer, camels, and rats have demonstrated the ability to cross species barriers and infect humans, although the mechanisms underlying zoonotic transmission remain poorly understood [12,13].

Bats, which are known reservoirs for a wide range of viral pathogens, have played a crucial role in the ecology and transmission of emerging infectious diseases [14,15]. Their unique biological traits, including their ability to fly, their migratory behavior, and their distinctive immune system, allows them to harbor a diverse array of viruses, many with zoonotic potential [16]. Bats also live in large colonies and have extensive migratory patterns, facilitating the spread of viruses both within bat populations and between bats and other species, including humans. Their immune systems are particularly noteworthy, as bats can mount an effective antiviral response without inducing the harmful inflammation seen in other species, allowing them to carry viral pathogens without severe diseases [17]. Bats' exceptional ability to tolerate viral infections has implicated them as reservoirs for a number of high-profile zoonotic viruses, including SARS-CoV-1, SARS-CoV-2, MERS-CoV (family *Coronaviridae*), Ebola and Marburg viruses (family *Filoviridae*), and Hendra and Nipah viruses (family *Paramyxoviridae*) [18,19]. Despite the risks associated with bat-borne diseases, bats play crucial ecological roles, such as controlling insect populations, pollinating plants, and dispersing seeds, which complicates their relationship with human and animal health [20].

HEV-related viruses were first discovered in bats in 2012 and were found to be genetically distinct from human-infecting HEV strains within the genus *Paslahepevirus* [21].

Over the past decade, advances in high-throughput sequencing and metagenomic studies, particularly involving bat viromes, have uncovered a growing number of HEV-related viruses in a variety of bat species. Although these bat-derived viruses are genetically diverse, they share genomic similarities with human-infecting HEV strains [11]. Currently, bat-derived HEV strains are classified within a novel genus, *Chirohepevirus*, which includes three distinct species (*C. desmodi*, *C. eptesici*, and *C. rhinolophi*) based on phylogenetic relationships [10]. While there is currently no direct evidence of chirohepevirus spillover into humans, the genetic similarity between chirohepeviruses and human-infecting HEV strains raises concerns about its zoonotic potential, especially the recent case of zoonotic rat HEV infection [22]. In 2022, we analyzed 18 bat-derived HEV sequences from 12 bat species [11]. Since then, additional chirohepeviruses has been discovered in more bat species. The present study aims to analyze all globally available chirohepevirus sequences to assess the genetic diversity and molecular evolution of these bat-associated HEV strains. By understanding the genetic variability and evolutionary patterns of chirohepeviruses, we aim to better understand the potential risks of zoonotic spillover and expand our knowledge of the broader *Hepeviridae* family.

2. Materials and Methods

2.1. Dataset Collection

To compile a comprehensive dataset of chirohepevirus sequences, a series of search terms were used to query the NCBI GenBank database up until 15 November 2024. The search terms included "bat hepatitis E virus", "*bat hepevirus*", "*chirohepevirus*", "*chirohepevirus* sp.", "*chirohepevirus desmodi*", "*chirohepevirus eptesici*", "*chirohepevirus rhinolophi*", and "*unclassified orthohepevirus*". Additionally, sequences of chirohepevirus were retrieved from the database of bat-associated viruses (DBatVir). A total of 64 sequences were retrieved, including partial genomic sequences and complete or nearly complete genomes of chirohepevirus strains (Table 1).

Table 1. Detection of chirohepeviruses in diverse bat species.

Host Family	Host Species	Host Common Name [a]	Sampling Country (Collection Year)	Sample Source	Genomic Sequence (No.)	GenBank Accession No.	Reference
Rhinolophidae	*Rhinolophus ferrumequinum*	greater horseshoe bat	China (2013)	Anal swab	Complete (1)	KJ562187	[23]
Rhinolophidae	*Rhinolophus sinicus*	Chinese rufous horseshoe bat	China (2016, 2019)	Anal swab	Complete (2)	MT210622, OR951173	N.A. [b], [24]
Hipposideridae	*Hipposideros abae*	Aba roundleaf bat	Ghana (2009)	Feces	Partial (2)	JQ001744, JQ071861	[21]
Phyllostomidae	*Vampyrodes caraccioli*	great stripe-faced bat	Panama (2009)	Blood	Partial (1)	JQ001745	[21]
Phyllostomidae	*Desmodus rotundus*	common vampire bat	Peru (2016)	Anal swab	Complete (4)	MW249011–MW249014	[25]
Miniopteridae	*Miniopterus magnater*	Western long-fingered bat	China (2017)	Pooled animal	Complete (1)	OQ715533	[26]
Miniopteridae	*Miniopterus pusillus*	small bent-winged bat	China (2017)	Anal swab	Partial (1)	OR951184	[24]
Mystacinidae	*Mystacina tuberculata*	New Zealand lesser short-tailed bat	New Zealand (2013, 2020, 2021)	Bat guano	Partial (9)	KM204384, KM204385 [c], OR248814–OR248820	[27,28]
Vespertilionidae	*Chalinolobus tuberculatus*	New Zealand long-tailed bat	New Zealand (2020)	Bat guano	Partial (2)	OR248813, OR248821	[28]
Vespertilionidae	*Myotis daubentonii*	Daubenton's bat	Germany (2009)	Feces	Partial (2)	JQ001746, JQ001747	[21]

Table 1. *Cont.*

Host Family	Host Species	Host Common Name [a]	Sampling Country (Collection Year)	Sample Source	Genomic Sequence (No.)	GenBank Accession No.	Reference
Vespertilionidae	*Myotis bechsteinii*	Bechstein's bat	Germany (2008)	Feces	Partial (1)	JQ001748	[21]
Vespertilionidae	*Myotis davidii*	David's myotis	China (2011)	Liver	Complete (1)	KX513953	[29]
Vespertilionidae	*Myotis ricketti*	Rickett's big-footed myotis	China (2017)	Pooled animal	Complete (1)	OQ715534	[26]
Vespertilionidae	*Eptesicus serotinus*	common serotine bat	Germany (2009)	Liver	Complete (1)	JQ001749	[21]
Vespertilionidae	*Eptesicus japonensis*	Japanese short-tailed bat	Japan (2015)	Feces	Partial (2)	LC340968, LC340970	[30]
Vespertilionidae	*Pipistrellus nathusii*	Nathusius's pipistrelle	Switzerland (2019)	Feces	Nearly complete (1) [d]	MT815970	[31]
Vespertilionidae	*Pipistrellus pygmaeus*	soprano pipistrelle	Sweden (2020)	Feces	Partial (1)	ON513427	N.A.
Vespertilionidae	*Pipistrellus abramus*	Japanese house bat	China (2016–2020)	Anal swab	Partial (18)	OR951187–OR951204	[24]
Vespertilionidae	*Tylonycteris pachypus*	lesser bamboo bat	China (2016–2017)	Anal swab	Partial (8)	OR951174–OR951181	[24]
Vespertilionidae	*Tylonycteris robustula*	greater bamboo bat	China (2017)	Anal swab	Partial (2)	OR951185–OR951186	[24]
Vespertilionidae	*Plecotus sacrimontis*	Japanese long-eared bat	Japan (2015)	Feces	Partial (1)	LC340969	[30]
Vespertilionidae	*Scotophilus kuhlii*	lesser Asiatic yellow house bat	China (2018)	Anal swab	Partial (2)	OR951182–OR951183	[24]
Total	22 species				64 sequences		

[a] Host common name is according to the GenBank Taxonomy Browser (https://www.ncbi.nlm.nih.gov/taxonomy/, accessed on 15 November 2024); [b] N.A. denotes not available. These GenBank accession numbers do not yet appear in any publication which reports or discusses these data; [c] These two viral fragments derived from a single sample with different sequencing method; [d] Viral genome lacks 5′ end.

2.2. Sequence Analyses

The genomic sequences of chirohepevirus strains were annotated to indicate the presence of the three typical open reading frames (ORFs) that are characteristic of HEV genomes using Geneious Prime software, version 2025.0.3 (Biomatters Ltd., Auckland, New Zealand). The sequences were then aligned using MAFFT as implemented in Geneious Prime. To assess the genetic variation of chirohepevirus strains, complete genomic sequences were compared with representative strains from the five genera within the family *Hepeviridae*. Pairwise nucleotide distances for the chirohepevirus strains were calculated using Molecular Evolutionary Genetics Analysis (MEGA) software, version 11 [32]. Maximum nucleotide distances were calculated across different host genera and species to evaluate the extent of genetic divergence within the family. The reference sequences for the phylogenetic comparison were selected based on the latest ICTV Virus Taxonomy Profile for *Hepeviridae*. These included the following reference strains: the human HEV prototype Burma strain (GenBank accession no. M73218) for the genus *Paslahepevirus*, the avian HEV prototype F93-5077 strain (GenBank accession no. AY535004) for the genus *Avihepevirus*, the rat HEV prototype R63 strain (GenBank accession no. GU345042) for the genus *Rocahepevirus*, the bat HEV BS7 strain (GenBank accession no. JQ001749) for the genus *Chirohepevirus*, and the fish HEV prototype Heenan Lake strain (GenBank accession no. HQ731075) for the genus *Piscihepevirus* [10].

2.3. Phylogenetic and Evolutionary Analyses

Phylogenetic relationships of chirohepevirus strains were reconstructed based on complete genomes using maximum-likelihood (ML) methods in IQ-TREE version 2.3.6, with 1000 ultrafast bootstrap replicates to assess statistical support. The phylogenetic tree was rooted using the piscihepevirus Heenan88 strain from cutthroat trout (GenBank accession no. HQ731075). The phylogenetic inference was visualized using FigTree version 14.4. To specifically examine the phylogenetic relationships within the genus *Chirohepevirus*, separate ML phylogenetic analyses were performed using either partial ORF1 (1600 nucleotides) of 46 chirohepevirus or nearly complete genomes (5600 nucleotides) of 31 chirohepevirus strains. An avian HEV strain (GenBank accession no. AY535004) was used as the outgroup for these analyses to root the trees. Additionally, to specifically investigate the phylogenetic relationships of six HEV-related variants identified in bats in New Zealand, phylogenetic analysis was performed using partial ORF1 (900 nucleotides). A Tuatara cloacal-associated hepevirus strain (GenBank accession no. OP080571) was included for this analysis.

To investigate the potential co-speciation relationship of bat chirohepeviruses and their chiropteran hosts, ML topologies were reconstructed and compared between the partial complete genomes (4400 nucleotides) of 33 chirohepevirus strains and their corresponding 13 bat species, based on mitochondrial cytochrome B (CYTB) gene sequences (1140 nucleotides). The mitochondrial genomes of the bat species were downloaded from the NCBI GenBank database (Supplementary Table S1). The host species' taxonomy was cross-referenced from the NCBI Taxonomy database and Bats of the World: A Taxonomic and Geographic Database version 1.6 [33].

3. Results

3.1. Detection and Distribution of Chirohepeviruses

A comprehensive search was conducted to identify all publicly available bat-associated HEV and chirohepeviruses from the PubMed, GenBank, and DBatVir databases. In total, 64 chirohepevirus sequences were retrieved, comprising both partial and complete genomic sequences (Table 1). These chirohepeviruses were found in 22 bat species across six bat families: Rhinolophidae, Hipposideridae, Phyllostomidae, Miniopteridae, Mystacinidae, and Vespertilionidae. They are distributed in different geographic regions, including Switzerland, Germany, Sweden, Panama, Peru, Ghana, China, Japan, and New Zealand (Figure 1). The samples were collected between 2008 and 2021, with diverse sample types, including anal swab, feces, blood, liver, bat guano, and pooled animal samples (Table 1).

Notably, our partial RdRp phylogenetic tree revealed a monophyletic group of related HEV strains (shaded in orange in Supplementary Figure S1) that are closely related to the family *Hepeviridae*. This well-supported clade (ML bootstrap support = 95%) comprises HEV-related strains identified in New Zealand from the lesser short-tailed bat (*Mystacina tuberculata*) and long-tailed bat (*Chalinolobus tuberculatus*). Within this clade, the bat viruses shared >90% amino acid sequence identity with a Tuatara cloacal-associated hepevirus (GenBank accession no. OP080571), detected in cloacal swabs from the tuatara (*Sphenodon punctatus*), a reptile species endemic to New Zealand. Interestingly, one chirohepevirus strain from *M. tuberculata* (7470 nt) (GenBank accession no. OR248815) consists of only one single predicted ORF (2486 aa), albeit lacking the typical three HEV ORFs. This truncated ORF shared the highest amino acid identity of up to 36.8% with the RdRp gene of a Swiper virus (GenBank accession no. QQR34432) identified in red fox (*Vulpes vulpes*) in Australia. Whether this unique sequence represents a spillover from an unknown animal species, given the sample's origin from bat guano, or a possible sequencing artefact remains unclear. Moreover, this clade is distinct from the family *Hepeviridae*, which consists of a diverse

range of HEV strains from various hosts, including bats, humans, swine, rodents, birds, and fish HEV strains (shaded in red in Supplementary Figure S1).

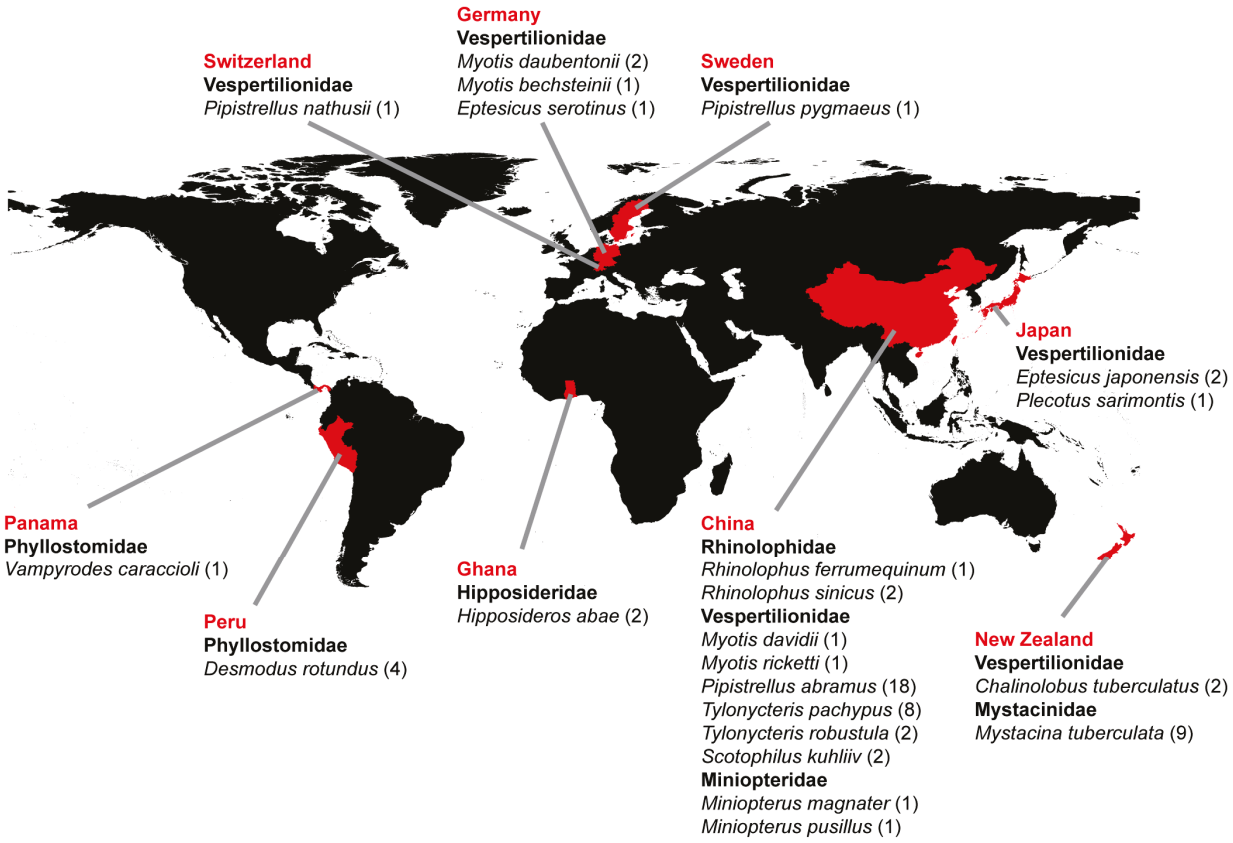

Figure 1. Worldwide distribution of chirohepeviruses in bats. Countries where chirohepevirus genomic sequences have been detected are marked in red. Bat families that are positive for chirohepeviruses are highlighted in bold. The number of chirohepevirus sequences detected in each bat species is shown in brackets. The world map was created using a free and open-source quantum geographic information system (QGIS) version 3.38, with raster map data from Natural Earth.

3.2. Genomic Characterization of Bat Chirohepeviruses

While most of the bat chirohepevirus sequences were partial genomic fragments, 12 sequences correspond to complete or nearly complete viral genomes (>6410 nt) (Figure 2 and Table 1). These included strains Rf-HEV/Shanxi/2013 from a Chinese greater horseshoe bat (*Rhinolophus ferrumequinum*), GD2019 and LQB_Rsin from Chinese rufous horseshoe bats (*Rhinolophus sinicus*), API17_F_DrHEV, AYA11_F_DrHE, AYA14_F_DrHEV, and LR3_F_DrHEV from Peruvian common vampire bats (*Desmodus rotundus*), BtHEVMd2350 from a Chinese David's myotis (*Myotis davidii*), JM_My.ricketti.hev from a Chinese Rickett's big-footed myotis (*Myotis ricketti*), BS7/GE/2009 from a German common serotine bat (*Eptesicus serotinus*), BtHEV-Ps1/CH/2019 from a Swiss Nathusius's pipistrelle (*Pipistrellus nathusii*), and JM_Mi.magnater.hev from a Chinese Western long-fingered bat (*Miniopterus magnater*). Notably, the viral genomes of GD2019 and BtHEV-Ps1/CH/2019 lacked the sequences of the 5′ end of the untranslated region (UTR).

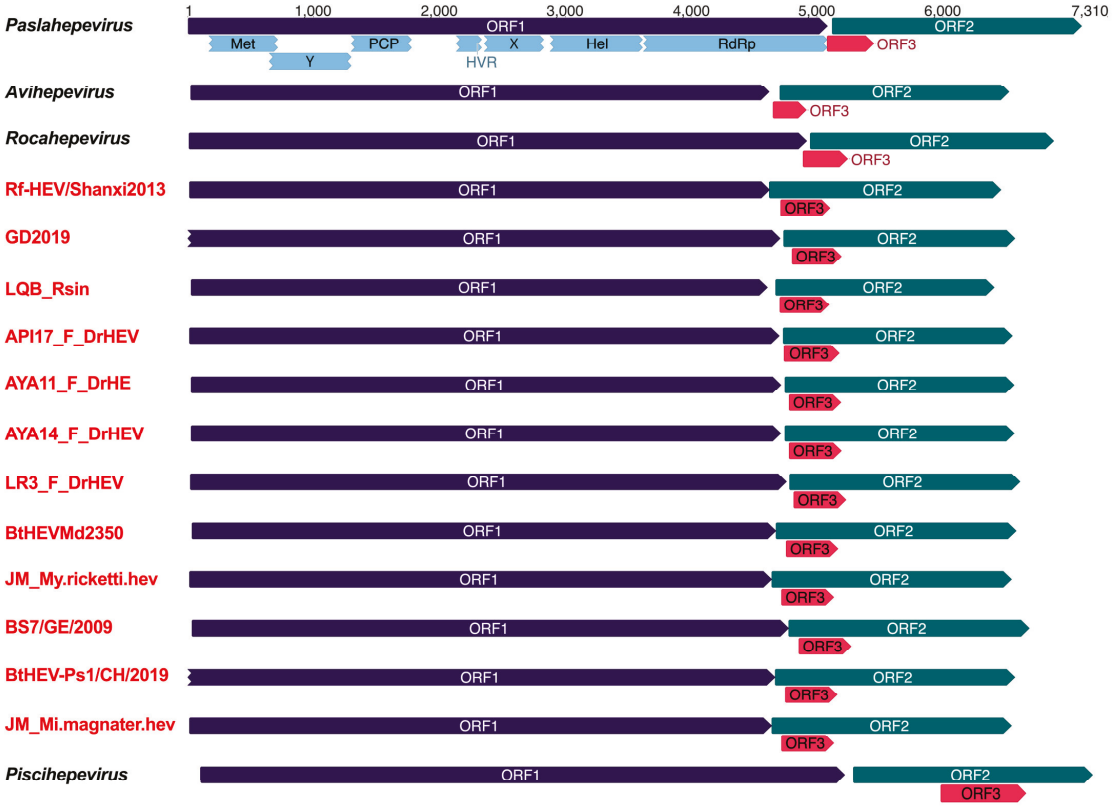

Figure 2. Genome organization comparisons of chirohepeviruses and related genera. The typical three HEV open reading frames (ORFs) and putative functional domains within the ORF1 of *Paslahepevirus* are depicted, including methyltransferase (Met), Y domain, papain-like cysteine protease (PCP), hypervariable region (HVR), X domain, helicase (Hel), and RNA-dependent RNA polymerase (RdRp). The genome scale in nucleotides is shown at the top. GenBank accession numbers for the virus strains presented: M73218 (*Paslahepevirus*), AY535004 (*Avihepevirus*), GU345042 (*Rocahepevirus*), KJ562187 (Rf-HEV/Shanxi2013), MT210622 (GD2019), OR951173 (LQB_Rsin), MW249011 (API17_F_DrHEV), MW249012 (AYA11_F_DrHEV), MW249013 (AYA14_F_DrHEV), MW249014 (LR3_F_DrHEV), KX513953 (BtHEVMd2350), OQ715534 (JM_My.ricketti.hev), JQ001749 (BS7/GE/2009), MT815970 (BtHEV-Ps1/CH/2019), OQ715533 (JM_Mi.magnater.hev), and HQ731075 (*Piscihepevirus*).

In analogy to other mammalian, avian, and fish HEV variants within the family *Hepeviridae*, all bat chirohepeviruses exhibit a similar genomic organization and ORF composition, containing ORF1, ORF2, and ORF3 regions. Excluding the highly divergent piscihepevirus within the *Parahepevirinae* subfamily, some genomic features of bat chirohepeviruses are notably distinct from the three other genera within the subfamily *Orthohepevirinae*. Specifically, chirohepeviruses (~6.5 kb) have a shorter genome length compared to paslahepeviruses (~7.2 kb), avihepevirues (~6.7 kb), and rocahepeviruses (~7.0 kb). Despite their smaller genome size, chirohepeviruses possesses a markedly longer ORF3 (~138 amino acids) compared to paslahepeviruses (~113 amino acids), avihepevirues (~87 amino acids), and rocahepeviruses (~118 amino acids). Additionally, while other HEV strains have distinct translation frames for ORF2 and ORF3, the ORF3 of bat chirohepeviruses is entirely located within the N-terminal region of ORF2. In contrast, the ORF3 of other mammalian and avian orthohepeviruses only partially overlaps with the N-terminal region of ORF2 (Figure 2).

3.3. Genetic Diversity of Chirohepeviruses

Maximum likelihood phylogeny based on nearly whole genomes revealed that all bat-derived chirohepeviruses (n = 12) formed a well-supported monophyletic clade (BS = 100%),

which is distinct from the four other genera *Paslahepevirus*, *Avihepevirus*, *Rocahepevirus*, and *Pischhepevirus* (Figure 3A). All chirohepeviruses exhibited substantial genetic divergence from paslahepeviruses, avihepeviruses, and rocahepeviruses, with pairwise nucleotide sequence distances of 55%, 53%, and 54%, respectively. They were even more distantly related to piscihepevirus, with a pairwise nucleotide sequence distance of up to 67% (Figure 3B).

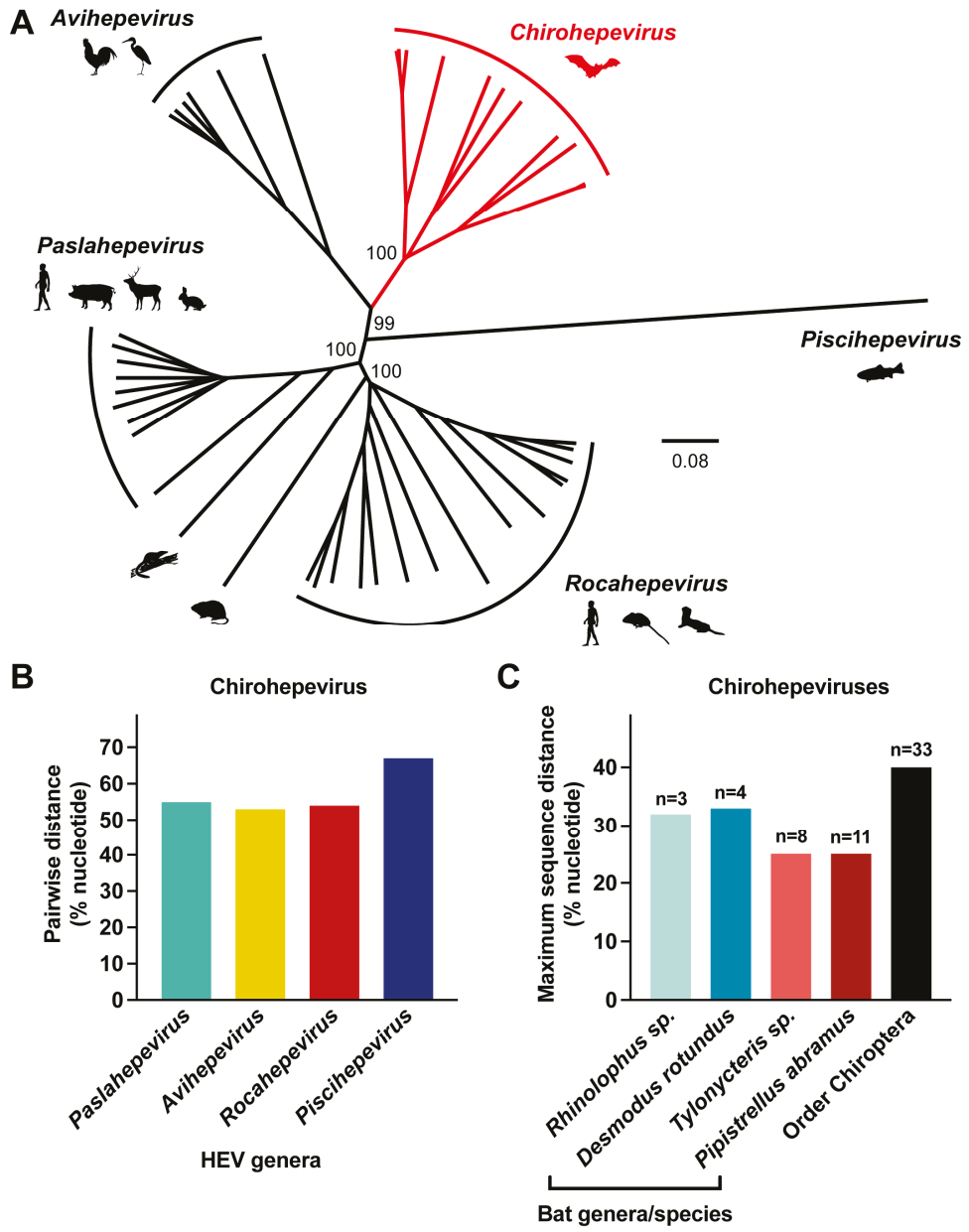

Figure 3. Genetic diversity of chirohepeviruses and other HEV genera. (**A**) Phylogenetic tree based on complete genomes of representative members of the family *Hepeviridae*. Virus classification follows the ICTV Virus Taxonomy Profile: *Hepeviridae* 2022. Virus strains within the genus *Chirohepevirus* are highlighted in red. Major host tropisms for each virus genus are indicated using animal icons. The tree is rooted using the divergent cutthroat trout *Piscihepevirus*. Bootstrap support values are indicated at relevant nodes. The scale bar corresponds to number of nucleotide substitutions per site. (**B**) Pairwise nucleotide sequence distances between *Chirohepevirus* and four other HEV genera. (**C**) Comparison of nucleotide sequence distances based on partial HEV genomes (at least >4400 nucleotides) identified in known bat genera or within the Order Chiroptera.

Moreover, chirohepeviruses from different bat genera displayed genetic variability, with notable divergence even within the same host species (Figure 3C). The maximum nucleotide sequence distances among horseshoe bats (*Rhinolophus* sp.), common vampire bats (*Desmodus rotundus*), bamboo bats (*Tylonycteris* sp.), and Japanese house bats (*Pipistrellus abramus*) were 32%, 33%, 25%, and 25%, respectively, suggesting long-term evolutionary associations between these HEV viruses and their bat host species [34]. Overall, the nucleotide sequence divergence among all bat-derived chirohepeviruses reached 40%.

3.4. Phylogenetic Relationships of Chirohepeviruses

To investigate the phylogenetic relationships among the chirohepeviruses identified in bats, we constructed two maximum likelihood phylogenetic trees: one based on partial ORF1 region (1600 nucleotides; Figure 4) and another using nearly complete genomes (5600 nucleotides, Figure 5). An avian HEV strain (GenBank accession no. AY535004) within the genus *Avihepevirus* was used as an outgroup for these analyses. Among the 46 chirohepevirus strains using partial ORF1 sequences, we identified seven distinct subclades within the genus *Chirohepevirus* (Figure 4). Notably, five of these subclades (5/7) were closely associated with specific bat genera or species, including *Tylonycteris* sp., *Myotis* sp., *Pipistrellus abramus*, *Rhinolophus* sp., and *Desmodus rotundus*. In contrast, one subclade contained chirohepeviruses from several bat genera and species, including *Pipistrellus pygmaeus*, *Pipistrellus nathusii*, *Eptesicus serotinus*, *Eptesicus japonensis*, and *Plecotus sacrimontis*. Another subclade included viruses from *Miniopterus pusillus* and *Scotophilus kuhlii*. Two chirohepevirus strains – one from *Miniopterus magnate* (GenBank accession no. OQ715534) and another from *Myotis ricketti* (GenBank accession no. OQ715533), detected in the same study, clustered together with an exceptionally high nucleotide identity of 99.9% (6556 nt). Whether this chirohepevirus from *Miniopterus magnate* represents a natural infection through a potential cross-species transmission event, or a case of contamination during field sampling or sequencing, remains uncertain. Given these uncertainties, we have cautiously excluded this virus strain from further analysis.

The phylogenetic tree based on nearly complete genome of 31 chirohepevirus strains exhibited a similar topology (Figure 5). According to the latest classification proposed by the ICTV *Hepeviridae* Study Group, three distinct *Chirohepevirus* species have been defined based on virus phylogeny and host tropism: *C. eptesici*, *C. rhinolophi*, and *C. desmodi*, [10]. Specifically, *C. eptesici* includes BS7/GE/2009 from *Eptesicus serotinus* and BtHEVMd2350 from *Myotis davidii*; *C. rhinolophi* includes Rf-HEV/Shanxi2013 from *Rhinolophus ferrumequinum*; and *C. desmodi* includes several strains from *Desmodus rotundus* (API17_F_DrHEV, AYA11_F_DrHE, AYA14_F_DrHEV, and LR3_F_DrHEV) (Figure 2). Based on our phylogenetic inference, here we propose that each subclade may represent a distinct *Chirohepevirus* species. Following ICTV guidelines, which suggest that HEV species names be Latinized descriptors derived from the binomial nomenclature of the virus and its host, we designate the following seven species: *C. tylonicteris* from *Tylonycteris* sp., *C. eptesici* from *Eptesicus* sp., *C. myotis* from *Myotis* sp., *C. pipistrelli* from *Pipistrellus abramus*, *C. miniopteri* from *Miniopterus pusillus* and *Scotophilus khulii*, *C. desmodi* from *Desmodus rotundus*, and *C. rhinolophi* from *Rhinolophus* sp., noting that the virus from *Miniopterus pusillus* was detected earlier than that from *Scotophilus kuhlii*.

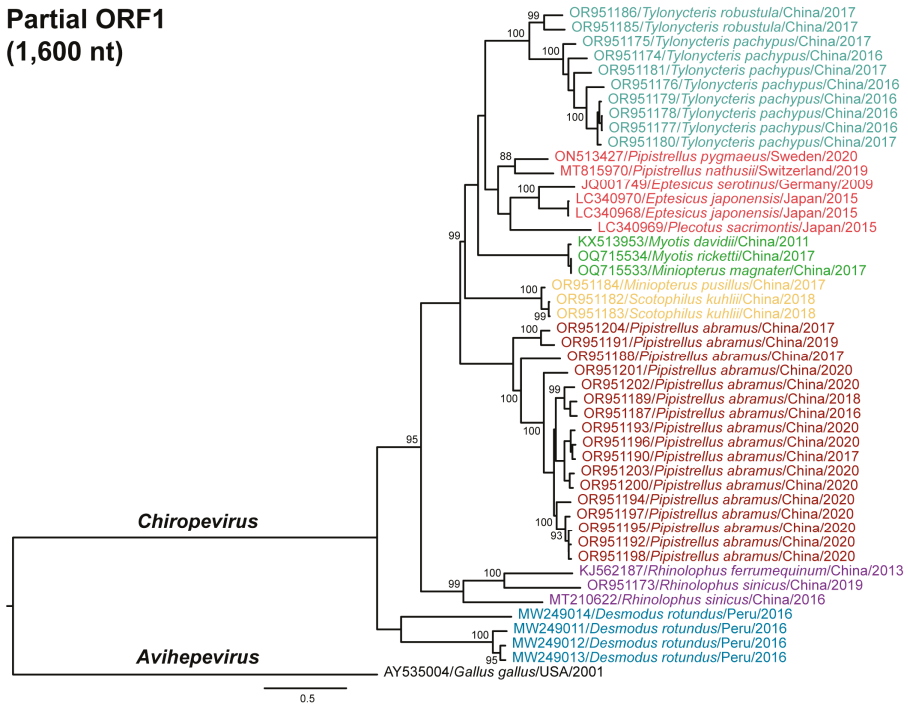

Figure 4. Maximum likelihood phylogeny of partial ORF1 of chirohepeviruses. The tree is based on approximately 1600-nucleotide region of ORF1 and is rooted using an avian HEV strain (GenBank accession no. AY535004) from the genus *Avihepevirus*. Bootstrap support values are indicated at relevant nodes. The scale bar represents the number of nucleotide substitutions per site. Viruses from distinct subclades are color-coded, with designations including GenBank accession number, host species, and sampling location and year.

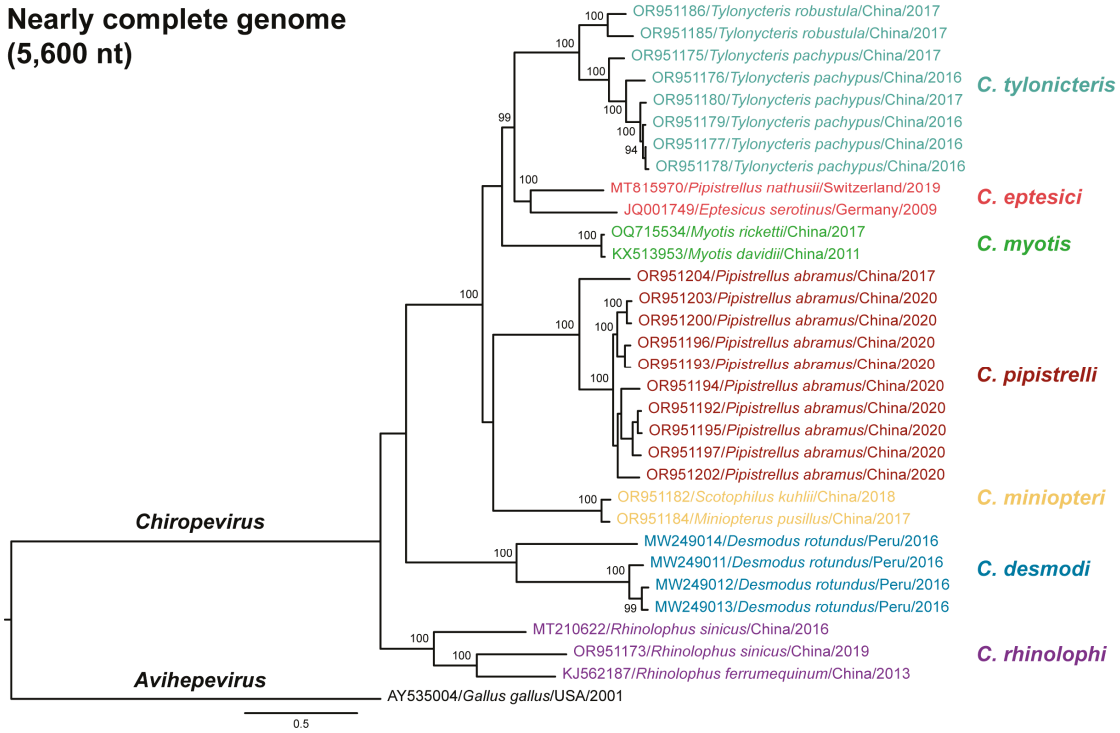

Figure 5. Maximum likelihood phylogeny of nearly complete genomes of chirohepeviruses. The nearly complete genomes consist of approximately 5600 nucleotides. The tree is rooted using an avian HEV strain (GenBank accession no. AY535004) from the related genus *Avihepevirus*. Bootstrap

support values are indicated at relevant nodes. The scale bar corresponds to number of nucleotide substitutions per site. Viruses from distinct subclades are highlighted in various colors. Virus designations include GenBank accession number, host species, sampling location, and collection year. Seven potentially designated bat *Chirohepevirus* species are shown on the right.

3.5. Co-Evolution of Chirohepeviruses and Bat Species

To assess the degree of co-evolution between chirohepeviruses and their bat hosts, we performed independent phylogenetic analyses of both the viruses and their host species and examined their co-evolutionary relationships (Figure 6A). The host phylogeny was constructed based on the cytochrome B (CYTB) gene sequences from 13 bat species across four families: Vespertilionidae, Miniopteridae, Phyllostomidae, and Rhinolophidae, that have been identified as hosts for selected chirohepeviruses. The viral phylogeny closely mirrored that of the host phylogeny, suggesting a long history of co-speciation between chirohepeviruses and their bat hosts (Figure 6A). Importantly, although chirohepevirus strains from *Eptesicus serotinus* (Germany) and *Eptesicus japonensis* (Japan) were identified in separate continents, Europe and Asia, respectively, they were grouped together, indicating the wide geographical transmission of their common ancestral chirohepeviruses.

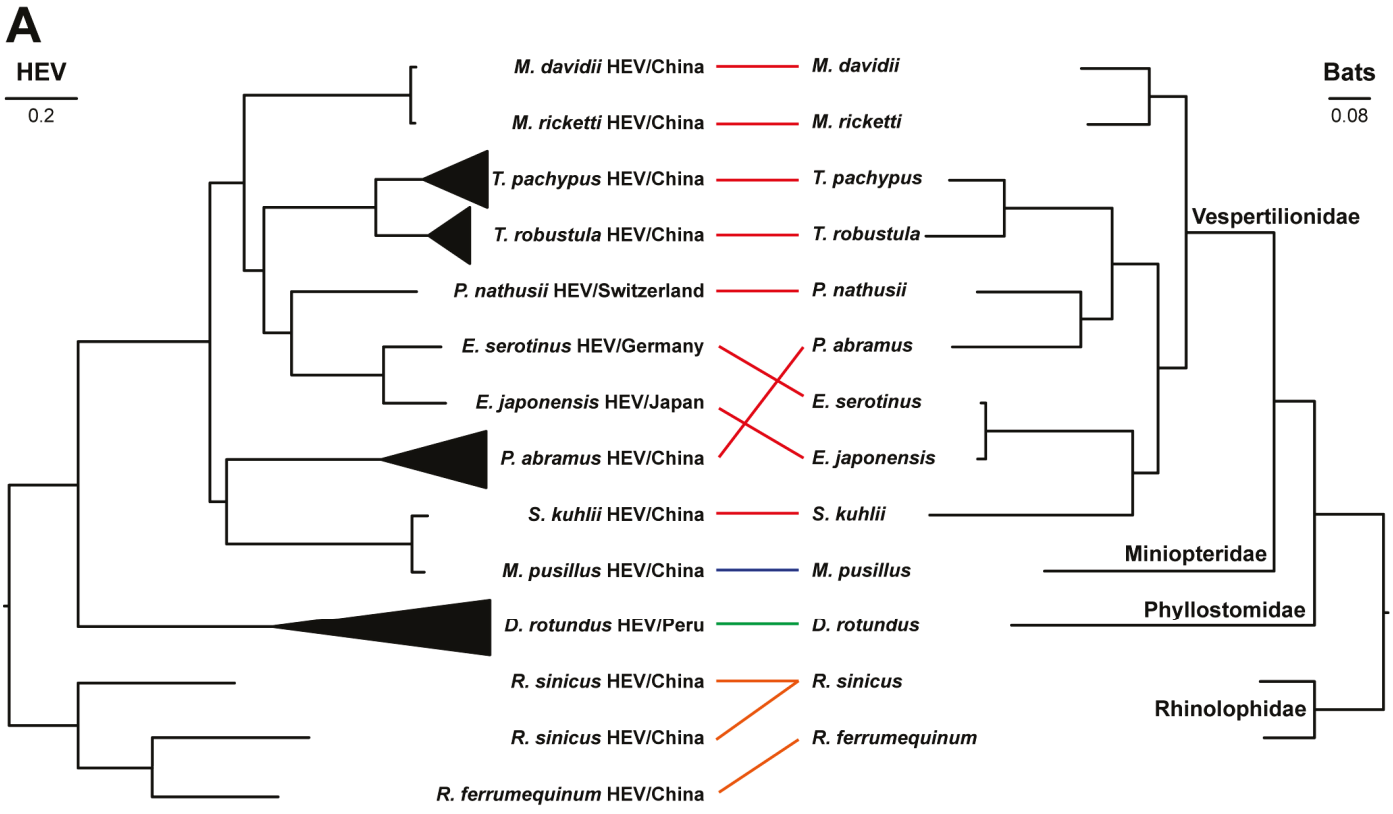

Figure 6. *Cont.*

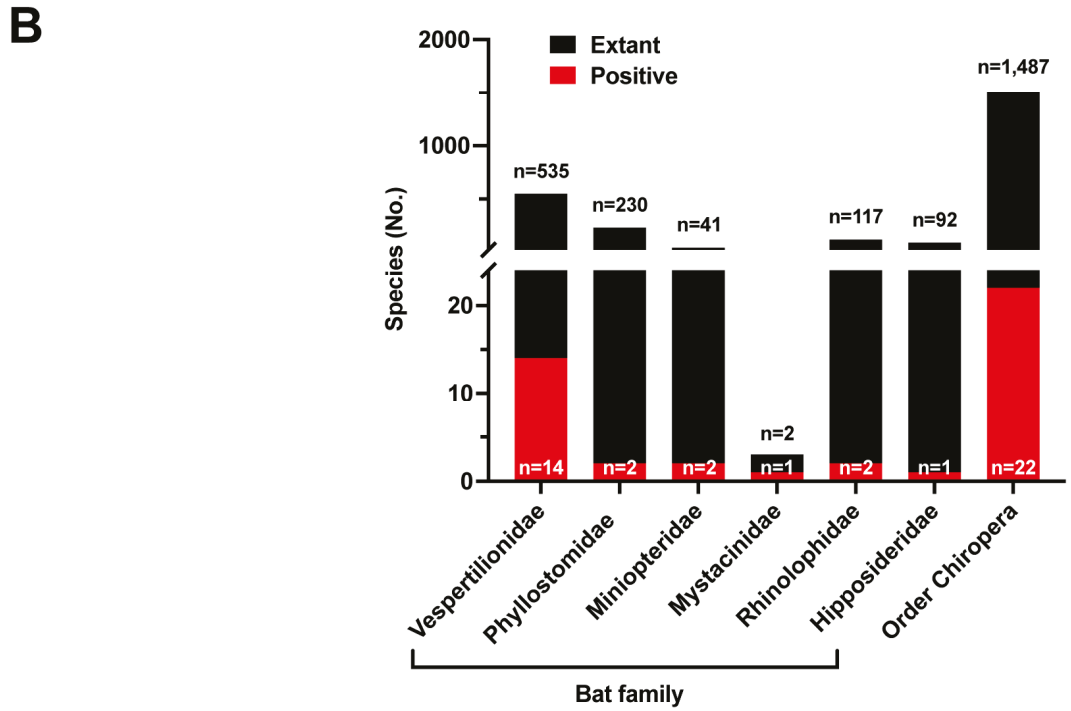

Figure 6. Co-evolution of chirohepeviruses and bat species. (**A**) Maximum likelihood trees generated based on approximately 4400 nucleotide sequences of chirohepeviruses in various bat species (left) and nucleotide sequences of the cytochrome B (CYTB) gene of corresponding hosts (right). Host taxonomy (family) is specified on the phylogenetic tree. Lines between chirohepeviruses and their corresponding host species are color-coded according to the four bat families. The scale bar corresponds to the number of nucleotide substitutions per site. (**B**) Comparison of the numbers of bat species testing positive for chirohepevirus versus the total number of extant bat species in each family or within the Order Chiroptera. The numbers of taxonomically described chiropteran species in each family are derived from the Bat Species of the World databases.

Some chirohepevirus strains display discordant evolutionary patterns compared to their respective bat hosts. For instance, although *Pipistrellus nathusii* and *Pipistrellus abramus* taxonomically belong to the same bat genus, the chirohepevirus detected in *P. nathusii* (Switzerland) clustered with viruses from *Eptesicus* species, forming a distinct group from the strain detected in *P. abramus* (China). Additionally, the chirohepevirus found in *Scotophilus kuhlii* (China) is closely related to a strain from *Miniopterus pusillus* (China), despite the hosts belonging to different families, Miniopteridae and Phyllostomidae. Since both viral strains were identified in the same study and country, it remains unclear whether these bat populations are interconnected and share similar viral strains, especially given the considerable evidence of frequent cross-species transmission events within the *Hepeviridae* family.

While it is evident that chirohepeviruses may have co-evolved with certain bat species, these results must be interpreted with caution. Bats are an extraordinarily diverse group, with more than 1400 species recognized to date, although only a small proportion have been studied. To date, chirohepeviruses have been detected in six bat families (Vespertilionidae, Phyllostomidae, Miniopteridae, Mystacinidae, Hipposideridae, and Rhinolophidae), spanning 22 species. This only represents less than 2% of the 1487 recognized bat species (Figure 6B), highlighting that a large number of bat species remain unexplored for the presence of HEV variants.

4. Discussion

Bats are unique among wildlife in their ability to carry viruses without exhibiting symptoms, positioning them as crucial reservoirs for emerging infectious diseases. Bat-associated viruses are particularly concerning due to the vital role bats play as natural hosts for a wide range of pathogens capable of spilling over into humans, such as coronaviruses, Ebola and Marburg viruses, and Hendra and Nipah viruses [17,20,35]. Recent discoveries of HEV-like viruses in multiple bat species worldwide have led to the establishment of a new genus, *Chirohepevirus*, within the family *Hepeviridae* [21,25,30]. This finding significantly broadens our understanding of HEV-related virus diversity, suggesting that bats may harbor a wider range of HEV-like viruses than previously recognized [11].

HEV is distinctive among hepatotropic viruses due to its zoonotic potential, with the ability to be transmitted between humans and animals. HEV ranks sixth in spillover risk among 887 wildlife viruses, underscoring its potential for cross-species transmission [36]. First identified in domestic pigs in the United States in 1997, swine HEV demonstrated zoonotic potential, prompting the identification of various HEV strains closely related to human variants in over a dozen animal species [37]. Among these, *Paslahepevirus balayani* and *Rocahepevirus ratti* species are known to infect humans. Specifically, HEV-1 and HEV-2 (within *P. balayani*) infect only humans, while HEV-3 and HEV-4 can infect both humans and a variety of animal species [12]. Additionally, HEV-5 and HEV-6 have been found in wild boars, and HEV-7 and HEV-8 in camels, with potential implications for human infection [38]. Furthermore, strains of *R. ratti* HEV-C1 in rats have been linked to zoonotic transmission, with over 20 documented cross-species infections globally [39]. The continuous emergence of novel HEV strains in wildlife, particularly those genetically similar to human HEV, highlights the importance of studying viral transmission dynamics and evolution to prevent future outbreaks [13]. Conceivably, the discovery of chirohepevirus strains in bats has raised important public health concerns regarding their zoonotic potential. Our phylogenetic analyses reveal that all known chirohepevirus strains from bats form a distinct monophyletic clade within the family *Hepeviridae*, separate from the clades of HEV variants found in other animal groups. Furthermore, extensive testing of 93,146 plasma samples from blood donors in Germany and 453 serum samples from HIV-infected patients in Cameroon yielded no evidence of bat chirohepevirus RNA, suggesting that these viruses do not currently pose a direct threat to humans [21]. Nevertheless, ongoing surveillance of bat populations and continued research into the zoonotic potential of chirohepeviruses remains important. Understanding the evolutionary processes within bat populations is essential for assessing potential spillover risks, especially as these viruses may evolve over time.

The discovery of novel HEV-related viruses across various animal species has significantly expanded the taxonomy within the family *Hepeviridae* [40]. Based on our phylogenetic inference and in accordance with the ICTV guidelines, we propose that at least seven distinct species, tentatively named *C. tylonicteris* from *Tylonycteris* sp., *C. eptesici* from *Eptesicus* sp., *C. myotis* from *Myotis* sp., *C. pipistrelli* from *Pipistrellus abramus*, *C. miniopteri* from *Miniopterus pusillus* and *Scotophilus khulii*, *C. desmodi* from *Desmodus rotundus*, and *C. rhinolophi* from *Rhinolophus* sp., could be assigned in the genus *Chirohepevirus*. These species correspond to specific chirohepevirus strains found in different bat species. Although 64 chirohepevirus sequences from six bat families and 22 bat species have been reported, only a few represent full-length or nearly complete viral genomes, with many others consisting of partial fragments. Phylogenetic analyses based on partial viral sequences may affect the robustness of viral evolutionary patterns. Therefore, acquiring more complete chirohepevirus genomes from additional bat species is essential to refining the classification of the genus *Chirohepevirus*. Furthermore, with the growing interest in the global bat virome

and advancements in sequencing technologies, we anticipate that novel chirohepeviruses will be discovered in other bat species.

Nevertheless, our study highlights the substantial genetic diversity within chirohepevirus strains, even within the same host species or genus, suggesting that bats have a long-term association with a diverse pool of genetically related chirohepeviruses. The observed genetic diversity raises the possibility that specific amino acid substitutions may be associated with strain-specific characteristics, such as viral virulence, transmission efficiency, or host specificity, similarly to the observations in human HEV strains [41]. A more detailed comparative analysis of amino acid substitutions across chirohepevirus strains from different host species and geographical regions may help determine whether certain genetic variations correlate with host species or environmental factors [42,43]. Despite the emergence of genetically diverse chirohepevirus strains, there remains a significant gap in research regarding their molecular virology and virus–host interactions. This is largely due to the absence of essential virological tools, such as reverse genetic systems and cell culture models, for these newly identified viruses [44]. Recent studies on paslahepevirus suggest that the papain-like cysteine protease (PCP) domain of ORF1 functions as either a metal-binding domain or a fatty acid binding domain [45,46]. Further structural analysis of HEV, particularly the ORF1 protein of chirohepevirus, would be useful for comparing the binding domain among different genera. Additionally, studying the infectivity and receptor-binding profiles of different chirohepevirus strains requires appropriate animal models. While bats may serve as a natural host for studying these viruses, technical difficulties remain, particularly given the limited understanding of bat immunology and immunopathology. Further research into the functional and structural properties of chirohepeviruses could yield valuable insights into the replication mechanisms and disease processes associated with human HEV.

Co-evolutionary analyses suggests that chirohepevirus strains exhibit a close evolutionary relationship with their chiropteran hosts, indicating virus–host co-speciation. While genetically diverse chirohepevirus strains have been identified in bats, the spillover risks into other mammalian hosts, such as humans, rodents, and pigs, are not known. In contrast, HEV-related viruses recently detected in various rodents may be ancestral to human- and swine-associated HEV variants [47–49]. The close phylogenetic relationship between the genera *Paslahepevirus* and *Rocahepevirus* suggests a shared ancestry, providing valuable context for understanding the broader evolutionary landscape of the *Hepeviridae* family [34,50]. As more novel HEV-related viruses continue to be discovered, they are likely to fill our knowledge gaps in understanding HEV evolution and transmission.

In summary, the discovery of chirohepeviruses in bats has significantly expanded the host range and diversity within the *Hepeviridae* family, providing new insights into the molecular characterization and evolutionary origins of HEV across different host species. Further detection and genomic characterization of chirohepevirus variants in additional bat species will be crucial for accurately mapping the evolution of genus *Chirohepevirus* and assessing potential zoonotic risks. Future functional research is essential to fully elucidate the ecology and molecular biology of bat chirohepeviruses.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/v17030339/s1, Figure S1: Maximum likelihood phylogeny of partial RdRp of chirohepeviruses identified in New Zealand; Table S1: GenBank accession numbers of the mitochondrial genomes of the bat species used in this study.

Author Contributions: B.W., P.C. and M.G.M. analyzed the data. B.W. and X.-L.Y. designed the study. B.W. drafted the original manuscript. Y.C.F.S. commented on and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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The Prevalence, Risk Factors, and Outcomes of Hepatitis E Virus Infection in Solid Organ Transplant Recipients in a Highly Endemic Area of Italy

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Abstract: Hepatitis E virus (HEV) infection can become chronic in immunocompromised patients, like solid organ transplant recipients (SOTRs). We evaluated HEV prevalence, risk factors, and outcomes among SOTRs in a hyperendemic HEV area. Three hundred SOTRs were enrolled from April to July 2019 and tested for anti-HEV IgM and IgG and HEV RNA. Sixty-three recipients (21%) were positive for any HEV marker. HEV infection was independently associated with older age and pork liver sausage consumption. Three viremic recipients harbored genotype 3e and 3f according to HEV RNA sequencing and phylogenetic analysis. Overall, 10 recipients had markers of active/recent infection (HEV RNA and/or anti-HEV IgM) and were followed up prospectively. Five of them spontaneously resolved their HEV infection. In two recipients, HEV clearance was achieved only through immunosuppression reduction, while three needed ribavirin therapy to achieve virologic resolution. We observed a chronic course in 30% of SOTRs with active/recent HEV infection. No association was found between tacrolimus assumption and chronicization. In conclusion, we found a high prevalence of infection among SOTRs attending a transplant center in a hyperendemic Italian HEV region. Systematic screening for all HEV markers and dietary education for infection control are needed for transplant recipients.

Keywords: HEV; HEV–host interactions; HEV genotypes; antivirals

1. Introduction

Hepatitis E virus (HEV) belongs to the Hepeviridae family. The taxonomy of this family has been recently updated [1]. Members of the family are now assigned to two subfamilies, five genera, and ten species. The HEV species Paslahepevirus balayani (subfamily Orthohepevirinae; genus Paslahepevirus) includes eight genotypes (HEV-1 to HEV-8) that can infect humans and wild and domestic animals. HEV-1 and HEV-2 infect only humans. The other genotypes also infect other mammals like pigs, wild boars, and deer (HEV-3 and HEV-4) and camelids (HEV-7 and HEV-8) [2–5]

HEV-1 and HEV-2 are mainly found in low-income African, Central American, and South East Asian countries, where viral transmission is mainly fecal–oral [3–7]. HEV-3 has spread worldwide, whereas HEV-4 is prevalent in Asia but is also present in Europe. Usually, HEV-3 and HEV-4 are transmitted by food through the ingestion of raw or undercooked meat and organs (e.g., liver and offal) of infected animals or through direct contact with them. Food-borne transmission can also occur by consuming fecally contaminated vegetables, fruits, mollusks, and drinking water. Finally, inter-human transmission of HEV-3 and HEV-4 through the transfusion of blood products and via solid organ transplantation is also possible [4–7]. Most of these zoonotic HEV infections are asymptomatic and self-limiting but in solid organ transplant recipients (SOTRs) and other immunocompromised patients (e.g., those with hematological malignancies, HIV infection, or on autoimmune disease treatments) can become chronic (in most cases HEV-3, sometimes HEV-4 and HEV-7, and rarely HEV-8) and evolve rapidly into cirrhosis [8–13]. In these patients, reduction in immunosuppressive treatment, when possible, and antiviral therapy with ribavirin are indicated [5].

Studies conducted up to the early 2010s in Italy reported very variable data on HEV infection prevalence in the general population and blood donors [14]. In the following years, the availability of highly sensitive and accurate anti-HEV antibody assays allowed researchers to conduct some reliable studies, both at regional and national scales, which greatly contributed to clarifying the epidemiological picture of HEV infection in our country [13–16]. These studies indicated that the mean crude anti-HEV IgG prevalence among blood donors in Italy was around 8–9%. There was also considerable interregional prevalence variability, with very high prevalence levels in some regions (e.g., Abruzzo), mainly attributable to local eating habits [13,14,16].

Our research group was the first to report high HEV endemicity in the Abruzzo region by conducting a prevalence survey in February–March 2014 among 313 voluntary blood donors residing in L'Aquila, the Abruzzo regional capital [13]. The detected anti-HEV IgG prevalence was 49%, and the consumption of raw or poorly cooked pork liver sausages was the only independent predictor of HEV infection. Subsequently, we performed two different nationwide HEV prevalence surveys among blood donors [14,16]. In 2015–2016, the anti-HEV IgG prevalence figures among donors from the Abruzzo region and L'Aquila were 22.8% and 31.6%, respectively [14]; in 2017–2019, we detected rates of 30% and 40%, respectively [16]. Temporal variations in anti-HEV IgG prevalence among blood donors in the same geographical area and using the same assay have already been reported in other countries, even across a longer time span [16]. Furthermore, a prospective incidence study conducted among blood donors in L'Aquila during 2013–2014 found an incidence rate of 2.1/100 person/years [17]. Such an incidence figure is much higher than that observed in the general population and blood donors from other European countries and the United States and approached the incidence rates estimated in immunocompromised patients [17]

Despite this, thus far, few Italian studies have reported data on the prevalence of HEV infection and the persistence of viremia in immunocompromised patients and even fewer in SOTRs [18–25].

In this study, we aimed to determine HEV infection prevalence and risk factors among SOTRs attending a regional transplant center operating in a high HEV prevalence area (Abruzzo). Also, we aimed to assess the prevalence and outcomes of chronic HEV hepatitis in these patients, describing clinical aspects, laboratory features, and therapeutic approaches.

2. Patients and Methods

2.1. Study Population, Design, and Ethics

All SOTRs attending the Regional Transplant Center of Abruzzo and Molise at "San Salvatore" Hospital in L'Aquila (Abruzzo region) for post-transplant follow-up in 2019 who signed an informed consent form were eligible for participation in this study. At that time, patients were not routinely tested for HEV infection before organ transplantation. All enrolled patients provided signed informed consent and were administered a questionnaire collecting information regarding risk factors for HEV infection. The clinical and laboratory data and transplant history were collected from the outpatient records of the enrolled patients.

This study used a cross-sectional and prospective design. In the first phase, all participants were screened for all HEV infection markers: anti-HEV IgM, anti-HEV IgG, and HEV RNA. After the screening phase, patients who tested positive for IgM and/or HEV RNA underwent a virologic, biochemical, and clinical assessment, which would then be repeated every month for a year in order to assess the infection's evolution. According to Kamar et al., chronic hepatitis E in SOTRs is defined as viremia persistence for at least 3 months from its detection [26].

The study was conducted as part of a public health effort for the active surveillance of transplanted patients at risk of chronic HEV infection in an area with high HEV circulation.

2.2. Serologic Testing for Anti-HEV Antibodies

Anti-HEV IgM and anti-HEV IgG were detected using the Wantai HEV IgM ELISA and Wantai HEV IgG ELISA, respectively (Beijing WANTAI Biological Pharmacy Enterprise Co., Ltd., Beijing, China), according to the manufacturer's instructions. In order to make it easier to describe the results of this study, SOTRs reactive to any of these infection markers were considered as HEV-positive, regardless of whether the recipient had been infected in the past (anti-HEV IgG only) or had markers of recent/active HEV infection (anti-HEV IgM and/or HEV RNA-positive). The exact group of HEV-positive SOTRs has been specified in the text wherever confusion might arise.

2.3. Detection of HEV RNA

HEV RNA was detected as previously described [27]: RNA was extracted from 200 μL of plasma using the QIAmp MinElute Virus Spin kit (Qiagen, Hilden, Germany); then, one half extract was used as a template for Real-Time PCR using the RealStar HEV RT-PCR kit (Altona Diagnostics, Hamburg, Germany) according to the manufacturer's instructions.

2.4. Sequencing of HEV RNA

HEV RNA-positive extracts were used as a template to amplify a fragment from the ORF2 region of the HEV genome using a previously described nested Reverse Transcription PCR [27,28]. Purified PCR products were sequenced using the BigDye Terminator Cycle Sequencing Kit on an automated sequencer (Thermo Fisher/Applied Biosystems, Waltham, MA, USA). The obtained final sequences (493 nt) were deposited in GenBank (accession numbers PP898067, MZ274270, and MZ274271).

2.5. Phylogenetic Analyses

Phylogenetic analysis was used for sequence genotyping and sub-genotyping. A sequence dataset for genotyping was built, including the newly obtained sequences and established reference genotype sequences [27,29]. As all of the sequences of the present study proved to be genotype 3 (see Results), a separate dataset for sub-genotyping was built, including the newly obtained sequences and established reference 3 sub-genotypes (3a to 3m) [29,30]. Phylogenetic analysis was carried out using a Maximum Likelihood approach in MEGA version 12. The phylogenetic tree was constructed using the best substitution model preliminarily estimated using the Model tool in MEGA. The statistical significance of the tree was evaluated through bootstrap analysis; bootstrap values > 70 were considered significant.

All virologic analyses were performed in the Department of Infectious Disease of Istituto Superiore di Sanità (ISS) in Rome, Italy.

2.6. Other Laboratory Analyses

All patients underwent routine blood tests, including complete liver and kidney function, whole-blood cell count, and plasmatic determination of immunosuppressive drug levels.

2.7. Clinical Management of HEV RNA-Positive Recipients

HEV RNA-positive recipients underwent infectious disease consultation, liver ultrasound, and a fibroscan to assess liver damage. In patients who had a standard immunological risk, a reduction in immunosuppressive therapies was attempted, according to EASL guidelines. Specifically, the dose of calcineurin inhibitors was reduced by approximately 30% in most cases. Monthly HEV viremia monitoring was then performed in these SOTRs. Patients persistently viremic despite a four-month reduction in immunosuppression underwent antiviral therapy. These patients received a 3-month course of ribavirin at a dose of 600 mg per day, modified according to renal function, the development of side effects, and HEV RNA levels during therapy. HEV-RNA was monitored every month until the test was negative and then at one, three, and six months to confirm a sustained virologic response.

2.8. Statistical Analysis

The statistical analysis was performed with RStudio (version 1.3.959), IDE for R software (version 4.2.3.2). Odds Ratios and 95% Confidence Intervals for positive results for any HEV marker (i.e., HEV positivity, as reported above) were computed using the Wald Test. The association between HEV infection and other categorical variables was estimated using the *p*-value and chi-squared test. Variables with a *p*-value < 0.10 were evaluated in a multivariate logistic model. Backward selection (based on the AIC) was used to perform the multivariate model. The variables sex and age were included in the model independently of their *p*-value. The relationship between HEV infection and continuous variables was evaluated using the Mann–Whitney U test.

3. Results

3.1. Patients' Characteristics

In 2019, approximately 430 SOTRs attended the transplant center's outpatient clinic. Of these patients, 300 agreed to participate in the study and signed an informed consent form and were enrolled between April and July 2019, during the scheduled transplantation follow-up visit. They were mostly males (65.3%) with a mean age of 57.8 (±10.3) years and, on average, 8.6 (±7.5) years post-transplant. More than half of the recipients resided in the Abruzzo region, while the others were mostly from the two bordering regions of Lazio and

Molise. A few recipients resided in the Campania region. The vast majority of SOTRs had had a kidney transplant. (Table 1).

Table 1. Characteristics of the SOT recipients enrolled in the study.

	Total (300)	**HEV+**	**HEV−**	***p*-Value**
Sex	***n.***	***n.* (%)**	***n.* (%)**	
Female	104	18 (17.3)	86 (82.7)	0.253
Male	196	45 (23)	151 (77)	
Age	**(mean ± SD)**	**(mean ± SD)**	**(mean ± SD)**	
	57.8 (10.3)	62.6 (10.5)	56.5 (9.9)	0.00
Region of residence	***n.***	***n.* (%)**	***n.* (%)**	
Abruzzo	171	42 (24.6)	129 (75.6)	0.081
Molise	44	10 (22.7)	34 (77.3)	0.761
Lazio	77	10 (13)	67 (87)	0.045
Campania	8	1 (12.5)	7 (87.5)	0.55
Comorbidity	***n.***	***n.* (%)**	***n.* (%)**	
Yes	285	59 (20.7)	226 (79.3)	0.957
No	10	2 (20)	8 (80)	
Missing	5	2 (40)	3 (60)	-
Transplanted organ	***n.***	***n.* (%)**	***n.* (%)**	
Kidney	295	60 (20.3)	235 (79.7)	0.146
Liver	4	2 (50)	2 (50)	
Kidney + liver	1	1 (100)	0 (0)	-
Organ donor	***n.***	***n.* (%)**	***n.* (%)**	
Deceased	271	61 (22.5)	210 (77.5)	0.05
Living	29	2 (6.9)	27 (93.1)	
Years from transplant	**(mean ± SD)**	**(mean ± SD)**	**(mean ± SD)**	
	8.6 (7.5)	7.4 (6.2)	8.9 (7.8)	0.27
Laboratory data [1]	**(mean ± SD)**	**(mean ± SD)**	**(mean ± SD)**	
AST (UI/L)	18.9 (7.1)	19.6 (8.7)	18.7 (6.6)	0.75
ALT (UI/L)	17.2 (10.7)	18.6 (15.4)	16.89 (9.1)	0.99
ALP (UI/L)	79.2 (34.1)	83.4 (38.8)	78.1 (32.7)	0.33
GGT (UI/L)	34.3 (40.3)	39.2 (55.7)	33.0 (35.1)	0.64
Bilirubin (mg/dL)	0.72 (0.35)	0.73 (0.31)	0.72 (0.36)	0.34
Creatinine (mg/dL)	1.63 (1.03)	1.49 (0.53)	1.67 (1.12)	0.90
Platelets (μ/nL)	216.1 (61.9)	214.5 (60.3)	216.6 (62.5)	0.85
Leukocytes (μ/nL)	6.9 (2.02)	6.7 (2.01)	7.0 (2.03)	0.45
Lymphocyte (μ/nL)	1.77 (0.73)	1.67 (0.68)	1.8 (0.75)	0.30
Lymphocyte (%)	26.4 (8.7)	25.7 (8.2)	26.5 (8.9)	0.49
Immunosuppressants	***n.***	***n.* (%)**	***n.* (%)**	
Tacrolimus	219	50 (22.8)	169 (77.2)	0.2
Cyclosporine A	65	12 (18.5)	53 (81.5)	0.56
MPA/MMF	246	48 (19.5)	198 (80.5)	0.17
Methylprednisolone	272	57 (21)	215 (79)	0.95

[1] ALT, alanine transaminase; AST, aspartate transaminase; ALP, alkaline phosphatase; GGT, gamma-glutamyl transferase; MPA, mycophenolic acid; MMF, mycophenolate mofetil.

The comparison between SOTRs positive for any HEV marker and negative ones showed that those positive were significantly older; organ donations came more often

from deceased donors, but this difference was only close to significance ($p = 0.05$); between the two groups there were no differences regarding sex, region of residence, presence of comorbidities, type of transplanted organ, biochemical parameters and immunosuppressive medications (Table 1).

3.2. HEV Infection Prevalence and Risk Factors

In total, 63 SOTRs (21%) were positive for any HEV marker: 61 were anti-HEV IgG-positive (of whom 8 were also anti-HEV IgM-positive and 3 were HEV RNA-positive); 1 was positive for both anti-HEV IgM and HEV RNA but anti-HEV IgG-negative; and 1 tested HEV RNA-positive but negative for both anti-HEV IgM and anti-HEV IgG (see also Supplementary Table S1). Thus, in total, there were nine anti-HEV IgM-positive patients and five HEV RNA-positive patients. Out of the nine anti-HEV IgM-positive SOTRs, four were simultaneously HEV RNA-positive and anti-HEV IgM-positive, while five recipients were IgG-positive and HEV RNA-negative. All anti-HEV IgM-positive patients and the one positive for only HEV RNA were considered as actively/recently infected and were followed up prospectively. Three HEV sequences were obtained (Figure 1). The 53 recipients who tested positive for anti-HEV IgG only had a past HEV infection and were not followed up.

By analyzing the responses to the socio-demographic and risk factors questionnaire through univariate and multivariate logistic analyses (Table 2), we found that the only variables independently associated with HEV positivity were age over 65 years and eating pork liver sausages.

Table 2. Univariate and multivariate analyses of sociodemographics and risk factors [a] associated with hepatitis E virus infection in solid organ transplant recipients.

					Univariate Analysis			Multivariate Analysis		
		N. Tested	**HEV+ (N)**	**HEV+ (%)**	**OR**	**95% CI**	***p***	**AdjOR**	**95% CI**	***p***
Sex	Female	104	18	17.3	1	-	-			
	Male	196	45	22.9	1.42	0.77–2.61	0.253	1.300	0.73–2.31	0.370
Age (yrs)	21–54	99	10	10.1	1	-	-			
	55–64	124	21	16.9	1.81	0.81–4.06	0.143			
	>65	77	32	41.6	**6.33**	**2.86–14.21**	**0.000**	**2.55**	**1.32–4.94**	**0.004**
Place of residence	Urban area	194	41	21.1	1	-	-			
	Rural area	106	22	20.7	0.97	0.55–1.75	0.936			
Years of schooling	0–8 yrs	140	28	20.0	1	-	-			
	≥9 yrs	157	35	22.3	1.12	0.61–1.96	0.691			
Work with animals	No	271	57	21.0	1	-				
	Yes	29	6	12.1	0.98	0.38–2.52	0.966			
Swine contact	No	279	61	21.9	1	-	-			
	Yes	17	2	11.8	0.48	0.11–2.14	0.323			
Contact with other animals [b]	No	107	25	23.4	1	-	-			
	Yes	189	38	20.1	0.82	0.47–1.46	0.511			
Hunting	No	277	59	21.3	1					
	Yes	14	4	28.6	1.48	0.45–4.88	0.519			
Vegetable gardening	No	241	54	22.4	1	-	-			
	Yes	47	9	19.1	0.82	0.37–1.80	0.621			
Eating vegetables from own or friends' gardens	No	120	22	18.3	1	-	-			
	Yes	174	40	23.0	1.33	0.74–2.38	0.336			
Using manure to fertilize the garden	No	217	44	20.3	1	-	-			
	Yes	52	13	25.0	1.31	0.64–2.66	0.454			
Eating pork sausage [c]	No	126	23	18.2	-					
	Yes	164	36	21.9	1.26	0.70–2.43	0.438			

Table 2. *Cont.*

					Univariate Analysis			Multivariate Analysis		
		N. Tested	**HEV+ (N)**	**HEV+ (%)**	**OR**	**95% CI**	***p***	**AdjOR**	**95% CI**	***p***
Eating pork liver sausages [c]	No	240	42	17.5	1	-	-	-	-	-
	Yes	50	17	34.0	**2.43**	**1.24–4.76**	**0.008**	**2.025**	**1.11–3.68**	**0.024**
Eating wild boar sausages [c]	No	264	53	20.1	1	-	-			
	Yes	26	6	23.1	1.19	0.46–3.12	0.717			
Eating pork seasoned sausages	No	171	29	17.0	1	-	-			
	Yes	119	30	25.2	1.651	0.93–2.93	0.086			
Eating homemade sausages [c]	No	191	39	20.4	1	-	-			
	Yes	99	20	20.2	0.98	0.54–1.80	0.965			
Eating game meat [c]	No	264	56	21.2	1	-	-			
	Yes	29	6	20.7	0.97	0.37–2.49	0.948			
Eating raw seafood	No	224	50	22.3	1	-	-			
	Yes	70	12	17.1	0.720	0.36–1.44	0.354			
Drinking usually non-bottled water	No	177	36	20.3	1	-	-			
	Yes	123	27	21.9	0.81	0.46–1.41	0.450			
Blood or blood product transfusion	No	133	25	18.8	1	-	-			
	Yes	164	38	23.2	1.30	0.74–2.29	0.359			
Travelling abroad	No	146	27	18.5	1	-	-			
	Yes	149	36	24.2	1.404	0.80–2.46	0.235			

[a] Exposure to risk factors (except for demographic variables) was assessed over a lifetime; [b] wild boar, deer, deer, etc.; [c] raw or undercooked.

3.3. Prospective Follow-Up of Patients with Evidence of Active or Recent HEV Infection

The explosion of the COVID-19 pandemic in Italy in early 2020 heavily conditioned the second phase of our study. In particular, the restrictions imposed by the lockdown prevented the SOTRs from complying with the follow-up visit schedule. Furthermore, several months elapsed between the execution of the cross-sectional survey and the start of the prospective study. This time was needed for sample collection, their shipment to Istituto Superiore di Sanità in Rome, and the execution of all virologic tests.

Table 3 shows in detail the results of the prospective virologic follow-up of the ten actively/recently infected SOTRs. It was evident that the five recipients that were anti-HEV IgM- and IgG-positive but HEV RNA-negative at the screening survey, namely, recipient 36 (a 67-year-old male), recipient 92 (a 63-year-old male), recipient 141 (a 65-year-old male), recipient 218 (a 69-year-old male), and recipient 287 (a 64-year-old male), all had a recently resolved HEV infection, as documented by their decreasing anti-HEV IgM and IgG OD values. Thus, follow-up of these patients ceased.

For recipient 133 (a 63-year-old female), resulting in anti-HEV IgM positivity with low-level viremia (the sequencing attempt failed) at the screening survey, the lack of any subsequent follow-up prevented us from ascertaining the virologic resolution until 11 months later, when new serum samples from this patient became available. At time zero, transaminases were normal, while GGT values were slightly increased. This recipient was non-compliant, and their tacrolimus dosage had already been reduced several times during standard follow-up, maintaining a very low tacrolimus trough level during any control test. We can hypothesize that viral clearance was influenced by this behavior.

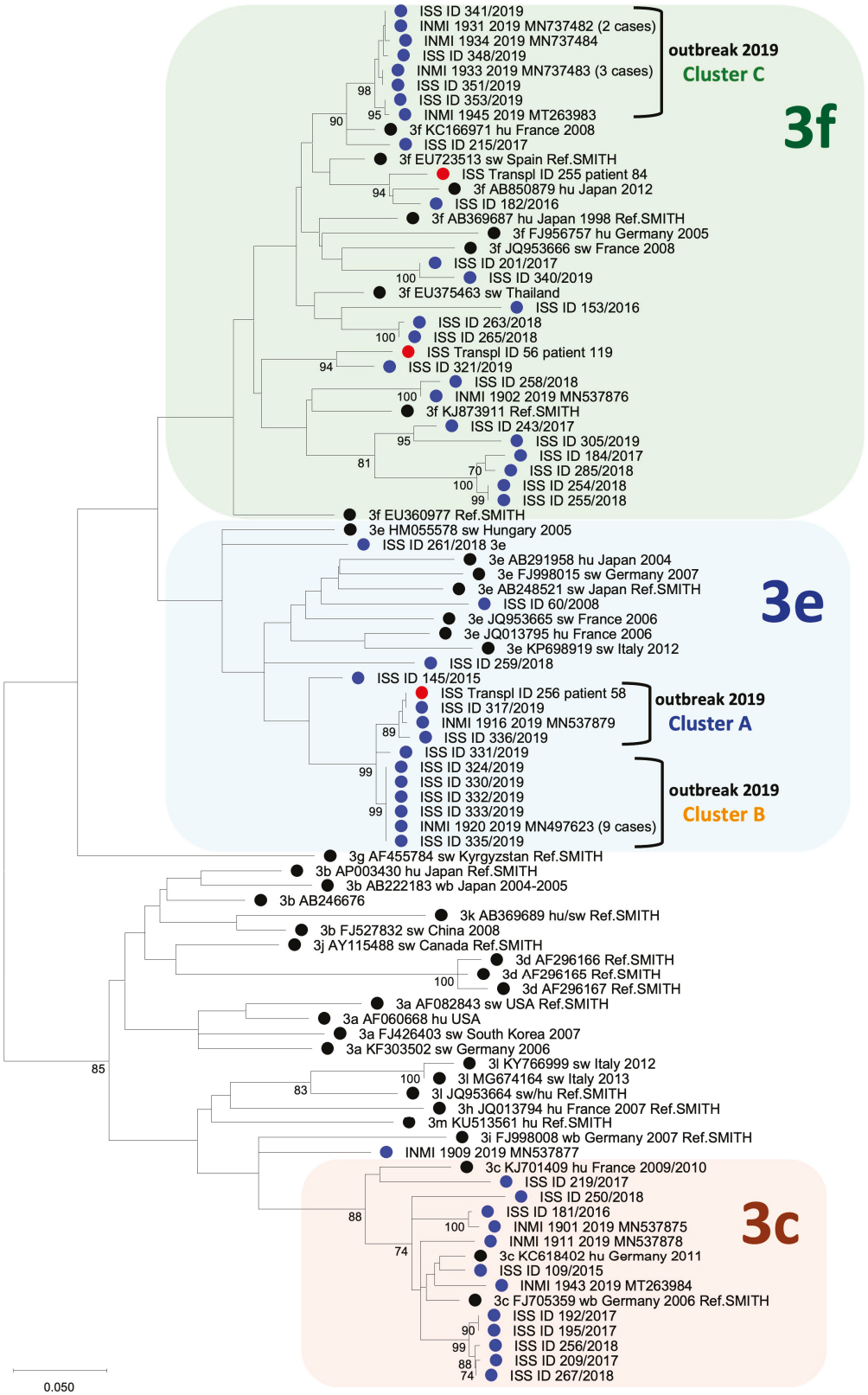

Figure 1. Phylogenetic tree from the analysis of the HEV sequences from the three SOTR patients (red circles) together with sequences from HEV-positive cases detected during an outbreak in Abruzzo and Lazio in 2019 (blue circles) and subtype reference sequences of HEV genotype 3 (black circles); the suffix "Ref.SMITH" in the sequence name marks the references recommended by international expert agreement [29,30]. The three molecular clusters (A, B, and C) identified in the 2019 outbreak are shown [27].

Table 3. Prospective follow-up of SOT recipients with evidence of recent and/or viremic HEV infection.

Code	Date *	IgM †	IgG †	HEV RNA †	Date	IgM	IgG	HEV RNA	Date	IgM	IgG	HEV RNA	Date	IgM	IgG	HEV RNA	Date	IgM	IgG	HEV RNA
IN	m/y	OD	OD	cp/mL	m/y	OD	OD	cp/mL	m/y	OD	OD	copies/mL	m/y	OD	OD	cp/mL	m/y	OD	OD	cp/mL
36	4/19	**1.202**	**1.940**	NR	10/19	0.002	**1.493**	NR	FUI											
58	6/19	0.001	0.008	**10^4–10^5**	10/19	**2.484**	**2.319**	**10^6**	11/19	**2.299**	**2.494**	**>10^5**	12/19	**2.507**	**2.428**	**10^3**	1/20	**1.980**	**2.449**	**10^3**
84	6/19	**2.312**	**3.000**	**>10^5**	10/19	**2.154**	**2.490**	**10^6**	11/19	**2.475**	**2.366**	**10^4**	12/19	**2.321**	**2.462**	**10^3–10^4**	1/20	**2.462**	**2.188**	NR
92	7/19	**0.523**	**2.479**	NR	10/19	**0.536**	**2.503**	NR	FUI											
119	4/19	**2.550**	**2.545**	**10^5**	5/19	**2.517**	**2.566**	**10^5**	6/19	**2.524**	**2.514**	**10^4**	7/19	**2.513**	**3.000**	**>10^3**	9/19	**2.523**	**2.490**	**10^4**
133	4/19	**1.270**	0.001	**<10^2**	6/19	0.001	0.001	NR	5/20	0.012	0.002	NR	9/20	0.012	0.049	NR	FUI			
141	4/19	**0.538**	**1.526**	NR	9/19	**0.483**	**1.245**	NR	FUI											
218	5/19	**0.350**	**1.746**	NR	11/19	**0.343**	**0.810**	NR	FUI											
228	5/19	**2.535**	**1.190**	**10^3**	6/19	**2.511**	**2.525**	NR	4/20	**0.588**	**2.425**	NR	FUI							
287	5/19	**0.834**	**2.361**	NR	10/19	**0.683**	**2.115**	NR	FUI											

Code	Date	IgM	IgG	HEV RNA	Date	IgM	IgG	HEV RNA	Date	IgM	IgG	HEV RNA	Date	IgM	IgG	HEV RNA	Date	IgM	IgG	HEV RNA
	m/y	OD	OD	cp/mL	m/y	OD	OD	cp/mL	m/y	OD	OD	cp/mL	m/y	OD	OD	cp/mL	m/y	OD	OD	cp/mL
58	2/20	**1.575**	**2.455**	NR	4/20	**1.497**	**2.436**	NR	9/20	**0.908**	**2.350**	NR	FUI							
84	3/20	**2.467**	**2.172**	NR	4/20	**2.445**	**2.443**	NR	10/20	**2.285**	**2.372**	NR	FUI							
119	10/19	**2.484**	**2.497**	NR	11/19	**2.476**	**2.470**	NR	12/19	**2.469**	**2.481**	**<10^2**	1/20	**2.452**	**2.457**	NR	2/20	**2.432**	**2.464**	NR

Code	Date	IgM	IgG	HEV RNA	Date	IgM	IgG	HEV RNA	Date	IgM	IgG	HEV RNA
	m/y	OD	OD	cp/mL	m/y	OD	OD	cp/mL				cp/mL
119	4/20	**2.449**	**2.456**	NR	6/20	**2.416**	**2.438**	NR	8/20	**2.328**	**2.388**	NR	FUI

FUI, follow-up interruption; IN, identification number; IgM, anti-HEV immunoglobulin M; IgG, anti-HEV immunoglobulin G; NR, non-reactive, m/y, month/year; OD, optical density; cp/mL, copies/mL. * The first date reported in this table corresponds to that of the cross-sectional screening execution. † Positive test results are highlighted in bold.

Recipient 228 (a 54-year-old female) showed low-level HEV RNA (the sequencing attempt failed) and positivity for both anti-HEV IgM and IgG at the baseline survey. Her transaminases were normal with GGT values moderately increased. Before HEV screening, she had already received a low dose of a cyclosporine regimen because of the development of breast cancer and intolerance to m-TOR inhibitors. After screening, she underwent a further slight reduction in immunosuppression in consideration of the foreseeable rapid failure of the kidney transplant. She became HEV RNA-negative a month later. The patient returned to dialysis 5 months after the diagnosis of HEV infection.

Patients 58 (a 72-year-old male) and 84 (a 29-year-old female), both HEV RNA-positive at the screening survey, were still viremic 4 months later. They harbored genotype 3e and 3f, respectively (Figure 1). The strain detected in patient 58 was one of the three strains involved in an HEV outbreak in Abruzzo in 2019 [27]. After an initial and unsuccessful reduction in immunosuppression, they were both treated with ribavirin (see below).

Recipient 119 (a 56-year-old male) tested HEV RNA-positive (genotype 3f) at the screening survey (Figure 1). Despite immunosuppression reduction, he continued to have low-level viremia for 4 months, and then, due to the detection of liver fibrosis on his fibroscan, he was administered ribavirin therapy (see below).

Figure 2 summarizes all serovirologic results and the infection evolution in all HEV-infected patients. Overall, we observed a chronic course in 30% of SOTRs followed up prospectively. No association was found between a chronic course of infection and the use of tacrolimus rather than cyclosporine A as immunosuppressive treatment. Four out of the five (80%) recipients experienced a spontaneous resolution of infection and were treated with tacrolimus. A non-significant different proportion (two out of three recipients were administered tacrolimus) was found among those that developed chronic hepatitis ($p = 1.0$). Likewise, among our recipients, no association was found between a chronic course of HEV infection and low ALT/AST levels or a low platelet count upon the diagnosis of HEV infection.

3.4. Clinical–Laboratory Features and Outcome of Chronically Infected SOTRs

- Recipient 58 (a 72-year-old male) underwent a kidney transplant from a deceased donor 9 years earlier. He had a complex medical history: chronic HCV infection, successfully treated with interferon 18 years earlier, and bilateral kidney cancer surgically removed before the transplant, in addition to skin cancer, cardiovascular diseases, and recurrent lithiasic cholangitis post cholecystectomy after transplant. His e-GFR was 53 mL/min/1.73 mq, remaining stable over time. He was on maintenance immunosuppressive therapy with a low dose of prednisone, calcineurin inhibitors (cyclosporine), and m-TOR inhibitors (everolimus) because of his cancer history. Four months after his anti-HEV-positive screening test, he presented a three-fold elevation of transaminases and GGT values. Liver ultrasound documented mild–moderate steatosis and mild hepatomegaly. The fibroscan described non-constant calculated stiffness (6.9 and 13.6 KPa). As he was considered a standard immunological risk patient, a 25% reduction in cyclosporine dose was first attempted, but given the persistence of viremia, he underwent ribavirin therapy (400 mg/day) for three months. Liver enzyme levels returned to the baseline range, and a control fibroscan showed stiffness reduction (3.6 Kpa). Viral clearance was confirmed one month and one year after the end of therapy.
- Recipient 84 (a 29-year-old woman) was kidney transplanted from a deceased donor 5 years earlier. She was on maintenance immunosuppressive therapy with prednisone, tacrolimus, and mycophenolate mofetil. Her e-GFR was 88 mL/min/1.73 mq, remaining stable over time. She had no previous rejection episodes or pregnancy, and she

was considered a standard immunological risk patient. She was HEV-RNA-positive at the screening survey and still viremic 4 months later. She showed a two-fold liver enzyme and GGT level elevation and normal liver ultrasound and fibroscan. Tacrolimus dose reduction of approximately 30% was applied, but given the persistent viral load, 3 months later, ribavirin therapy was introduced at a dose of 600 mg per day for 3 months. During therapy, she experienced mild anemia, but a dose reduction was not necessary. Viral clearance was achieved at the end of therapy and confirmed 12 months later.

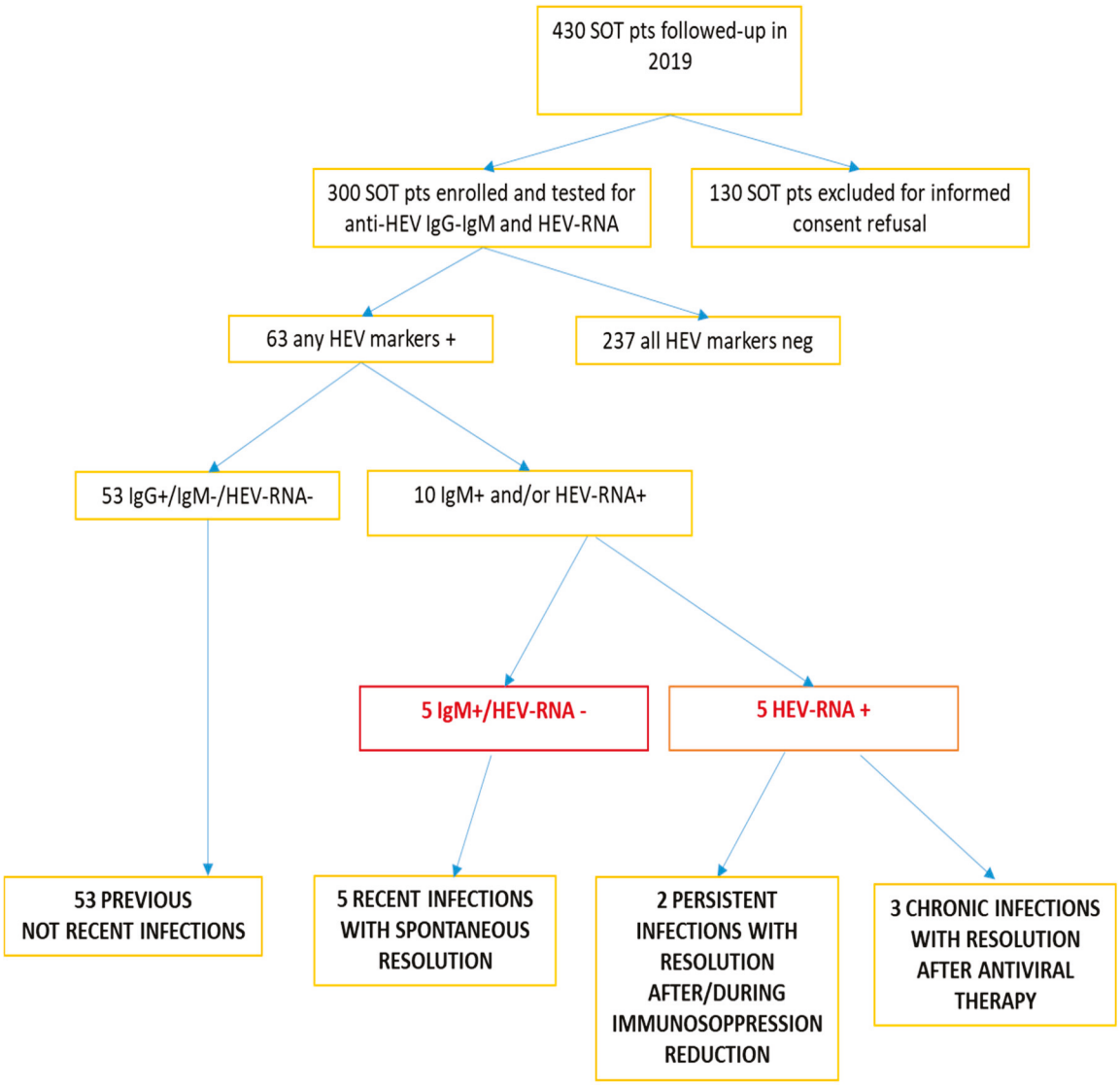

Figure 2. Summary of serovirological results and infection evolution in HEV-infected patients.

- Recipient 119 (a 57-year-old man) underwent a kidney transplant from a deceased donor 2 years earlier. He was on maintenance immunosuppressive therapy with prednisone, mycophenolate mofetil, and tacrolimus, and his e-GFR was 70 mL/min/1.73 mq. One year before the screening test, he had shown a two–three-fold increase in liver enzyme values, which remained unchanged over time. Liver ultrasound at that time revealed moderate steatosis, screening for HBV and HCV was negative, and HEV testing was not performed because it was not available. In April 2019, he was enrolled in the study, and the screening test was positive for HEV-RNA IgM and IgG. He complained of muscle pain in his neck and shoulders. He was

considered a standard immunological risk patient, and tacrolimus dose reduction of approximately 30% was promptly applied. A fibroscan showed a stiffness of 7.2 Kpa (F1–F2 fibrosis). After 3 months, he showed persistent viral load and started a 3-month therapy with ribavirin at a dose of 600 mg per day. After 45 days and 35 days, he needed two ribavirin dose reductions (400 mg and then 200 mg per day) for symptomatic anemia. Follow-up HEV-RNA tests performed after 30 and 60 days were negative, but at the end of treatment, low-level HEV RNA was once again detected. Therefore, he underwent a further one-month course of ribavirin 400 mg per day, achieving a sustained virologic response confirmed during 1-, 3-, and 6-month follow-up tests. The short length of this second course was due to ribavirin toxicity. Liver enzyme levels returned to the baseline range after the first month of ribavirin therapy.

Two of the three chronically HEV-infected patients (recipients 58 and 119) and both patients who resolved their infection after immunosuppression modulation (recipients 133 and 228) declared joint or muscle pain with resolution after healing. All patients had normal liver enzyme levels and cholestasis indices at the end of follow-up, while eGFR remained stable in all patients with active infection, except for recipient 228, who lost the graft.

4. Discussion

In this study, 63 out of 300 SOTRs were positive for any HEV marker, giving an overall prevalence of 21%. The prevalence of past infection was 17.6% (53/300), while that of active/recent infection was 3.3% (10/300).

In a recent systematic review and meta-analysis of 18 studies (4557 SOTRs), the pooled estimated prevalence of HEV infection (any HEV marker) in SOTRs was about 20%, while that of acute HEV infection (anti-HEV IgM+ and/or HEV RNA+) was about 4.5%. The approximate pooled prevalence according to the different solid organs were as follows: liver 27%, kidney 15%, heart 13%, lung 5.5%, and undetermined organ 29.5% [31]. In this meta-analysis, anti-HEV IgG prevalence was significantly higher in studies that used the Wantai assay compared to studies employing other assays. The highest HEV seroprevalence was reported by studies performed among liver transplant recipients in south/south-east France (about 36–38%) and Thailand (about 56%) [32–34].

To the best of our knowledge, only four studies have thus far investigated HEV prevalence among SOTRs in Italy [22–25]. The study by Scotto et al. was performed in southern Italy (Apulia region) during 2012–2013 and investigated 120 kidney transplant recipients (mean age 48 years) using a non-Wantai assay, detecting an anti-HEV IgG prevalence of 3.3%. Puttini et al., during 2011–2013, investigated 118 kidney transplant recipients (mean age 51 years) in central Italy (Siena, Tuscany region) by using a non-Wantai assai and found an HEV prevalence of 10.2%. In 2017, in Turin, Piedmont region (northern Italy), Zanotto et al. analyzed 120 liver transplant recipients (mean age 51 years) using a non-Wantai assay, detecting an HEV prevalence of 8.3%. Finally, De Nicola et al., during 2010–2014, tested 79 liver transplant recipients (mean age 55 years) in Milan (Lombardy, northern Italy) using a Wantai assay and found a prevalence of 33%. Considering the characteristics and results of our study and those of the above-mentioned Italian studies, it is likely that the differences in prevalence are attributable to the antibody assay used and the type of transplanted organ, but the geographical location and the mean age of the study population may also play an important role. However, a limitation of most of these studies, including the present one, is the lack of pre-transplant HEV serological surveillance data, which prevents the possibility of assessing post-transplant incidence.

Indeed, most of the recipients enrolled in our study resided in Abruzzo, a region of Italy where very high levels of HEV incidence and prevalence have been constantly

documented in blood donors [13,14,16,17]. The detected prevalence of 21% among SOTRs in this study was higher than that found among Italian blood donors during 2017–2019 (8.3%) but was significantly lower than that found among blood donors in the Abruzzo region in the same years (30%). This epidemiological picture was attributed to the widespread habit among Abruzzo residents of consuming raw or poorly cooked pork liver sausages [13,14, 16,17]. In fact, in the present study, the only two independent risk factors for HEV infection among SOTRs were the consumption of raw or poorly cooked pork liver sausages and age over 65 years. A similar scenario was documented in south-west France, where among blood donors, an HEV prevalence of 52% was reported [35] and a prevalence of 38.4% was found (with an annual incidence of 3.3%) in kidney and liver transplant recipients [32].

It is well known that immunocompromised patients, particularly SOTRSs, are usually strongly and steadily advised by doctors to avoid eating any kind of raw or undercooked food. How can we then explain the high HEV prevalence found among them? Eating raw or poorly cooked pork liver sausages is a hard habit to break in certain populations (like those living in the Abruzzo region or in south-west France) and is closely linked to their traditions and history. Furthermore, we know that inter-human transmission of HEV-3 and HEV-4 through the transfusion of blood products is possible and that chronic kidney or liver disease patients, as far as immunocompromised ones are concerned, are prone to need them. However, in our study, no association was found between the transfusion of blood products and HEV infection (Table 2). In consideration of the low-to-moderate anti-HEV IgG seroprevalence in blood donors and, above all, the absolute lack of donors positive for HEV RNA in two different nationwide surveys [14,16], Italy has deemed the introduction of universal HEV RNA blood donation screening unnecessary.

One of the three HEV strains isolated in this study was involved in an outbreak in Abruzzo and Lazio in 2019 caused by multiple strains and due to the consumption of raw or undercooked pork products. The molecular data suggested that the outbreak was sustained by newly imported strains, possibly through the import of pork products or live animals from outside of Abruzzo [27].

In 3 out of 10 recipients (30%) with active/recent HEV infection, a chronic course was observed, while five patients showed a spontaneous resolution of their infection. The chronicity rate reported by us was much less than that reported by Kamar (66%) and by other authors [36–38]. Also, differing from Kamar, no association was found between a chronic infection course and the use of tacrolimus rather than cyclosporine A or a low platelet count or a low transaminases level upon the diagnosis of HEV infection. This is probably attributable to the small number of patients with active/recent HEV infection observed in our study compared with the number observed by Kamar (85 SOTRSs) [36].

None of our patients with viremic infection complained of gastroenteric symptoms or jaundice, while the majority (four out of five) complained of joint or muscle pain, mainly in the neck or upper arms. This suggests that extrahepatic manifestation can be predominant in chronic as well as in acute HEV infection [39]. All three patients with chronic HEV infection had altered liver enzyme levels, but this was not observed in all of the viremic infections. Thus, HEV surveillance in SOT patients should be systematic and not only guided by specific symptoms and/or laboratory test alterations.

Immunosuppressive treatment reduction of about 30% was performed as suggested by guidelines in all patients with standard immunologic risk but was effective in just one out of four patients, while another patient acquired and cleared the infection during a low-dose regimen. The decision to reduce treatment can be challenging in this type of patient but can avoid the use of antiviral therapy [40].

Ribavirin is the only antiviral therapy available against HEV infection [41]. It was mainly used in the past for anti-HCV treatment. Ribavirin is a drug with a low therapeutic

index, mainly for anemia occurrence, and this can be enhanced in SOT patients, especially in those with impaired kidney function. Moreover, its efficacy can be widely affected by dose reduction. In our study, two out of three patients treated with ribavirin developed anemia and one needed a dose reduction. This same patient needed a longer course of therapy because of a viremic recurrence of infection.

5. Conclusions

We found a high HEV infection prevalence among SOTRSs attending a transplant center in a highly endemic HEV region in Italy. Considering the immunocompromised status of these patients and the related risk of chronicization and liver damage, all SOTRSs should be systematically tested for all HEV markers, including HEV RNA, upon transplant center admission and periodically during their post-transplant follow-up, especially if residing in hyperendemic areas. The prevention of HEV infection must be pursued, together with enhancing dietary education for these patients. Further studies are needed to assess the prevalence, clinical course, and outcome of this infection in other groups of patients with immunodeficiency beyond SOT ones, especially in hyperendemic regions.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/v17040502/s1. Table S1. Distribution of the different HEV markers in SOT recipients.

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

Abbreviations

The following abbreviations are used in this manuscript:

SOTRs	Solid Organ Transplant Recipients
HEV	Hepatitis E Virus
ALT	Alanine transaminase
AST	Aspartate transaminase
ALP	Alkaline phosphatase
GGT	Gamma-glutamyl transferase
MPA	Mycophenolic acid
MMF	Mycophenolate mofetil

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viruses

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Communication

Recent Hepatitis E Virus Infection in Wild Boars and Other Ungulates in Japan

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Abstract: Hepatitis E virus (HEV) is a zoonotic pathogen with multiple hosts, posing significant public health risks, especially in regions like Japan where game meat consumption is prevalent. This study investigated HEV infection and viral shedding in wild boars, sika deer, and Japanese serows across Japan. A total of 1896 serum samples were tested for anti-HEV antibodies, 1034 for HEV RNA, and 473 fecal samples for viral shedding. Anti-HEV antibodies were detected in wild boars from all seven prefectures studied, while HEV RNA was detected in wild boars from Fukuoka, Oita, and Miyazaki in southern Japan, as well as Yamaguchi prefecture. Genetic analysis revealed subtypes 3b, 4a, and 4g, with 3b being the most prevalent. Subtype 3b exhibited distinct geographical clustering, whereas 4g persisted exclusively in Yamaguchi for over 12 years. Infectious HEV particles were confirmed in wild boar feces, highlighting the risk of environmental contamination and zoonotic transmission. Sika deer showed no evidence of HEV infection, and only one Japanese serow tested positive for antibodies without detectable RNA. These findings underscore the importance of ongoing surveillance to assess the zoonotic risks from game meat consumption and prevention of HEV transmission to humans.

Keywords: hepatitis E virus; game meat; wild boar; sika deer; Japanese serow

1. Introduction

Hepatitis E virus (HEV) is a single-stranded positive-sense RNA virus that causes an estimated 20 million infections worldwide [1]. HEV virions exist in two forms: quasi-enveloped particles in the bloodstream and non-enveloped particles in feces [2]. Belonging to the *Hepeviridae* family, HEV comprises four genera: *Paslahepevirus*, *Rocahepevirus*, *Chirohepevirus*, and *Avihepevirus* [3]. The *Paslahepevirus* genus includes two species, *P. alci* and *P. balayani*, with the latter comprising eight genotypes. Genotypes 1 and 2 are transmitted via

the fecal–oral route, primarily through contaminated water, and exclusively infect humans, posing significant fetal, neonatal, and maternal mortality risks [4,5]. In contrast, genotypes 3 and 4 are zoonotic, primarily transmitted through foodborne routes, and are prevalent in industrialized countries. Although domestic pigs are the main reservoirs, these genotypes have been detected in various species and, more recently, in wastewater, shellfish, and seawater [6–10]. Genotypes 5 and 6 have been identified exclusively in wild boars in Japan, while genotypes 7 and 8 have been found in dromedary and Bactrian camels, respectively [11,12].

In Japan, genotypes 3 (HEV-3) and 4 (HEV-4) are the most prevalent, with transmission primarily occurring through zoonotic foodborne routes and, to a lesser extent, via blood or organ transfusions [13,14]. The first zoonotic case in Japan was linked to the consumption of sika deer [15]. Since then, HEV-3 and HEV-4 have been detected in various wild animals, including wild boars, mongooses, rabbits, and monkeys [16–19]. Additionally, HEV-3 has been detected in environmental samples, such as sewage and seawater, indicating its environmental circulation in Japan [20,21].

HEV exhibits broad host adaptability, infecting various ungulates, as evidenced by the detection of HEV RNA and anti-HEV antibodies in species such as moose, red deer, and roe deer [22–25]. While wild boars are widely recognized as the primary HEV reservoir in wildlife in Japan [12], the possibility of cross-species spillover infections and environmental transmission of HEV remains poorly understood. In Japan, where game meat consumption is widespread, wild ungulates represent a significant risk factor for HEV transmission to humans. Moreover, the recent increase in diagnosed HEV cases highlights the urgent need for continuous monitoring of HEV reservoirs and zoonotic transmission routes [13]. Therefore, this study aimed to assess HEV prevalence and viral shedding in wild ungulates across Japan to understand HEV circulation in wildlife and evaluate zoonotic transmission risks to humans.

2. Materials and Methods

2.1. Serum Sample Collection

In total, 1896 samples were collected from three species: wild boar (*Sus scrofa*), deer (*Cervus nippon*), and serow (*Capricornis crispus*), amounting to 952, 909, and 35 samples, respectively. Wild boar samples were collected from 2017 to December 2024 from seven prefectures, including Aomori (n = 13), Toyama (n = 57), Ishikawa (n = 173), Wakayama (n = 539), Yamaguchi (n = 51), Kagawa (n = 47), and Nagasaki (n = 72). Deer samples were collected between 2022 and 2024 from the prefectures of Aomori (n = 17), Gunma (n = 10), Gifu (n = 116), Wakayama (n = 328), Yamaguchi (n = 126), Kagawa (n = 30), and Nagasaki (n = 282). All samples from Nagasaki Prefecture were collected on Tsushima Island. A total of 35 serow samples were collected from Yamagata from 2017 to 2023. These wild animals were mainly captured as countermeasures under the official population control program. All samples were collected without overlap with those in our previous study [26]. Animal experiments were approved by the Japan National Institute of Infectious Diseases (NIID) Institutional Animal Care and Use Committee (Approval No. 122212).

2.2. Fecal Sample Collection

A total of 473 fecal samples were collected from wild ungulates across multiple prefectures in Japan, including 186 samples from wild boars and 287 from deer. Wild boar samples were collected from 2021 to January 2024 across 13 prefectures: Aomori (n = 10), Yamagata (n = 14), Chiba (n = 3), Toyama (n = 2), Shizuoka (n = 1), Nara (n = 4), Tottori (n = 1), Okayama (n = 3), Fukuoka (n = 6), Kumamoto (n = 7), Oita (n = 95), Miyazaki (n = 38), and Kagoshima (n = 2). Deer samples collected between 2021 and 2022 came from

the prefectures of Hokkaido (*n* = 9), Aomori (*n* = 9), Iwate (*n* = 4), Yamagata (*n* = 1), Gunma (*n* = 7), Kanagawa (*n* = 1), Yamanashi (*n* = 3), Shizuoka (*n* = 39), Aichi (*n* = 7), Kyoto (*n* = 6), Osaka (*n* = 29), Hyogo (*n* = 7), Tottori (*n* = 26), Fukuoka (*n* = 1), Oita (*n* = 33), and Miyazaki (*n* = 54).

2.3. Sample Processing and Storage

Serum samples were transported to the laboratory in cooling boxes maintained at 4 °C and stored at −20°C. Fecal samples were diluted in phosphate-buffered saline (PBS) to a final concentration of 10% (*w*/*v*) and stored at −80 °C. They were subsequently clarified via centrifugation at 10,000× *g* for 1 min at 4 °C before RNA extraction.

2.4. Detection of Anti-HEV Antibodies in Wild Ungulate Sera

Anti-HEV antibodies in wild animal sera were detected using our previously reported highly sensitive enzyme-linked immunosorbent assay (ELISA) for various species [27,28]. Briefly, animal sera (100 µL per well) were added as the primary antibody in a 1:100 dilution, and detection was performed using peroxidase-conjugated protein AG (Thermo Fisher Scientific, Waltham, MA, USA). The absorbance was measured after adding ABTS 2-Component Microwell Peroxidase Substrate (SeraCare Life Sciences, Milford, MA, USA) and shaking the plates for 30 min at room temperature. The reaction was stopped with 100 µL of 1% sodium dodecyl sulfate, and absorbance was measured at 405 nm using a spectrophotometer (Bio-Rad, Hercules, CA, USA). As reported in our previous study on different mammalian species, the cut-off value was set at 0.437 for wild boar sera and 0.500 for other species [27,28].

2.5. HEV Genome Detection in Sera and Fecal Samples from Wild Animals

As previously described [29–31], RNA was extracted from fecal and serum samples using the MagMAX Pathogen RNA/DNA Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Due to the sample availability, 116 µL of serum and 116 µL of fecal samples were processed separately, resulting in a 50 µL elution each.

Nested reverse transcription polymerase chain reaction (RT-PCR) was performed to detect HEV RNA using the OneStep RT-PCR Kit (QIAGEN, Germantown, MD, USA) and KOD-Plus-NEO (Toyobo, Osaka, Japan). Primers targeting the conserved open reading frame 2 (ORF2) region of HEV genotypes 1, 3, and 4 were used for amplification, as previously described [27,32]. The resulting 378 bp amplicon was purified with the FastGene Gel/PCR Extraction Kit (Nippon Genetics, Tokyo, Japan), and sequencing was performed using BigDye Terminator v3.1 chemistry (FASMAC, Atsugi, Japan). Following primer removal, the final 338 bp sequences were deposited in the DNA Data Bank of Japan under accession numbers LC857165–LC857174.

2.6. Quantitative Real-Time RT-PCR (RT-qPCR)

The copy numbers of viral RNA were quantified using a one-step RT-qPCR assay with TaqMan Fast Virus 1-Step Master Mix (Applied Biosystems, Foster City, CA, USA) on a LightCycler 480 II (Roche, Vienna, Austria). A broadly reactive one-step RT-qPCR assay was performed using the forward primer JVHEVF (5′-GGTGGTTTCTGGGGTGAC-3′), reverse primer JVHEVR (5′-AGGGGTTGGTTGGATGAA-3′), and probe JVHEVP (5′-FAM-TGATTCTCAGCCCTTCGC-TAMRA-3′), following a previously described protocol [33,34]. The thermal cycling conditions were as follows: reverse transcription at 50 °C for 5 min, initial denaturation at 95 °C for 20 s, followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. A 10-fold serial dilution of HEV-3 RNA (10^1 to 10^7 copies) was used to generate a standard curve for quantification [34,35].

2.7. Cell Culture and Virus Inoculation

The human hepatocarcinoma cell line PLC/PRF/5 (JCRB0406) was obtained from the Health Science Research Resources Bank, Japan. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, Saint Louis, MO, USA) and 1% penicillin-streptomycin (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan), at 37 °C in a humidified 5% CO_2 atmosphere.

For virus isolation, fecal samples were filtered through 0.45-μm filters (Corning, Corning, NY, USA). PLC/PRF/5 cells were seeded in 6-well plates (Sumitomo Bakelite, Tokyo, Japan) and inoculated with 100 μL of the filtered samples with 1% antibiotic-antimycotic solution (Gibco, Grand Island, NY, USA). The cells were incubated at 37 °C for 24 h. After 24 h, the medium was removed, and the cells were washed two times with DMEM. The culture medium was then replaced with fresh DMEM containing 2% heat-inactivated FBS and antibiotics. The medium was replaced every 3–4 days and supernatants were collected every 7 days for further detection of HEV RNA. Cells were observed daily for cytopathic effects.

2.8. Immunofluorescence Assay

HEK-293T cells were transfected with the plasmids pCAGGS-HEVcap (112–660) and pCAGGS using polyethyleneimine (PEI; Thermo Fisher Scientific, USA), as previously described [28]. Two days post-transfection, the cells were fixed with 10% formalin (Fujifilm, Osaka, Japan), permeabilized with 0.5% (*v*/*v*) Triton X-100 (Sigma-Aldrich, Saint Louis, MO, USA) for 30 min, and washed three times with PBS. Blocking was performed at 37 °C for 1 h using PBS containing 10% FBS, followed by three washes with PBS. Serow serum was used as the primary antibody at a dilution of 1:100 in PBS containing 1% FBS. For the positive controls, rabbit anti-HEV-1 VLPs hyperimmune serum (kindly provided by Dr. Tian-Cheng Li from the National Institute of Infectious Diseases) and monoclonal mouse anti-HEV ORF2 antibody (clone 1E6, Sigma-Aldrich, Saint Louis, MO, USA) were diluted at 1:1000 and 1:100, respectively. After 1 h of incubation at 37 °C, the cells were washed with PBS. For secondary staining, Protein A and G conjugated with Alexa Fluor 488 (Thermo Fisher Scientific, Waltham, MA, USA) was used for serow samples at a 1:100 dilution, while Alexa Fluor 488-labeled anti-rabbit IgG (Life Technologies, Chicago, IL, USA) and FITC-conjugated mouse IgG, IgM, and IgA antibodies (ICN Pharmaceuticals, Bryan, OH, USA) were used as positive controls. Fluorescence was visualized by indirect immunofluorescence microscopy, using pCAGGS-transfected cells as negative controls.

2.9. Phylogenetic Analysis

Phylogenetic analysis was performed using the MEGA7 software (version 7.0) based on partial ORF2 sequences (338 nucleotides). A phylogenetic tree was generated using the neighbor-joining method with 1000 bootstrap replicates, and branches with bootstrap values > 70% were grouped [36]. The analysis included reference sequences from the *Paslahepevirus* genus (genotypes 1 to 8), as well as the outer groups of *Rocahepevirus*, *Chirohepevirus*, *Avihepevirus,* and unclassified HEV sequences. For *Paslahepevirus* genotypes 3 and 4, the proposed subtypes were incorporated [36], including at least five strains per subtype when available, with the strains closest to our isolates available in NCBI up to January 2025 included in the analysis.

2.10. Statistical Analysis

All analyses, including the calculation of prevalence rates and corresponding confidence intervals, were performed in R version 4.4.3 (R Core Team, 2021). Chi-square analysis was used where appropriate, with statistical significance set at $p < 0.05$.

3. Results

3.1. Detection of Anti-HEV Antibodies and HEV RNA in Serum Samples of Wild Ungulates

A total of 952 wild boar serum samples were collected from seven prefectures, with 53 animals testing positive for anti-HEV antibodies, yielding an overall seroprevalence of 5.6% (95% confidence interval (CI) 4.20–7.22) (Table 1). The seroprevalence rates were 21.6% (11/51, 95% CI 11.29–35.32), 11.1% (8/72, 95% CI, 4.92–20.73), 10.6% (5/47, 95% CI 3.55–23.11), 7% (4/57, 95% CI 1.95–17.00), 7.7% (1/13, 95% CI 0.19–36.02), 3.5% (19/539, 95% CI 2.14–5.45), and 2.9% (5/173, 95% CI 0.95–6.62) for the Yamaguchi, Nagasaki, Kagawa, Toyama, Aomori, Wakayama, and Ishikawa prefectures, respectively (Table 2). In Yamagata, one of the 35 serow samples tested positive for anti-HEV antibodies, yielding a seroprevalence of 2.8% (95% CI 0.07–14.92). The positivity was confirmed using indirect immunofluorescence. All 909 deer serum samples from various prefectures were negative for anti-HEV antibodies (Table 1).

Table 1. HEV prevalence in wild ungulates in Japan.

Samples	Species	Collection Year	% of Anti-HEV Antibody-Positive Animals (No. of Positive Animals/No. of Examined Animals)	% of HEV RNA-Positive Animals (No. of Positive Animals/No. of Examined Animals)
Serum	Wild boar	2017–2024	5.6 (53/952)	0.4 (3/703)
	Deer	2022–2024	0 (0/909)	0 (0/296)
	Serow	2017–2023	2.8 (1/35)	0 (0/35)
Feces	Wild boar	2021–2024	-	3.8 (7/186)
	Deer	2021–2022	-	0 (0/287)

Table 2. Detection of anti-HEV antibodies and HEV RNA in serum samples.

Species	Prefecture	Collection Year	% of Anti-HEV Antibody-Positive Animals (No. of Positive Animals/No. of Examined Animals)	% of HEV RNA-Positive Animals (No. of Positive Animals/No. of Examined Animals)
Wild boar	Aomori	2022–2023	7.7 (1/13)	0 (0/13)
	Toyama	2022–2024	7 (4/57)	0 (0/57)
	Ishikawa	2017–2023	2.9 (5/173)	ND *
	Wakayama	2022–2024	3.5 (19/539)	0 (0/539)
	Yamaguchi	2022–2024	21.6 (11/51)	6.4 (3/47)
	Kagawa	2022–2024	10.6 (5/47)	0 (0/47)
	Nagasaki	2022–2023	11.1 (8/72)	ND
Deer	Aomori	2022–2023	0 (0/17)	0 (0/17)
	Gunma	2022	0 (0/10)	0 (0/10)
	Gifu	2022–2023	0 (0/116)	0 (0/116)
	Wakayama	2022–2023	0 (0/328)	ND
	Yamaguchi	2022–2024	0 (0/126)	0 (0/123)
	Kagawa	2022–2024	0 (0/30)	0 (0/30)
	Nagasaki	2022–2023	0 (0/282)	ND
Serow	Yamagata	2017–2023	2.8(1/35)	0 (0/35)

* ND: No data.

HEV genome detection was performed using nested RT-PCR, and HEV RNA was detected exclusively in wild boar samples collected from Yamaguchi Prefecture, with a positivity rate of 6.4% (3/47, 95% CI 1.34–17.54). All three detected strains belonged to genotype 4, subtype 4g (Figure 1). HEV RNA was not detected in deer serum samples. Serow samples were analyzed using nested RT-PCR and real-time RT-PCR to ensure the accuracy and sensitivity of our results, but no RNA was detected in either assay (Table 2).

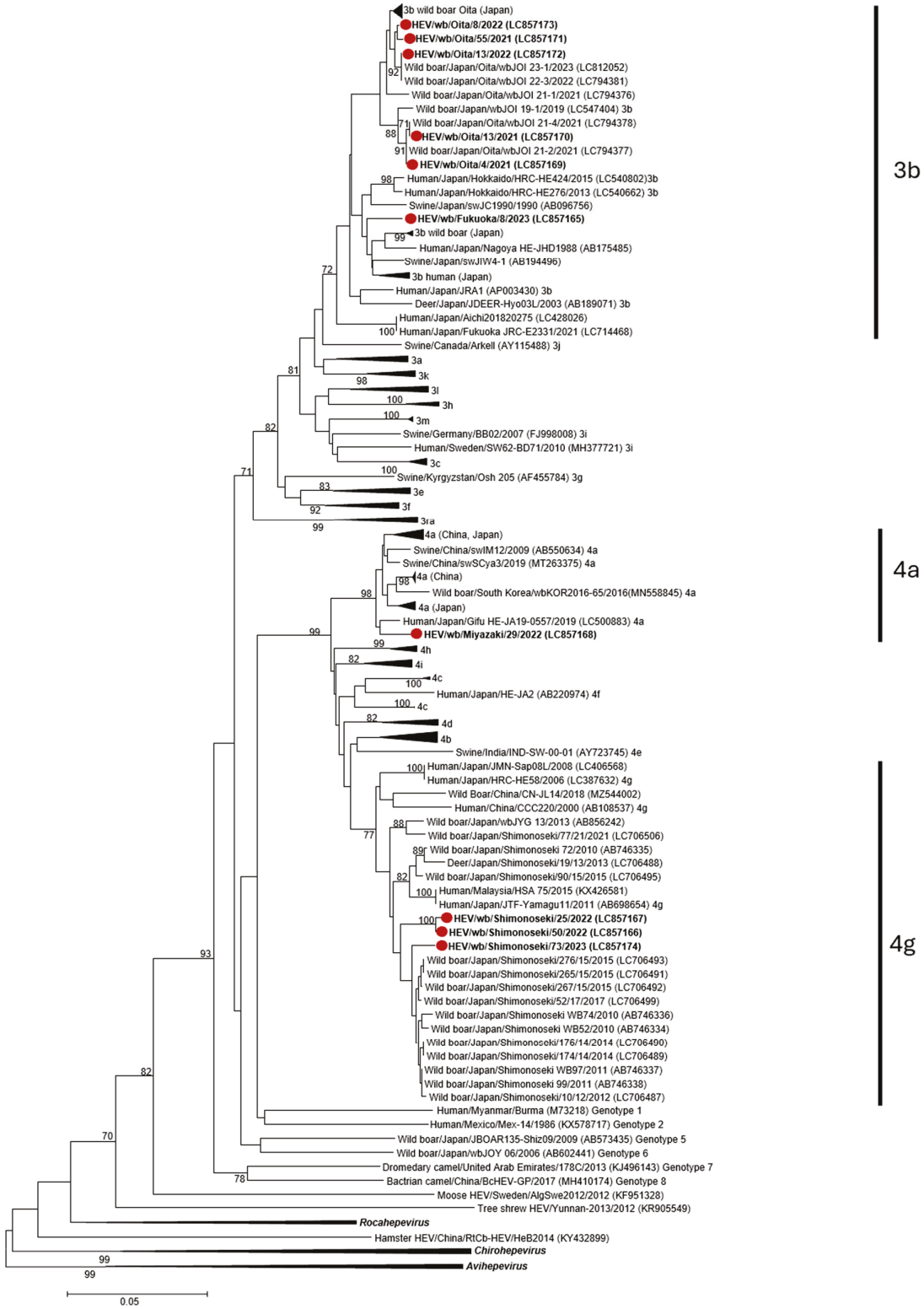

Figure 1. Phylogenetic analyses based on the partial ORF2 sequences (338 nucleotides) using the genotype 3 and 4 subtype reference strains proposed by Smith et al., 2020 [36] and the closest strains available in GenBank. The 10 wild boar strains obtained in the current study are highlighted with closed red circles (●). Subtypes 3b, 4a, and 4g are marked with vertical lines. Similar sequences are grouped by black triangles. For homogeneous groups, species and country of origin are indicated. The phylogenetic tree with 1000 bootstrap replicates was generated using the neighbor-joining method, and values less than 70% were removed. Sequences were labeled as "host/country/region/strain/year (GenBank accession number) subtype". Scale bar indicates 0.05 nucleotide substitutions per site.

3.2. HEV RNA Detection in Fecal Samples of Wild Boar and Deer

HEV genome detection in fecal samples was performed using nested RT-PCR, which showed positive results only in wild boar samples. The overall genome detection positivity rate was 3.8% (7/186, 95% CI 1.53–7.60) (Table 1). HEV RNA was detected in fecal samples from Fukuoka, Oita, and Miyazaki, with positivity rates of 16.7% (1/6, 95% CI 0.42–64.12), 5.3% (5/95, 95% CI 1.73–11.86), and 2.6% (1/38, 95% CI 0.07–13.81), respectively, whereas fecal samples from the remaining 10 prefectures were negative for genome screening (Table 3). Six of the detected strains belonged to genotype 3, subtype 3b, and one strain belonged to genotype 4, subtype 4a (Figure 1).

Table 3. Detection of HEV RNA in fecal samples.

Species	Prefecture	Collection Year	% of HEV RNA-Positive Animals (No. of Positive Animals/No. of Examined Animals)
Wild boar	Aomori	2023	0 (0/10)
	Yamagata	2021–2023	0 (0/14)
	Chiba	2023	0 (0/3)
	Toyama	2023	0 (0/2)
	Shizuoka	2021	0 (0/1)
	Nara	2021–2022	0 (0/4)
	Tottori	2021	0 (0/1)
	Okayama	2022	0 (0/3)
	Fukuoka	2022–2023	16.7 (1/6)
	Kumamoto	2021–2023	0 (0/7)
	Oita	2021–2023	5.3 (5/95)
	Miyazaki	2021–2023	2.6 (1/38)
	Kagoshima	2023–2024	0 (0/2)
Deer	Hokkaido	2021–2022	0 (0/9)
	Aomori	2021–2022	0 (0/9)
	Iwate	2022	0 (0/4)
	Yamagata	2022	0 (0/1)
	Gunma	2021	0 (0/7)
	Kanagawa	2022	0 (0/1)
	Yamanashi	2022	0 (0/3)
	Shizuoka	2021–2022	0 (0/39)
	Aichi	2022	0 (0/7)
	Kyoto	2022	0 (0/6)
	Osaka	2021–2022	0 (0/29)
	Hyogo	2022	0 (0/7)
	Tottori	2021	0 (0/26)
	Fukuoka	2022	0 (0/1)
	Oita	2021–2022	0 (0/33)
	Miyazaki	2021–2022	0 (0/54)

3.3. Infectivity of Wild Boar HEV Strains in PLC/PRF/5 Cells

Of the 10 strains, only the wb/Oita/8 strain (accession number LC857173) could infect PLC/PRF/5 cells. The cells were inoculated with 100 µL of the original stool suspension, which contained 2.39×10^8 copies/mL of viral RNA. Viral RNA in the infected PLC/PRF/5 cells was first detected on day 7 post-infection (p.i.) at 1.73×10^4 copies/mL. The viral load progressively increased to 5.93×10^6 copies/mL by day 49 p.i. and reached 1.89×10^7 copies/mL on day 84 p.i. (Supplementary Figure S1). To confirm the infectivity of the isolated strain, 0.5 mL of supernatant from day 84 p.i. was used to infect a 25T flask

of PLC/PRF/5 cells, resulting in a viral RNA concentration of 3.25×10^6 copies/mL by day 77 p.i. No cytopathic effects were observed in any of the passages.

4. Discussion

This study evaluated HEV infection and shedding in three wild ungulate species: wild boar, sika deer, and Japanese serow. We analyzed 1896 serum samples for anti-HEV antibodies, 1034 serum samples for HEV RNA, and 473 fecal samples from wild boars and deer for HEV RNA shedding. Anti-HEV antibodies were detected in wild boars across all seven prefectures, indicating that wild boars are likely the primary HEV reservoir in wildlife, as previously observed [26]. Seropositivity rates varied by prefecture, with Yamaguchi showing a rate of 21.6%, followed by Ishikawa (11.1%), Kagawa (10.6%), Aomori (7.7%), Toyama (7%), Wakayama (3.5%), and Nagasaki (2.9%).

Among the three ungulates, sika deer showed no evidence of HEV infection, with neither seroprevalence nor HEV RNA detected in serum or fecal samples, consistent with previous findings suggesting a low risk of HEV infection in this species [26,37]. In contrast, one Japanese serow tested positive for HEV antibodies using both ELISA and immunofluorescence assay; however, no HEV RNA was detected, making it difficult to conclusively classify the Japanese serow as a susceptible species for HEV infection. Although our HEV ELISA has demonstrated high specificity across various animal species [27,28], limited sample availability precluded further confirmation using western blot analysis. Other ungulate species, including roe deer, moose, red deer, and fallow deer [22–25,38–42], in Europe and Asia, have shown susceptibility to HEV infection. Additionally, antibodies against HEV but not HEV RNA have been reported in wild ruminants [43,44], suggesting that other mammals may be exposed to HEV. Although the absence of detectable HEV RNA in the Japanese serow limits definitive conclusions, the lack of previous research on HEV infection in this species highlights the importance of continued surveillance of wild ungulates to assess their susceptibility and role in HEV transmission.

The detection of HEV RNA in three wild boar serum samples and seven fecal samples from adjacent prefectures in southern Japan and Yamaguchi, including sub-genotypes 3b, 4a, and 4g, demonstrates the co-circulation of multiple HEV strains in wild boar populations. Sub-genotype 3b was the most frequently detected (6 out of 10 samples), which aligns with its established prevalence in Japanese swine, humans, and wild boars [13,45]. Subgenotype 4a, initially linked to China [36], has been identified in autochthonous Japanese cases since 2019 [13,46]. The 4a strain from Miyazaki wild boar clustered with other Asian isolates and more closely with a human-derived strain (LC500883) from a Japanese patient with a history of consuming wild boar and raw horse meat before HEV infection [46]. However, the strains shared only 95.86% identity, precluding confirmation of direct zoonotic transmission. Interestingly, subgenotype 4g, which is currently prevalent in humans in Japan, has only been detected in wild boars from the Yamaguchi prefecture [26,47]. The three 4g strains from wild boars clustered with the historical strains, indicating their persistence in the Yamaguchi prefecture for over 12 years. The absence of 4g strains in wild boars elsewhere in Japan suggests a geographically restricted circulation, underscoring the ability of HEV strains to establish long-term wildlife reservoirs.

Phylogenetic analysis revealed distinct clustering patterns within subtypes, reflecting geographical and host-specific variations, as observed in the distinct clusters formed by the 3b subtype strains from the adjacent Oita and Fukuoka prefectures in the Kyushu district. The 3b strains from Oita, detected in fecal samples, clustered with previously detected wild boar strains circulating in the same area [48], suggesting a unique strain endemic to Oita wild boars. These Oita strains did not cluster with a previously identified 3b strain isolated from deer in the same prefecture, indicating that the two strains may be spread among wild

animals in Oita. In contrast, the 4g strains identified in this study grouped closely with the endemic Yamaguchi prefecture 4g strains from humans, deer, and wild boars, suggesting that cross-species transmission must occur in this region. These findings underscore the complex dynamics of HEV circulation in wildlife.

The successful isolation of the genotype 3b strain from a wild boar fecal sample, achieving a viral concentration of 1.89×10^7 copies/mL by 84 days p.i along with the successful passage of the progeny virus, confirms the presence of infectious HEV particles in wild boar feces. This finding suggests that wild boars may contribute to environmental contamination, potentially exposing other wildlife and natural habitats, and could pose a risk to hunters handling game meat, as previously documented in studies on meat handlers [44,49,50].

Detection of anti-HEV antibodies and HEV RNA in serum and fecal samples from wild boar did not reveal a clear association between HEV infection and animal sex, in contrast to the gender-influenced HEV infection observed in human populations [14,45]. Additionally, wild boars weighing less than 30 kg showed lower rates of seroconversion than adults (>50 kg), with one animal exhibiting viremia and two showing fecal shedding (Supplementary Table S1). These findings highlight the importance of assessing the risk of HEV transmission from wild boars, particularly piglets.

This study showed the circulation of multiple HEV subtypes among wild boars in adjacent prefectures of southern Japan, with viral shedding detected in feces and antibodies identified in wild boars and a Japanese serow. Endemic strains were observed in wild boar populations, indicating that wild boars may be an important reservoir of HEV in wildlife in Japan. The emergence of new HEV subtypes and their host adaptation, along with the stronger association of genotype 4 with fulminant hepatitis than genotype 3 [51], highlights the need for continuous surveillance of wildlife. Genotyping and monitoring HEV in wild animals are essential for tracking disease dynamics and assessing zoonotic risks from game meat, ultimately guiding strategies to prevent HEV transmission to human populations.

5. Conclusions

Our study demonstrates the circulation of multiple HEV subtypes among wild boars in southern Japan. Infectious HEV particles were detected in wild boar feces, and antibodies were identified in both wild boars and a Japanese serow, although HEV RNA was not detected in the serow. These findings emphasize the importance of continuous surveillance and a one-health approach for managing the potential risk of zoonotic diseases.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/v17040524/s1, Figure S1: Geographical distribution of wild boar HEV strains in Japan and in vitro growth kinetics of the wb/Oita/8 strain; Table S1: Detection of anti-HEV antibodies and RNA from wild boars.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The HEV sequences detected in this study were deposited in GenBank (accession numbers: LC857165–LC857174).

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The Epidemiology of Hepatitis E in Israel and Potential Risk Factors: A Cross-Sectional Population-Based Serological Survey of Hepatitis E Virus in Northern Israel

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Abstract: Hepatitis E Virus (HEV) has gained public health attention as one of the causative agents of viral hepatitis. Our study aimed to provide data about HEV seropositivity in the Israeli general population, including its seroprevalence geographical distribution, and to identify variables as possible risk factors for HEV exposure. A seroprevalence cross-sectional study was conducted: HEV serological status was determined in 716 blood samples collected from the routine check-up blood samples. Demographic information was available for all samples. The overall prevalence of HEV IgG in an apparently healthy population in the north of Israel was 10.5%, with no evidence of positive HEV IgM. There was a significant association between HEV seropositivity and elderly age and low socioeconomic status (SES). The age-adjusted seroprevalence was significantly lower among Jews compared to Arabs with a rate ratio of 2.02. We identified clusters (hot spots) of HEV infection in three regions under study. Our results confirmed a high prevalence of anti-HEV in the country where clinical hepatitis E is not endemic. For the first time, this study showed that a hot spot analysis was able to provide new knowledge about actual exposure zones. As HEV infection is not a notifiable disease, it is probably underdiagnosed. Thus, better awareness among physicians is warranted.

Keywords: hepatitis E virus; general population; seroprevalence; hot spot analysis

1. Introduction

Hepatitis E Virus (HEV), a member of the Heperviridae family, is globally classified into eight genotypes (1–8) [1,2]. HEV strains affecting humans are classified into genotypes 1, 2, 3, 4, and, most recently, 7; the other three genotypes can infect animals but are not transmissible to humans. Genotypes 1 (G1) and 2 (G2) are limited to human hosts. Genotypes 3 and 4 (G3 and G4) have multiple hosts and can also be transmitted to humans. Genotypes 5 (G5) and 6 (G6) are known to infect wild boar; however, it is unknown whether these genotypes can be transmitted to humans. Finally, genotypes 7 (G7) and 8 (G8) infect dromedary and Bactrian camels, respectively [1–3]. Recently, G7, isolated from a dromedary camel, was also associated with chronic viral hepatitis in a transplant recipient [4].

HEV is mainly feco-orally transmitted, usually by contaminated drinking water. It can also be transmitted through food contaminated with HEV or via animal-to-human transmission through feces or direct contact. Other possible modes of transmission such as parenteral, human blood supply, and vertical transmission from mother to child have

been reported [3,5–7]. HEV usually causes an acute, self-limiting infection with non-existent/mild symptoms that can evolve even to fulminant hepatitis; infections can also induce acute liver failure in pregnant women. Over the past decade, there have been new aspects associated with HEV infection, mainly the possibility of chronic hepatitis caused by G3 in immunosuppressed individuals [8–10]. In developing countries, the epidemiology of HEV seroprevalence among the general population varies extensively; the wide range of results appears to depend on many variables, including the serological assay used, the geographical region, and the study cohort [11]. Recently, a meta-analysis study aiming to assess global HEV seroprevalence estimated that 12.47% of the global population have experienced past infection of HEV based on the anti-HEV IgG antibody. Africa and Asia have been previously recognized for the high prevalence of HEV [12]. An epidemiological study from Germany found an overall HEV seroprevalence of 16.8% among adults in Germany, and it was determined that seroprevalence increased with age [13]. Similarly, high seroprevalence was found in blood donors in Denmark (20.6%) and southwestern England (16%) [14,15]. Related to recent European reports, some data have shown that blood donors' HEV seroprevalence rates are higher than those reported in patients with acute hepatitis [16]. In addition to this high prevalence in blood donors, transmission of HEV by blood transfusion has also been observed [17].

In the last 20 years, only one HEV seroprevalence study has been performed among the Israeli general population. The study was performed during 2009–2010 by the Israeli Center for Disease Control (ICDC) in the central area of Israel, using anonymous diagnostic laboratories' residual samples and healthy blood donor samples stored at the national serum bank of the ICDC. The study found an overall prevalence of 10.6% HEV IgG, as well as a significant association between HEV seropositivity and advanced age, low socioeconomic status (SES), Arab ethnicity, and being born in Asia, Africa, or the former Soviet Union [18]. HEV was also found to be circulating in environmental sewage samples in Israel. Most of the positive samples were identified in the region of Haifa, in the northwest area of Israel [19]. Subsequently, two additional studies showed a high HEV IgG prevalence among swine and camels in Israel [20,21]. One of the most effective methods for investigating prevalence is hot spot analysis.

Aiming to address epidemiological gaps in our understanding of HEV as an emerging pathogen that contributes to public health concerns, we investigated HEV seroprevalence in the "apparently healthy population" in North Israel, identified potential risk factors for seropositivity, and looked for the relationship between geographic locations of features and HEV seroprevalence. One of the most effective methods for investigating prevalence is hot spot analysis. While a basic comparison of seroprevalence across cities or villages provides a general overview, it does not account for spatial dependencies or clustering effects, treating each location as an isolated unit and potentially overlooking important epidemiological patterns. To address these limitations, the present study incorporates hot spot analysis to identify statistically significant clusters of high and low seroprevalence. This approach enables the detection of spatial trends that may be influenced by underlying factors such as population density, mobility patterns, healthcare accessibility, and environmental conditions.

2. Methods

2.1. Study Population

The study was a cross-sectional seroprevalence study based on the probability sampling of retrospective routine blood examinations from an "apparently healthy population". This population included individuals who are generally healthy without any underlying medical conditions according to their medical records, who undergo regular annual

blood tests as part of a "check-up" procedure in the department of routine chemistry in the Central Laboratory of Clalit Health Services (CHS) in the Haifa and Western Galilee district. CHS is the largest and leading healthcare organization in Israel, serving 52% of the Israeli population across 1500 clinics and throughout 200 municipalities. The Haifa and Western Galilee district is one of the nine districts served by CHS and is considered the largest in Israel, serving almost 800,000 people from both Jewish and Arab populations and receiving about 5000 tests per day for routine chemistry check-ups. In order to ensure a sample representative of both the Jewish and Arab populations and the different age groups, proportional stratified sampling was used. During the year 2017, 50 blood samples from each stratum were selected randomly each day from the routine check-up blood sample results list. If not ordered initially, the samples were further tested for HCV Ab, HBs Ag, and hepatitis B core Ab (HBc Ab), in addition to conducting the main liver function profile tests (aspartate transaminase (AST), alanine transaminase (ALT), gamma-glutamyl transferase (γ-GT), alkaline phosphatase (ALP), albumin, and bilirubin). Only sera from individuals over the age of 18 years and without laboratory evidence of chronic or acute liver disease were included in the study. Demographic information (age, gender, population group, residency) was collected for each sample using CHS Auto-Reports software (https://autoreport.info/). SES was assessed by socioeconomic rank, as defined by the Israel Central Bureau of Statistics 2017, calculated using multiple sociodemographic and economic factors, including the financial resources of the residents, their housing conditions, their motorization level, and their education and employment profile [22]. Ranks ranged on a scale from one to ten, with lower ranks representing a lower SES.

2.2. HEV Laboratory Diagnosis

All serum samples were tested once for HEV IgG using a DS EIA-ANTI-HEV G kit from Diagnostics Systems (Diagnostic Systems Italy, Saronno, Italy). HEV IgG-confirmed-positive samples were tested for HEV IgM with a DS EIA-ANTI-HEV M kit from Diagnostics Systems (Diagnostic Systems Italy, Saronno, Italy). Positive samples were re-tested in duplicate. Samples that were reactive two or three times were reported as seropositive.

Both IgG and IgM kits are based on Recombinant ORF2 and ORF3 antigen coating and capable of detecting antibodies against 4 HEV genotypes (1–4). According to the manufacturer, the relative sensitivity and specificity of both assays are 100%. An earlier evaluation study conducted by the U.S. Centers for Disease Control and Prevention (CDC) of 6 serologic assays for IgM antibodies against HEV identified the assay manufactured by Diagnostics Systems as having the best performance in terms of diagnostic sensitivity and specificity of 95.2% and 98% [23].

2.3. Hot Spot Analysis

In order to depict the relationship between the geographic locations and HEV seroprevalence in the Haifa and Western Galilee area, we employed an approach of spatial autocorrelation and cluster mapping: Getis-Ord hot spot analysis [24] using ArcMap software (version 10.6.1) [25]. According to this approach, a hot spot is a cluster of data with a high attribute value surrounded by lower-value data. An area is considered to be a statistically significant hot spot when the sum of the attributed data of neighboring features is different from the expected sum if the data were randomly divided over the space. When the found difference is too large to be the result of random chance, a very low p-value is present, indicating the statistical significance of the cluster. Clusters of significantly high values are considered hot spots, while clusters of low values are considered cold spots. During the data collection and processing, all study sites (cities or villages) were given geographical coordinates based on the government mapping website and were manually

checked for (typing) errors. A further selection criterion was applied before the data were analyzed: only sites that had more than two collected samples were included in the final analysis.

In order to reflect any type of false discovery rate (FDR) correlation, we calculated the Gi-Bin field to identify statistically significant hot spots. Features in the +/−3 bins reflect statistical significance with a 99% confidence level, features in the +/−2 bins reflect a 95% confidence level, features in the +/−1 bins reflect a 90% confidence level, and the clustering for features in bin 0 do not form a statistically significant hot or cold spot regardless of whether or not the FDR correction is applied. Furthermore, to reject the assumption that close points are affected by the same phenomenon more strongly and to demonstrate that it is not the geographical proximity that makes certain hot spots more exceptional than the other examined locations (rather, it is the high prevalence of HEV IgG findings), we used the inverse distance weighting (IDW) method [26]. The IDW method assumes that each measured point has a local influence that diminishes with distance and gives greater weight to the points closer to the prediction location than to those farther away. The spatial distribution was also normalized for age to improve the identification of hot spots through the "mining" of spatial patterns. The mean age for each study site was entered as a weight factor.

2.4. Statistical Analysis

Data were analyzed using SAS version 9.4 (SAS Institute, Cary, NC, USA). *p*-Values of 5% were considered statistically significant. Categorical data were reported as frequencies and percentages. The overall HEV IgG seroprevalence was standardized (per 100) to age, sex, and ethnicity according to the direct method using the 2017 Israel standard population from the Central Bureau Of Statistics "lamas" (age groups of 15–19, 20–24, 25–34, 35–44, 45–54, 55–64, 65–74, 75). In order to explore the associations between study characteristic variables (sex, age, ethnicity, living status, and socioeconomic status) and HEV IgG, we used logistic regression models (a univariable model for each variable and a multivariable model that included all study characteristic variables in the same model). The sample size for each group was determined based on the expected prevalence of HEV antibodies according to similar data in previous studies from Israel and was calculated using the "sample size for proportions". As commonly accepted, two-sided significance was determined as 5%, and a power of 80% was used for the calculations.

2.5. Ethical Aspects

The study was performed in accordance with the approval of the Helsinki declaration by both CHS (approval no. 0177-15-COM) and the University of Haifa ethics committee (approval no. 028/19).

3. Results

3.1. Description of the Study Population

Table 1 represents the demographic characteristics of all participants including sex, age, ethnicity, place of residence, and socioeconomic status. Overall, 716 blood samples were collected: 42.5% were men, the age range was from 18 to 80 years, and the mean age was 44.3 ± 17.5.

Table 1. Characteristics of the study population.

Variables		**Total Sample N = 716**
Sex	Male	304 (42.5)
	Female	412 (57.5)
Age (years)	18–34	260 (36.3)
	35–54	263 (36.7)
	55–74	145 (20.3)
	75+	48 (6.7)
Ethnicity	Arab	335 (46.8)
	Jewish	381 (53.2)
Place of residence	Village	314 (43.8)
	City	402 (56.2)
Socioeconomic status	Low rank 1–3	184 (25.7)
	Intermediate, high rank 4–10	532 (74.3)

Of the 716 study samples, 46.8% were obtained from Arabs. According to data available at the Israeli Central Bureau of Statistics, the Arab population constitutes about 20% of the Israeli population. The present study included oversampling of the Arab group (46.8% vs. 20%) to be able to analyze the data in this relatively small group and to ensure a good representation of the Arab population. However, the oversamples were statistically adjusted in the final results so that this group is represented in proportion to its actual share of the Israeli population.

3.2. HEV Seroprevalence Results

Tables 2 and 3 summarize the crude and standardized rate ratios in the different groups. Of 716 samples, 75 tested positive for HEV IgG 10.5 [8.2–12.7] with no evidence of positive HEV IgM, indicating past HEV infection. The overall HEV IgG seroprevalence standardized to age, sex, and ethnicity was 9.8 [6.9–12.8]. The overall HEV IgG seroprevalence standardized to age, sex, and ethnicity separately was 11.9 [9.2–14.6], 10.4 [8.3–12.8], and 9.4 [6.9–11.9], respectively. The age-adjusted seroprevalence in the Jewish group was 8.6 [5.7–11.6] and in the Arab group 17.5 [11.9–23.1]. Comparing the age-adjusted seroprevalence between the Arab and Jewish groups yielded a significant difference with a rate ratio of 2.02 (95% CI 1.26–3.22, $p < 0.001$). Comparing the age-standardized rate between females (12.6 [8.9–16.3]) and males (10.9 [6.9–14.8]) yielded no significant difference.

Table 2. Age-, sex-, and ethnicity-standardized and overall HEV IgG seroprevalence.

All	**Standardized Rate (per 100) 95% CI**
Standardized by age, sex, and ethnicity group [1]	9.8 (6.9–12.8)
Standardized by age [2]	11.9 (9.2–14.6)
Standardized by sex [3]	10.4 (8.3–12.8)
Standardized by ethnicity [4]	9.4 (6.9–11.9)

[1] Age-, sex-, and ethnicity-adjusted seroprevalence in the Israeli standard population as in "lamas", 2017. [2] Age-adjusted seroprevalence in the Israeli standard population as in "lamas", 2017 (15–19, 20–24, 25–34, 35–44, 45–54, 55–64, 65–74, 75+). [3] Sex-adjusted seroprevalence in the Israeli standard population as in "lamas", 2017. [4] Ethnicity-adjusted seroprevalence in the Israeli standard population as in "lamas", 2017.

Table 3. Age-standardized, male/female, and Arab/Jewish HEV IgG seroprevalence.

	Crude Rate (per 100) 95% CI	Standardized Rate [1] (per 100) 95%CI	Standardized Rate Ratio 95% CI
All	10.4 (8.5–13.0)	11.9 (9.2–14.6)	-
Female	10.9 (8.3–14.4)	12.6 (8. 9–16.3)	1.16 (0.73–1.85)
Male [2]	9.8 (6.9–13.7)	10.9 (6.9–14.8)	1
Arabs	12.5 (9.4–16.6)	17.5 (11.9–23.1)	2.02 (1.26–3.22)
Jews [2]	8.6 (6.3–12.0)	8.6 (5.7–11.6)	1

[1] Age-adjusted seroprevalence in the Israeli standard population as in "lamas", 2017 (15–19, 20–24, 25–34, 35–44, 45–54, 55–64, 65–74, 75+). [2] Reference group.

Table 4 show the associations between study characteristic variables and HEV IgG. Looking for potential risk factors, seropositivity was significantly associated with elderly age (OR = 59.05, 95%CI 21.1–165.1, $p = 0.001$) and low SES (OR = 0.41, 95%CI 0.21–0.82, $p = 0.01$). Within the different age groups, a significant gradual increase in the HEV seroprevalence rate was observed from the youngest to the oldest subjects. The odds of being anti-HEV-positive were higher in middle-aged individuals (aged 55–74) and in elderly individuals (aged 75 years and above) compared with individuals in the younger group (18–34 y/o). Anti-HEV IgG positivity in females and in the Arab population group was higher compared to males and the Jewish population group accordingly, but these differences were not significant.

Table 4. Associations between study characteristic variables and HEV IgG.

Variables		Negative IgG HEV N = 641	Positive IgG HEV N = 75	Univariable Models OR (95% CI)	Multivariable Model OR (95% CI)
Sex	Male	276 (43.1)	30 (40.0)	1	1
	Female	365 (56.9)	45 (60.0)	1.13 (0.69–1.85)	1.23 (0.74–2.12)
Age (years)	18–34	253 (39.4)	7 (9.3)	1	1
	35–54	249 38.9)	14 18.7)	2.03 (0.81–5.12)	2.46 (0.96–6.30)
	55–74	114 (17.8)	31 (41.3)	9.83 (4.20–22.98)	14.38 (5.87–35.25)
	75+	25 (3.9)	23 (30.7)	33.25 (12.98–85.17)	59.05 (21.11–165.13)
Ethnicity	Arab	293 (45.7)	42 (56.0)	1	1
	Jewish	348 (54.3)	33 (44.0)	0.66 (0.41–1.07)	0.57 (0.26–1.24)
Place of residence	Village	274 (42.8)	40 (53.3)	1	1
	City	367 (57.2)	35 (46.7)	0.65 (0.40–1.06)	0.96 (0.46–1.99)
Socioeconomic status	Low rank 1–3	160 (24.9)	24 (32.0)	1	1

3.3. Spatial Analysis

Figure 1 displays the HEV IgG hot spot classification in the study area, conducted using the Getis-Ord Gi* approach implemented via ArcMap. According to the hot spot analysis, three major sites were identified as hot spots for HEV IgG prevalence with a 99% confidence level (Haifa, Fassuta, and Hurfeish).

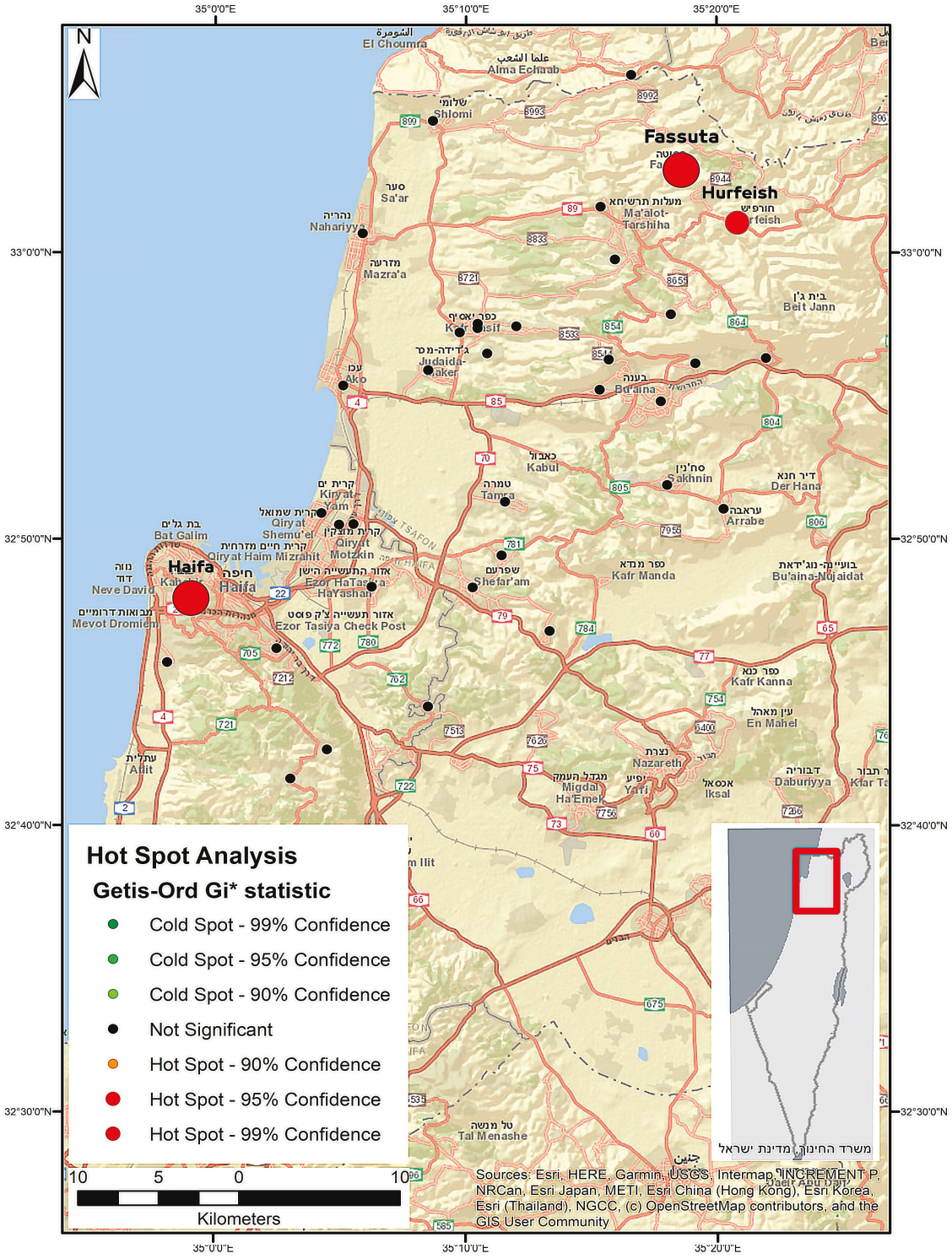

Figure 1. Hot spot classification by applying the Getis-Ord Gi* approach in the study area. Each circle represents a region, and the larger the circle, the higher the seroprevalence. The circle color represents the Gi* *p*-value ranges.

4. Discussion

This study presents a cross-sectional assessment of the HEV antibody prevalence among a representative sample of an apparently healthy population from northern Israel. Our overall prevalence (10.5%) is concordant with a previous seroprevalence study (10.6%) that used the same serological assays and analyzed healthy blood donors and anonymous diagnostic blood samples from people living in the central region of Israel. However, there

was a difference in the calculated age-adjusted rate in our study (11.9%) compared with the previous one (7.6%) [18]. The potential reasons for the difference in the rates may be attributed to the different representative samples for the population used in the studies. The outcome of the previous study was based on healthy blood donors and anonymous diagnostic blood samples—two groups that are not necessarily representative of the general Israeli population. Sampling blood donors may underestimate the real prevalence in a selectively healthier population. Furthermore, there was a poor representation of the Arab population in the previous study compared to our study. However, our sample was also adjusted for ethnicity, but still the age-adjusted rate within the Jewish group (8.6%) was higher than the overall age-adjusted prevalence in the previous study (7.6%).

The higher age-adjusted rate observed in our study may also stem from the different geographical regions that each study represents—the north of Israel in the current study versus the central region of Israel in the previous study. Northern parts of Israel are more exposed to HEV G3, which is possibly circulating in the country and was recently identified in local sewage facilities mainly in the north [19]. Furthermore, most of the swine slaughtering and breeding farms are located in the north of the country. Outside of Israel, HEV G3 strains were also recently detected in sewage and in environmental water samples in Germany [27] and France [28]. Three clusters (hot spots) of HEV infection were identified in three regions under this study: Haifa, Fassuta, and Hurfeish. The high HEV seropositivity rate observed in the Haifa region is consistent with the findings recently observed in a study aimed at investigating the occurrence of HEV infection in Israel through molecular screening of raw sewage samples [19]. According to the study, 14 of 160 sewage samples tested were positive for HEV RNA, with most of them identified in the Haifa region. Sequence analysis revealed HEV G3 sequences in these RNA-positive samples.

It is well known that swine are the main reservoir of HEV worldwide and that the virus is present on most swine farms [5,29–31]. A recent pilot study in Israel aiming to assess the status of HEV infection among swine farmers showed that domestic swine in Israel are infected with HEV G3 and three-quarters of all tested swine are anti-HEV-seropositive [20]. Fassuta is a local council in the north of Israel and all of its inhabitants are Christian. The Christian religion permits the eating of swine, and many members of the Christian community in Israel do eat it. Otherwise, swine production and breeding are limited in Israel, mainly located in communities with a significant Christian population. Fassuta is one such community. Our main hypothesis is that the high HEV seroprevalence in Fassuta is a result of zoonotic infection transmitted by swine and can be attributed to exposure to HEV G3, which is possibly circulating in this area. The higher HEV seroprevalence observed by a hot spot analysis in Hurfeish may be associated with its overall lower SES and poor sanitary conditions, which make HEV infection more likely. Hurfeish is a rural town categorized in socioeconomic cluster 3–4 out of 10 according to the Central Bureau of Statistics in Israel. This places it in a lower range of SES and it falls behind in terms of economic opportunities, infrastructure, and diversity. However, HEV G1 or G2 infections caused by contaminated water are linked to lower SES. As in Israel, no outbreak of HEV G1 or G2 infection had been reported so far. Our hypothesis regarding the association between higher HEV seroprevalence and the lower SES in Hurfeish must be supported by evidence of HEV G1 or G2 infection or by the presence of HEV G1 or G2 RNA in the sewage of Hurfeish. However, the cause of the high HEV seroprevalence in Hurfeish remains unknown.

The increase in HEV seroprevalence with age found in the present study is consistent with reports from other countries [8]. We believe that the increase in seropositivity with age seen here may be attributed to a cohort effect or cumulative exposure. Our thought is supported by the fact that one of the main sources of HEV transmission is through

the fecal–oral route, and improvements in sanitation and general living conditions can explain the decreasing rate of HEV transmission over time in the youngest groups and the cohort effect. The association of HEV exposure with lower SES is in agreement with other studies worldwide, which found that low SES is a major risk factor for increased prevalence of HEV infection [5,8]. Another risk factor associated with HEV seropositivity is ethnicity. Our study included oversampling of the Arab group (46.8% vs. 20%) in order to analyze the data in this relatively small group and to ensure a good representation of this population. However, the oversampling was statistically adjusted so that the group was represented in proportion to its actual percentage of the Israeli population. The overall calculated ethnicity-adjusted rate was 9.4%, and the age-adjusted seroprevalence was lower among Jews compared to Arabs. In our study, there was a strong correlation between ethnic categories and living residence. Within the Arab population, 78% live in a village compared to 14% of the Jewish population. We believe that the higher prevalence in the Arab population may be associated with lower level of sanitation and SES that are characteristic of their living areas.

Overall, the results of the present study confirm a high prevalence of anti-HEV in the country where clinical hepatitis E is not endemic with evidence of autochthonous infections and demonstrate that HEV circulates in the north district of Israel. Our results emphasize the importance of increasing the awareness of HEV infection among physicians and the necessity of establishing an algorithm for HEV diagnosis and screening in different Israeli populations.

However, this study has several limitations to consider. Being a cross-sectional study, a temporal association cannot be determined, and, thus, no causal inference can be made. To detect the seroprevalence in the different ethnic groups and to identify if these specific population groups are at high risk of HEV seroprevalence, samples were taken from within the Haifa and Western Galilee region, which is characterized by mixed ethnic populations. However, lack of representation of similar populations from other parts of Israel (center and south) decreases the external validity of our results. Further studies are necessary to define the clinical and epidemiologic burden of HEV infection in this area and to identify additional risk factors for HEV infection.

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Clinical Characteristics and Epidemiological Features of Hepatitis E Virus Infection Among People Living with HIV in Shanghai, China

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Abstract: Hepatitis E virus (HEV) poses a significant public health concern, particularly among immunocompromised populations. This study aimed to investigate HEV seroprevalence, clinical characteristics, and associated risk factors in people living with HIV (PLWH) in Shanghai, China. A retrospective analysis was conducted on serum IgG and IgM antibodies specific to HEV in 670 PLWH and 464 HIV-negative health-check attendees. The overall anti-HEV seropositivity rate among PLWH was 30.15% (202/670, 95% CI 26.68–33.62), with an IgG positivity rate of 30.00% (201/670, 95% CI 26.53–33.47). IgM positivity was observed in 1.19% (8/670, 95% CI 0.59–2.39) of PLWH, and dual IgM/IgG positivity was observed in 1.04% (7/670, 95% CI 0.50–2.16) of PLWH. The seropositivity rate of anti-HEV IgG in the HIV-negative health-check attendees was 17.67% (82/464, 95% confidence interval: 14.20–21.14), with no IgM positivity, which was significantly lower than that in PLWH ($\chi^2 = 22.84$, $p < 0.001$). Univariate and multivariate analyses identified advanced World Health Organization (WHO) HIV stage (III/IV) as an independent risk factor for HEV co-infection ($p < 0.05$). Notably, no significant associations were observed with age, gender, CD4 count, or liver function parameters. These findings underscore the importance of implementing HEV screening protocols and developing targeted preventive strategies for PLWH.

Keywords: hepatitis E virus; HIV; seroprevalence; risk factors

1. Introduction

Hepatitis E virus (HEV), a single-stranded, non-enveloped RNA virus that is transmitted primarily via the fecal-oral route, is a major etiological agent of acute viral hepatitis

globally [1,2]. Approximately 20 million HEV infections occur annually worldwide, resulting in around 70,000 fatalities [3]. Although immunocompetent hosts typically develop self-limiting hepatitis, immunocompromised patients—including organ transplant recipients [4], patients with hematological malignancies [5,6], and people living with HIV (PLWH) [7,8]—are at risk for chronic infection and cirrhosis.

At the same time, HIV remains a global public health challenge, with annual mortality still exceeding several hundred thousand [9]. The progressive immune depletion in PLWH increases vulnerability to opportunistic infections and atypical disease progression [10], potentially altering HEV persistence and clinical manifestations [11]. The geographical overlap between HIV-endemic regions and HEV-hyperendemic zones raises significant concerns about co-infection epidemiology. However, research on HIV and hepatitis E virus co-infection is currently limited. Although China is endemic for HEV, data on the serological prevalence patterns and associated risk factors among this vulnerable population in Shanghai remain scarce.

This study aims to (1) determine the seroprevalence of HEV among PLWH in Shanghai; (2) compare seroprevalence rates with HIV-negative health-check attendees; (3) characterize clinical profiles of co-infected individuals; and (4) identify epidemiological determinants of HEV infection. Through this comprehensive analysis, we seek to elucidate the complex interactions between HIV and HEV infections in Shanghai, informing targeted surveillance strategies and clinical management protocols for this vulnerable population.

2. Materials and Methods

2.1. Study Design and Population

Between 2018 and 2024, our clinic followed 8468 adult PLWH ($\geq$18 years); of these, 714 (8.4%) underwent anti-HEV testing as part of routine annual liver function surveillance, independent of clinical hepatitis symptoms. A total of 44 patients were excluded due to incomplete clinical data. Ultimately, 670 PLWH were included in this study (Figure 1). Controls comprised 464 HIV-seronegative adults who attended the hospital's annual health check program. All of these individuals voluntarily requested anti-HEV antibody testing as part of their health check due to increased awareness of hepatitis prevention. The control group was age- and sex-matched to the PLWH group, and all participants reported no chronic liver disease or immunodeficiency.

2.2. Data Collection

Demographic and clinical parameters were extracted from electronic medical records. These included epidemiological characteristics such as age, gender, ethnicity, and marital status. Hepatic profiles were also collected, comprising albumin (ALB), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total bilirubin (TBIL). HIV-specific markers were documented, including CD4 count, antiretroviral therapy (ART) regimen, and WHO clinical stage. Additionally, comorbidity data were gathered, focusing on hepatitis B virus (HBV)/hepatitis C virus (HCV) co-infection status and liver cirrhosis.

2.3. Serological Assays

Serum anti-HEV IgM and IgG antibodies were detected using commercial enzyme-linked immunosorbent assay (ELISA) kits (Wantai Biological Pharmacy, Beijing, China) according to the manufacturer's specifications. The assay demonstrated specific performance characteristics for each antibody type: Anti-HEV IgG showed high sensitivity at 97.7% and excellent specificity at 99.6%, while Anti-HEV IgM exhibited strong sensitivity at 93.2% and robust specificity at 97.8% [12]. Liver function parameters were analyzed on Hitachi 7600 automated analyzers (Hitachi High-Technologies Corporation, Tokyo, Japan)

using standard enzymatic methods. The CD4 count was quantified in peripheral blood specimens from PLWH using flow cytometric analysis.

HEV RNA was detected using the reverse transcription-quantitative polymerase chain reaction (RT-qPCR) technique for samples positive for anti-HEV IgM antibodies. RNA extraction and RT-qPCR procedures were essentially the same as described previously, with a lower limit of detection of 500 copies/mL [13,14]. For molecular detection, HEV RNA was extracted from the serum using a QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany). Quantitative RT-PCR was performed on Applied Biosystems 7500 platforms using Vazyme One-Step RT-PCR kits (Vazyme, Nanjing, China). Primer and probe sequences are detailed in Table S1.

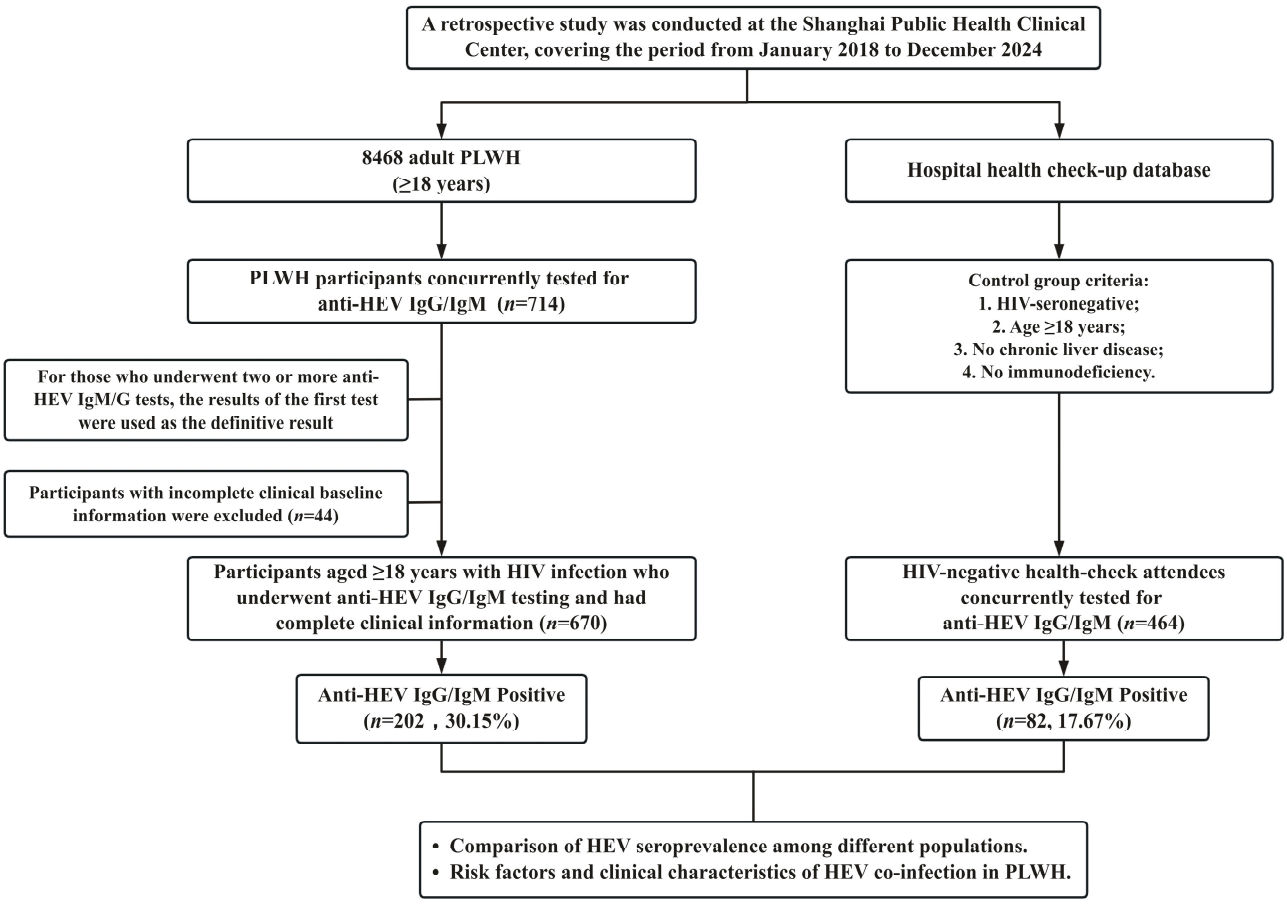

Figure 1. Flowchart of the study participants. HEV, Hepatitis E Virus; HIV, Human Immunodeficiency Virus; PLWH, People Living with HIV; IgM, Immunoglobulin M; IgG, Immunoglobulin G.

2.4. Statistical Analysis

The analyses were conducted using SPSS 20.0 and R 4.3.2. Continuous variables were expressed as mean ± standard deviation (SD) (for normally distributed data) or median [interquartile range (IQR)] (for non-normally distributed data) and were assessed by Student's *t*-test or Mann–Whitney U-test, respectively. Categorical variables are presented as counts and percentages and compared using a chi-square test or Fisher's exact test. Annual HEV seroprevalence trends (2018–2024) were examined using the Cochran–Armitage trend test. To mitigate detection bias from testing fluctuations during the COVID-19 pandemic, inverse-variance weighted linear regression was performed (weights = 1/variance of annual estimates). Age-specific patterns were analyzed using polynomial regression with quadratic term testing for non-linearity. Following significant non-linearity, Bonferroni-adjusted pairwise comparisons between age groups were conducted. Joinpoint regression

identified significant inflection points in age trends. Associations between anti-HEV antibody positivity and potential risk factors were evaluated in univariate and multivariate logistic regression models. Odds ratio (OR) and 95% confidence interval (95% CI) were calculated. We calculated variance inflation factors (VIFs) for all variables in our multivariate model. Results from two-sided tests with $p < 0.05$ were considered statistically significant.

3. Results

3.1. Demographic and Clinical Characteristics

The demographic, laboratory, and clinical characteristics of PLWH are shown in Table 1. The study cohort comprised 670 PLWH with a median age of 40 years (IQR 32–53), predominantly male (87.76%, 588/670) and of Han ethnicity (98.96%, 663/670). Clinical profiling revealed a median CD4 count of 211.9 cells/μL (IQR 45.2–473.7), with 61.34% (411/670) classified as WHO stage I/II. Viral hepatitis co-infections were observed in 6.87% (46/670; HBV) and 4.03% (27/670; HCV) of participants. Hepatic parameters demonstrated median levels of albumin (38.3 g/L, IQR 31.8–44.5), ALT (30.0 U/L, IQR 16.0–79.4), AST (30.0 U/L, IQR 20.0–63.8), and total bilirubin (10.4 μmol/L, IQR 7.0–16.7). Comparative analysis identified significant disparities between HEV-seropositive and seronegative groups, with the former exhibiting an older median age (47 vs. 37 years, $p < 0.001$) and higher total bilirubin levels (11.6 vs. 9.85 μmol/L, $p = 0.003$).

Table 1. Characteristics of hepatitis E virus infection in 670 PLWH in Shanghai, China, 2018–2024.

Characteristics	Overall, *n* = 670	Anti-HEV IgM/IgG		*p*-Value
		Negative, *n* = 468 (69.85%)	Positive, *n* = 202 (30.15%)	
Age (years)	40.00 [32.00, 53.00]	37.00 [31.00, 50.00]	47.00 [38.00, 57.00]	<0.001
Gender, *n* (%)				
Female	82 (12.24)	59 (12.61)	23 (11.39)	0.754
Male	588 (87.76)	409 (87.39)	179 (88.61)	
Marital Status, *n* (%)				0.076
Unmarried [1]	182 (27.16)	137 (29.27)	45 (22.28)	
Married	488 (72.84)	331 (70.73)	157 (77.72)	
Ethnicity, *n* (%)				0.207
Han	663 (98.96)	465 (99.36)	198 (98.02)	
Others	7 (1.04)	3 (0.64)	4 (1.98)	
WHO Stage, *n* (%)				0.677
Stage I/II	411 (61.34)	290 (61.97)	121 (59.90)	
Stage III/IV	259 (38.66)	178 (38.03)	81 (40.10)	
CD4 Count (cells/μL)	211.94 [45.20, 473.71]	196.90 [39.02, 491.21]	232.43 [77.45, 443.52]	0.273
<200, *n* (%)	323 (48.21)	235 (20.21)	88 (43.56)	
≥200 and <400, *n* (%)	139 (20.75)	92 (19.66)	47 (23.27)	
≥400, *n* (%)	208 (31.04)	141 (30.13)	67 (33.17)	
ART, *n* (%)				0.322
N + N + NN [2]	162 (24.18)	107 (22.86)	55 (27.23)	
N + N + PIs [3]	33 (4.93)	20 (4.27)	13 (6.44)	
N + N + NR [4]	308 (45.97)	219 (46.79)	89 (44.06)	
None	167 (24.93)	122 (26.07)	45 (22.28)	
Viral Hepatitis Co-infections				0.209
HBV co-infection, *n* (%)	46 (6.87)	27 (5.77)	19 (9.41)	
HCV co-infection, *n* (%)	27 (4.03)	18 (3.85)	9 (4.46)	
No virus, *n* (%)	597 (89.10)	422 (90.17)	175 (86.63)	
Chronic Liver Disease, *n* (%)				<0.001
Liver cirrhosis	19 (2.84)	4 (0.85) [5]	15 (7.43) [6]	
No liver cirrhosis	651 (97.16)	464 (99.15)	187 (92.57)	

Table 1. *Cont.*

Characteristics	Overall, *n* = 670	Anti-HEV IgM/IgG		*p*-Value
		Negative, *n* = 468 (69.85%)	Positive, *n* = 202 (30.15%)	
Liver Function Tests				
ALB (g/L)	38.25 [31.80, 44.48]	38.16 [31.64, 44.70]	38.33 [32.00, 43.37]	0.587
AST (U/L)	30.00 [20.00, 63.75]	29.00 [20.00, 57.00]	31.00 [19.85, 77.53]	0.354
ALT (U/L)	30.00 [16.00, 79.38]	29.40 [16.00, 75.00]	34.00 [16.00, 94.30]	0.112
TBIL (μmol/L)	10.40 [7.01, 16.70]	9.85 [6.90, 15.10]	11.60 [7.43, 19.65]	0.003
Anti-HEV IgM (S/CO)	0.07 [0.03, 0.10]	0.06 [0.02, 0.10]	0.10 [0.04, 0.16]	<0.001
Anti-HEV IgG (S/CO)	0.13 [0.07, 1.75]	0.10 [0.04, 0.16]	3.65 [2.02, 7.08]	<0.001

[1] Includes unmarried, divorced, and widowed; [2] includes two nucleoside reverse transcriptase inhibitors (NRTIs) and a non-nucleoside reverse transcriptase inhibitor (NNRTI); [3] includes two NRTIs and a protease inhibitor (PI); [4] includes two NRTIs and an integrase chain termination inhibitor (INSTIs); [5] 2 patients had HBV-related cirrhosis, 1 patient had HCV-related cirrhosis, and 1 patient had cirrhosis due to other causes; [6] 7 patients had HBV-related cirrhosis, 2 patients had HCV-related cirrhosis, and 6 patients had cirrhosis due to other causes. HEV, Hepatitis E virus; HIV, Human immunodeficiency virus; PLWH, People living with HIV; ART, Antiretroviral therapy; HBV, Hepatitis B virus; HCV, Hepatitis C virus; ALB, Albumin; AST, Aspartate aminotransferase; ALT, Alanine transaminase; TBIL, Total bilirubin; IgM, Immunoglobulin M; IgG, Immunoglobulin G; S/CO, Signal-to-cutoff ratio.

3.2. Anti-HEV Seroprevalence

The overall anti-HEV seropositivity rate among PLWH was 30.15% (202/670, 95% CI 26.68–33.62), with an IgG positivity rate of 30.00% (201/670, 95% CI 26.53–33.47). IgM positivity was observed in 1.19% of PLWH (8/670, 95% CI 0.59–2.39), and dual IgM/IgG positivity was observed in 1.04% of PLWH (7/670, 95% CI 0.50–2.16) (Table 2). Among IgM-positive cases (*n* = 8), 75.00% (6/8) presented with symptoms of acute hepatitis (jaundice and dark urine) and elevated liver transaminases, suggesting they might be in an acute or recent state of infection. HEV RNA was not detected in patients who were positive for anti-HEV IgM in this study. Detailed clinical data are presented in Table 3.

Among 464 HIV-negative health-check attendees, 82 individuals were positive for anti-HEV antibodies (all were positive for anti-HEV IgG), with a positivity rate of 17.67% (82/464, 95% CI 14.20–21.14%). No one in the HIV-negative health-check attendees was positive for anti-HEV IgM. There was a significant difference in HEV seropositivity rates between HIV-infected individuals and the HIV-negative health-check attendees ($\chi^2 = 22.84$, $p < 0.001$).

Table 2. HEV serology results.

Group	Anti-HEV Antibody	*n*	Percentage (%)
PLWH	Anti-HEV IgM-negative and lgG-negative	468	69.85
	Anti-HEV IgM-positive or IgG-positive	202	30.15
	Anti-HEV IgG-positive	201	30.00
	Anti-HEV IgG-positive and lgM-negative	194	28.96
	Anti-HEV IgM-positive	8	1.19
	Anti-HEV IgM-positive and IgG-positive	7	1.04
	Anti-HEV IgM-positive and IgG-negative	1	0.15
HIV-negative health-check attendees	Anti-HEV IgG-positive	82	17.67
	Anti-HEV IgM-positive	0	0

PLWH, People living with HIV; HEV, Hepatitis E virus; IgM, Immunoglobulin M; IgG, Immunoglobulin G.

Table 3. Demographic and clinical characteristics of patients with detectable HEV IgM.

No.	Date	Gender	Age	Anti-HEV IgM(S/CO)	Anti-HEV IgG(S/CO)	CD4 Count (Cells/uL)	ALB (g/L)	ALT (U/L)	AST (U/L)	TBIL (umol/L)
1	2018-05	Male	57	52.39	2.69	134.35	36.00	754.00	1294.00	141.60
2	2018-12	Male	30	39.06	4.14	106.18	36.00	553.00	753.00	156.40
3	2020-09	Male	52	28.00	5.47	319.99	32.72	673.00	63.00	100.30
4	2021-07	Male	31	25.43	3.56	156.15	38.35	1043.00	225.00	101.70
5	2021-12	Male	64	25.58	13.23	153.25	36.66	968.00	485.00	17.70
6	2022-08	Male	40	12.42	12.44	533.48	27.55	30.00	36.00	42.10
7	2024-07	Male	42	23.17	7.30	69.99	40.10	1618.90	1571.30	196.00
8	2024-09	Male	42	8.10	0.14	303.79	23.10	53.00	67.00	5.50

HEV, Hepatitis E Virus; HIV, Human Immunodeficiency Virus; ALB, Albumin; AST, Aspartate Aminotransferase; ALT, Alanine Transaminase; TBIL, Total Bilirubin; IgM, Immunoglobulin M; IgG, Immunoglobulin G; S/CO, Signal-to-Cutoff Ratio.

3.3. HEV Seroprevalence Patterns

Comprehensive analysis revealed stable HEV seroepidemiological patterns, without significant temporal, age-related, or gender-based variations (Figure 2). Annual HEV seroprevalence rates demonstrated stability from 2018 to 2024 (Cochran–Armitage trend test, $p = 0.15$), although the testing frequency exhibited notable fluctuations. The observed reduction in testing volume during 2020–2022 (71 tests in 2020, 64 in 2021, and 33 in 2022) coincided with the COVID-19 pandemic period, during which routine clinical surveillance activities were substantially disrupted. Despite this pandemic-related testing reduction, prevalence estimates remained consistent (2020: 33.8%, 95% CI: 23.0–46.0; 2021: 21.9%, 95% CI: 12.5–34.0; 2022: 27.3%, 95% CI: 13.3–45.5). To address potential detection bias arising from variable testing intensity, particularly during the pandemic years, we conducted inverse-variance weighted regression analysis. This sensitivity analysis confirmed trend stability ($\beta = -0.008$, 95% CI: -0.022–0.006, $p = 0.18$), supporting the absence of significant temporal variation independent of testing fluctuations.

Regarding age-specific patterns, seroprevalence distribution showed a non-monotonic configuration with peak rates in 40–49 year-olds (35.0%, 95% CI: 27.1–43.6) and those $\geq$60 years (31.4%, 95% CI: 22.7–41.2). Statistical validation through polynomial regression identified a significant quadratic component ($\beta = 0.021$, $p = 0.04$), confirming deviation from linearity. Post-hoc pairwise comparisons with Bonferroni adjustment revealed significantly higher seroprevalence in 40–49 year-olds compared to 30–39 year-olds ($\Delta = 6.0\%$, OR = 1.38, 95% CI: 1.02–1.87, $p = 0.04$). Joinpoint regression further identified significant inflection points at 45 years (95% CI: 42–48, $p = 0.03$) and 65 years (95% CI: 61–69, $p = 0.05$), statistically corroborating the observed bimodal pattern. Gender differences were non-significant both overall and within all age strata (all $p > 0.05$).

CD4 count was categorized into three levels: <200 cells/μL, $\geq$200 to <400 cells/μL, and $\geq$400 cells/μL. Stratification by CD4 count was performed to assess the relationship between CD4 count and HEV seropositivity rates. There was no significant difference in HEV seropositivity rates among the groups (CD4 count < 200 cells/μL: 88/323 [27.24%]; 200 $\leq$ CD4 count < 400 cells/μL: 47/139 [33.81%]; CD4 count $\geq$ 400 cells/μL: 67/208 [32.21%], $p = 0.26$). To further explore the potential association between CD4 count and HEV infection risk, we plotted the relationship between CD4 count and the estimated probability of HEV infection (Figure 3). The graph aimed to visualize whether changes in CD4 count influence HEV infection risk, particularly in immunocompromised individuals. A slight upward trend was observed as CD4 count increased, although this trend was not statistically significant.

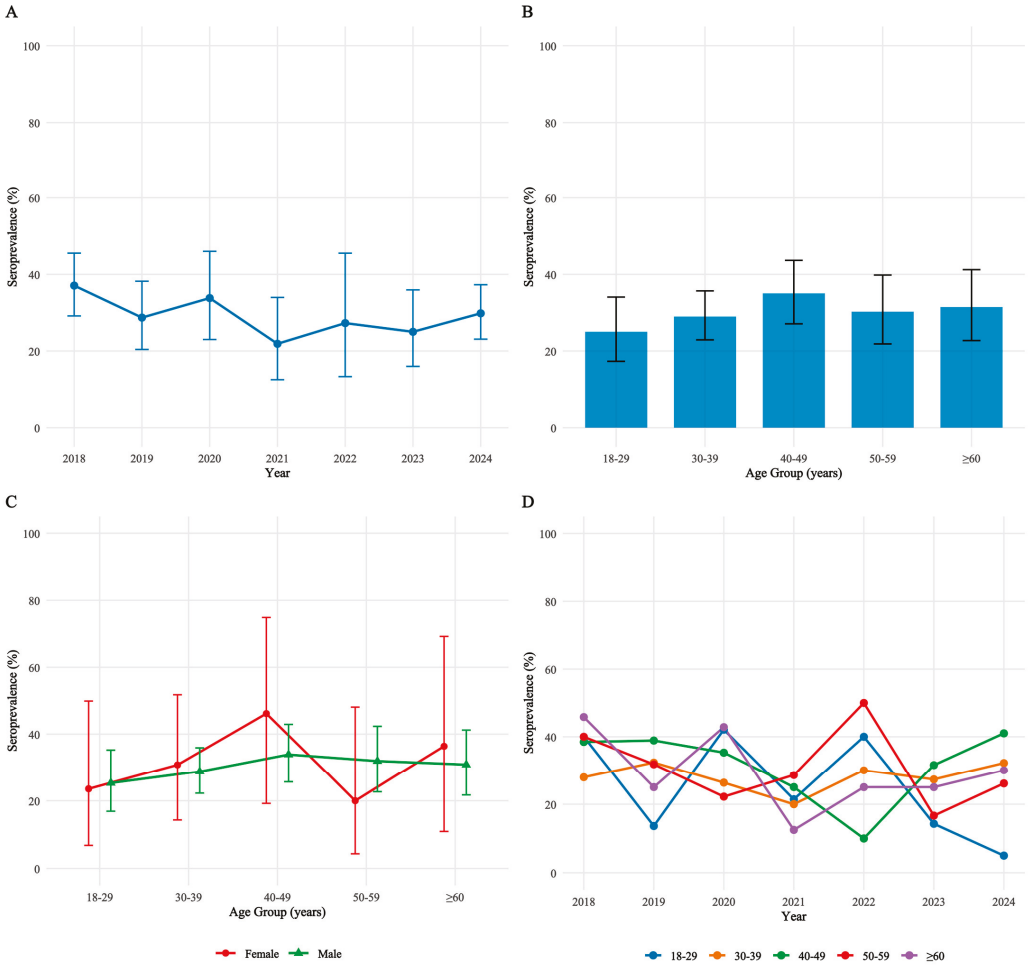

Figure 2. HEV seroprevalence patterns in PLWH. (**A**). Temporal trend of HEV seroprevalence from 2018 to 2024. Error bars indicate 95% confidence intervals. (**B**). Age-specific HEV seroprevalence. Error bars indicate 95% confidence intervals. (**C**). Age- and gender-stratified seroprevalence. Red: female; green: male. (**D**). Temporal trends by age cohort. HEV, Hepatitis E Virus; HIV, Human Immunodeficiency Virus; PLWH, People Living with HIV.

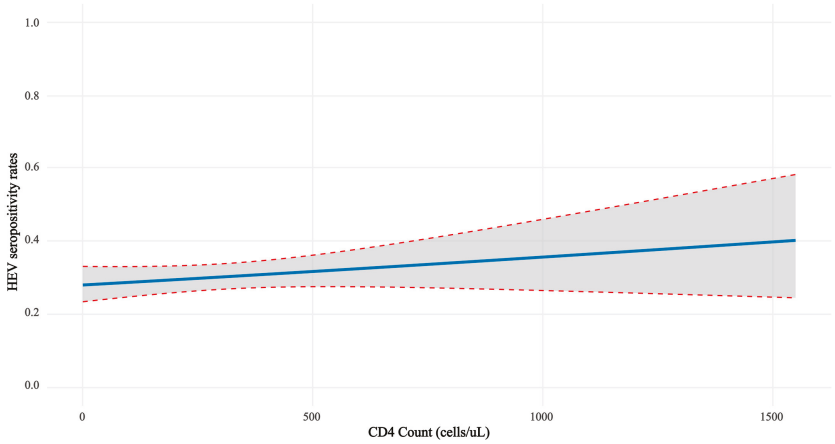

Figure 3. Relationship between CD4 count and estimated probability of HEV infection. The solid line represents the estimated probability of HEV infection across different CD4 counts. The dashed lines indicate the 95% confidence interval for this estimation. Model: logistic regression (glm, binomial family). Curve: predicted probability (solid line) with 95% Wald CI on logit scale back-transformed via inverse logit (shaded band). Fit: AIC = 822.8. CD4 effect: OR = 1.000 (95% CI: 1.000–1.001; $p = 0.23$). HEV, Hepatitis E Virus; PLWH, People Living with HIV; CD4, Cluster of Differentiation 4.

3.4. Risk Factor Analysis

We systematically evaluated risk factors for HEV co-infection in PLWH through univariate and multivariate logistic regression analyses (Table 4). Univariate analysis revealed a significant association between WHO HIV stage III/IV and anti-HEV antibody positivity (OR 1.422, 95% CI 1.016–1.988, $p = 0.040$). Anti-HEV positivity, age, gender, ethnicity, marital status, CD4 count, ART regimen, and co-infection were not associated with other hepatitis viruses or levels of ALB, ALT, AST, and total bilirubin ($p > 0.05$). Notably, these associations could not be determined (OR = 0.000) in non-Han ethnic groups due to a limited sample size, requiring cautious interpretation.

Table 4. Unifactorial and multifactorial analysis of HEV infections occurring in PLWH.

Characteristics	Univariate Analysis (n = 670)		Multivariate Analysis (n = 670)	
	Crude OR (95% CI)	***p*-Value**	**Adjusted OR (95% CI)**	***p*-Value**
Age	1.005 (0.993–1.017)	0.407	1.003 (0.990–1.016)	0.658
Gender				
Female	Base			
Male	0.982 (0.594–1.622)	0.943	0.876 (0.520–1.477)	0.620
Marital Status				
Married	Base			
Unmarried [1]	0.869 (0.596–1.266)	0.464	0.832 (0.541–1.281)	0.404
Ethnicity				
Han	Base			
Others	0.000 (0.000–∞)	0.979	0.000 (0.000–∞)	0.978
WHO Stage				
Stage I/II	Base			
Stage III/IV	1.422 (1.016–1.988)	0.040	1.566 (1.068–2.297)	0.022
CD4 Count (cells/μL)	1.000 (0.999–1.000)	0.380	1.000 (0.999–1.000)	0.284
ART				
None	Base			
N + N + NN [2]	0.987 (0.619–1.575)	0.957	0.913 (0.564–1.479)	0.712
N + N + PIs [3]	0.913 (0.606–1.375)	0.663	0.893 (0.577–1.382)	0.612
N + N + NR [4]	0.962 (0.427–2.165)	0.925	0.793 (0.339–1.857)	0.593
Viral Hepatitis Co-infections				
No virus	Base			
HBV co-infection	1.391 (0.746–2.593)	0.299	1.181 (0.600–2.322)	0.631
HCV co-infection	1.382 (0.621–3.073)	0.428	1.628 (0.674–3.928)	0.278
Chronic Liver Disease				
No liver cirrhosis	Base			
Liver cirrhosis	1.364 (0.529–3.517)	0.521	1.532 (0.575–4.082)	0.394
Liver Function Tests				
ALB (g/L)	1.002 (0.983–1.021)	0.851	1.020 (0.995–1.046)	0.123
AST (U/L)	1.000 (0.999–1.000)	0.928	1.000 (0.999–1.001)	0.614
ALT (U/L)	1.000 (1.000–1.000)	0.852	1.000 (0.999–1.001)	0.766
TBIL (μmol/L)	1.001 (0.999–1.004)	0.282	1.001 (0.998–1.004)	0.362

[1] Includes unmarried, divorced, and widowed. [2] Includes two nucleoside reverse transcriptase inhibitors (NRTIs) and a non-nucleoside reverse transcriptase inhibitor (NNRTI). c Includes two NRTIs and a protease inhibitor (PI). [3] Includes two NRTIs and an integrase chain termination inhibitor (INSTI); [4] includes two NRTIs and an integrase chain termination inhibitor (INSTIs). OR, Odds ratio; 95% CI, 95% Confidence interval; HEV, Hepatitis E virus; HIV, Human immunodeficiency virus; PLWH, People living with HIV; ART, Antiretroviral therapy; HBV, Hepatitis B virus; HCV, Hepatitis C virus; ALB, Albumin; AST, Aspartate aminotransferase; ALT, Alanine transaminase; TBIL, Total bilirubin; IgM, Immunoglobulin M; IgG, Immunoglobulin G; S/CO, Signal-to-cutoff ratio.

In the multivariate analysis conducted for all patients, WHO staging again emerged as an independent variable associated with anti-HEV antibody positivity (Table 4). Compared to early-stage patients, advanced WHO HIV stage (III/IV) was associated with a higher likelihood of prior HEV exposure (OR 1.566, 95% CI 1.068–2.297, $p = 0.022$). The VIFs for WHO stages and CD4 count were 1.3 and 1.6, respectively, indicating acceptable levels of collinearity (VIF < 5). No other variables in this model showed significant associations with anti-HEV antibody status ($p > 0.05$).

4. Discussion

This study provides key novel insights into HEV epidemiology among PLWH in Shanghai: (1) We report an anti-HEV IgG seroprevalence rate of 30.00%. (2) Despite this significant seropositivity, HEV RNA remained undetectable in all anti-HEV IgM-positive patients (1.19% of the cohort), suggesting acute/recent infection is uncommon and chronic infection, at least at detectable levels, is rare in this population. (3) Using Wantai ELISA, we demonstrated a significantly higher HEV seropositivity rate in PLWH (30.15%) compared to HIV-negative health-check attendees (17.67%), indicating increased susceptibility. (4) Advanced WHO HIV clinical stage (III/IV), but not CD4 count, emerged as a significant independent risk factor for prior HEV exposure. These findings fill a critical gap in the understanding of HEV co-infection in this major Chinese urban HIV cohort.

HEV has emerged as a leading cause of acute viral hepatitis globally [15–17]. While immunocompetent individuals typically experience asymptomatic seroconversion with minimal risk of chronic infection [18], immunosuppressed populations demonstrate a spectrum of clinical manifestations, including acute hepatitis and persistent chronic infection or viral reactivation [4,19,20]. Epidemiological data on HIV–HEV co-infection are scarce, with reported seroprevalence rates varying considerably across studies and geographical regions. These discrepancies likely stem from substantial differences in sample sources, study populations, and diagnostic methodologies. Previous cross-sectional studies report HEV seropositivity rates among PLWH ranging from 1.0% in Scotland to 71% in Zambia [21–26] (Table S2). Notably, HEV infection in immunosuppressed hosts can become chronic and rapidly progress to cirrhosis [27,28], and HEV seropositivity in PLWH may be underestimated due to impaired seroconversion kinetics [12]. Despite the significance of HEV in this context, research on HIV and HEV co-infection remains limited in Shanghai, China.

The observed IgG seroprevalence (30.0%) exceeds rates reported in European and North American populations but aligns with ranges documented across Africa and Asia [7,8,11,12,21–27,29–70]. Provincial comparisons within China reveal Shanghai's prevalence among PLWH resembles Anhui (31.3%) and exceeds Xinjiang (25.5%) but remains below Henan (44.2%), Yunnan (56.8%), and Zhejiang (41.1%) [71]. Crucially, anti-HEV antibody testing across these Chinese studies, including ours, utilized the same Wantai ELISA kits, eliminating inter-study variability due to assay differences. Consistent with these regional patterns, HEV RNA remained undetectable in all anti-HEV IgM-positive patients, mirroring reports from other Chinese cities of low IgM seropositivity (0.3–0.78%) and absent viremia [64,71]. Consequently, while HEV seropositivity is relatively common among PLWH, chronic HEV infection appears to be a rare manifestation of chronic liver disease in this population. However, the true prevalence of chronic HEV infection may be underestimated due to the limitations in detecting low-level viremia. Given the 500 copies/mL threshold, low-level viremia may have gone undetected; so, a negative RNA result does not definitively exclude chronic HEV infection in PLWH. Future studies would benefit from ultrasensitive PCR assays to detect occult viremia in immunocompromised populations.

The question of whether HIV infection heightens susceptibility to HEV remains inconclusive. Studies incorporating HIV-negative controls have yielded conflicting results regard-

ing differences in HEV exposure rates between PLWH and HIV-negative controls [21,26]. While some suggest PLWH may be more susceptible to HEV infection [8,29], others report no significant difference compared to HIV-negative controls [21,26,41]. In this study, the HEV seropositivity rate among HIV-negative health-check attendees was 17.67%, which is very close to the HEV seropositivity rate (18.02%) observed in the general population, according to the monitoring data from Chinese health examination centers from 2017 to 2022 [72]. Critically, we observed a significantly higher HEV seropositivity rate among PLWH (30.15%) compared to HIV-negative health-check attendees (17.67%). These findings suggest an increased vulnerability of PLWH to HEV infection, necessitating heightened clinical vigilance. This raises important questions regarding whether PLWH face higher exposure risks or possess increased biological susceptibility, warranting further mechanistic investigation.

HEV seroprevalence demonstrated relative stability across temporal, demographic, and immunological strata. No significant longitudinal trends were observed between 2018 and 2024 ($p = 0.15$), and no significant age-dependent gradients ($p = 0.53$) or gender disparities were detected. Although age-stratified analysis indicated a progressive increase in seroprevalence from 25.00% (18–29 years) to 35.00% (40–49 years)—consistent with established age-dependent exposure patterns in both immunocompetent individuals and PLWH [30,52,57,73]—multivariate regression failed to identify age as an independent risk factor ($p > 0.05$), suggesting potential cohort-specific effects.

Critically, advanced WHO HIV clinical stage (III/IV) emerged as an independent risk factor for HEV seropositivity in both univariate and multivariate analyses, highlighting that advanced WHO HIV stage (III/IV) was associated with a higher likelihood of prior HEV exposure. This association persisted even after adjustment for CD4 count in multivariate models, while CD4 count itself showed no independent correlation. This key dissociation implies that susceptibility is mediated by pathophysiological features of advanced HIV disease extending beyond CD4 count. WHO staging captures cumulative immune damage (e.g., chronic mucosal barrier disruption and gut-associated lymphoid tissue (GALT) impairment), whereas CD4 count primarily reflects current immunologic status [74]. Given the predominantly enteric transmission route of HEV, the persistent intestinal compromise characteristic of late-stage HIV may constitute a salient biological risk factor, irrespective of recent CD4 recovery following antiretroviral therapy [75,76]. However, the cross-sectional design precludes inference about directionality, and unrecognized chronic HEV infection could itself contribute to HIV disease progression. These findings underscore the clinical imperative for enhanced HEV vigilance in advanced HIV disease and warrant further investigation into the specific mechanisms underlying this association.

Several limitations of this study warrant careful consideration. The cross-sectional design, while providing valuable prevalence data, inherently limits causal inference regarding risk factors for HEV infection in PLWH, underscoring the need for longitudinal studies to establish temporal relationships and potential causality. Additionally, the absence of detailed exposure history data (e.g., dietary habits, zoonotic contact, water sources, transfusion history, occupation, and household income) hinders the identification of specific HEV transmission routes in this population and may introduce potential confounding. This limitation necessitates more comprehensive data collection in future studies. Critically, our complete reliance on serological diagnosis constitutes an important constraint. Immunocompromised individuals, including PLWH, may exhibit impaired or delayed humoral immune responses to HEV infection [5–7,77,78], potentially leading to false-negative serological results and an underestimation of true exposure rates. Furthermore, while HEV RNA testing was performed for all IgM-positive individuals, the lack of universal RNA testing across all seropositive (IgG+) participants precludes a comprehensive assessment of chronic HEV infection prevalence in this cohort. Finally, the single-center recruitment

strategy may limit the generalizability of the findings to broader populations or different geographical regions. These limitations highlight the importance of cautious interpretation and indicate key areas for improvement in future studies on HIV–HEV co-infection.

Future research directions emerging from this study are multifaceted. Prospective cohort studies are essential to elucidate the long-term outcomes of HIV–HEV co-infection. In-depth exploration of transmission routes and risk factors specific to HEV infection in PLWH is crucial for developing targeted prevention strategies. Given the availability of HEV vaccines with documented immunogenicity, evaluating their efficacy and safety in PLWH represents a promising approach to reducing co-infection rates. Additionally, future studies should incorporate HEV-specific T-cell assays or antigen detection methods to identify seronegative infections, thereby providing a more comprehensive assessment of HEV exposure in immunocompromised populations.

5. Conclusions

In summary, this study demonstrates that PLWH in Shanghai exhibit advanced WHO HIV stage (III/IV), which was associated with a higher likelihood of prior HEV exposure. This underscores the necessity of incorporating targeted HEV screening awareness and prevention strategies into HIV care programs, particularly for patients with advanced disease. These findings provide a foundation for future research and potential refinements in clinical practice, aiming to enhance care and prognosis for affected individuals.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/v17081038/s1, Table S1. List of primers used for HEV RNA quantification [13]. Table S2. Published studies on HEV and HIV coinfection [79,80].

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Abbreviations

The following abbreviations are used in this manuscript:

HEV	Hepatitis E virus
PLWH	People living with HIV
ALB	Albumin
ALT	Alanine transaminase

AST	Aspartate aminotransferase
TBIL	Total bilirubin
ART	Antiretroviral therapy
HBV	Hepatitis B virus
HCV	Hepatitis C virus
ELISA	Enzyme-linked immunosorbent assay
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
IQR	Interquartile range
CI	Confidence interval
VIFs	Variance inflation factors
NRTIs	Nucleoside reverse transcriptase inhibitors
NNRTI	Non-nucleoside reverse transcriptase inhibitors
PI	Protease inhibitor
INSTIs	Integrase chain termination inhibitor
GALT	Gut-associated lymphoid tissue

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