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TRANSLATIONAL RESEARCH IN HEPATITIS E

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REVIEW
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Hepatitis E Infection in HIV-Infected Patients

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Background: The hepatitis E virus (HEV) represents a major cause of acute hepatitis worldwide. The majority of HEV cases occur in low-income countries, mainly Asia and Africa, where HEV causes large outbreaks associated with the consumption of contaminated water and high mortality in specific populations. In high-income countries, HEV infection is considered a zoonotic disease that is linked to the consumption of contaminated food. Although a high proportion of cases have self-limiting asymptomatic or subclinical infections, immunosuppression may modify the pathogenesis and clinical impact of this emerging disease.

Results and Discussion: Here, we review the current knowledge about the epidemiology, diagnosis, clinical manifestations, management and prevention of HEV infection in HIV-infected subjects.

Conclusions: Despite the increasing knowledge about the pathogenesis, epidemiology and clinical impact of HEV infection, several major factors are faced by HIV-infected patients, including treatment recommendations, immunization and risk practices.

Keywords: HIV, HEV, epidemiology, zoonoses, treatment, prevention, diagnosis

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INTRODUCTION

The World Health Organization (WHO) estimates that, every year, more than 20 million people worldwide become infected with the hepatitis E virus (HEV) (World Health Organization [Who], 2017). Furthermore, it is estimated that 3.3 million of these cases are symptomatic and produce 44,000 deaths (World Health Organization [Who], 2017). Considering these statistics, the WHO ranks HEV as the leading cause of global acute hepatitis of viral origin. The majority of cases are reported in low-income countries, mainly Asia and Africa, where HEV causes large outbreaks associated with the consumption of contaminated water and is producing by HEV genotypes 1 and 2 (World Health Organization [Who], 2014). In contrast, in high-income countries, the majority of cases are produced by HEV genotype 3 and are linked to the consumption of contaminated food, including mainly pork-derived products and game meat (Faber et al., 2018). Because of the efficient transmission of the infection by this route, the European Food Safety Authority (EFSA) has indicated that HEV infection is a major public health problem in Europe (EFSA, 2017). Although a high proportion of these infections cause self-limiting asymptomatic or subclinical hepatitis (Kamar et al., 2012), there are clinical situations that can produce a worsened prognosis of the infection (Kamar et al., 2012; McPherson et al., 2018), and can even present with acute extrahepatic manifestations (Pischke et al., 2017); overall alterations in the central and peripheral nervous systems (Dalton et al., 2016).

HIV-infected patients encompass immunological, epidemiological, and clinical characteristics that can modify the pathogenesis of HEV. In this sense, after an acute HEV infection, the virus can persist and can develop into a chronic infection (Kenfak-Foguena et al., 2011). Furthermore, the immunosuppression derived from HIV infection can modify the immune response to HEV infection, causing a serological and virological pattern that implies a modification in the diagnosis algorithm (Pineda et al., 2014; Kuniholm et al., 2016). Additionally, in these patients, HEV reinfection has been suggested (Rivero-Juarez et al., 2017b); thus, extra preventive measures should be recommended in these subjects, even in those with evidence of past HEV infection. Furthermore, in patients with underlying chronic liver diseases (mainly by coinfection with other hepatotropic viruses such as hepatitis C or B), acute HEV infection has a worsened prognosis that is associated with a high mortality rate (Péron et al., 2006; Dalton et al., 2008). Thus, due to the high prevalence of hepatitis C and B coinfection among HIV infected patient, the risk of liver decompensation could be high in this population. Finally, HIV-infected subjects may be at higher risk for HEV acquisition due to HIV infection *per se* or to associated risk practices (Payne et al., 2013; Riveiro-Barciela et al., 2014). Thus, HIV-infected patients represent a population that is highly sensitive to HEV infection. For this reason, in this review, we describe the current knowledge about the epidemiology, diagnosis, clinical manifestations, management and prevention of HEV infection in HIV-infected populations.

RESULTS AND DISCUSSION

Search Strategy and Selection Criteria

The references used in this review were identified through searches of the PubMed database with the search terms "Hepatitis E," "chronic Hepatitis E," "HIV," and "viral hepatitis" from 1990 until September 2018. Articles were also identified through searches of the authors' own files. Only papers published in English were reviewed. The final reference list was generated on the basis of originality and relevance to the broad scope of this review.

Epidemiology of HEV in HIV-Infected Patients

Prevalence and Incidence

Studies evaluating the seroprevalence of IgG anti-HEV in HIV-infected subjects were conducted on all continents (**Figure 1**). Higher seroprevalence was reported in Africa and Asia (exceeding 40% in the majority of countries), followed by continental European Union countries (20–10%) and, finally, the Americas and Oceania (<10%). Full details of the studies evaluating HEV seroprevalence in HIV-infected individuals are presented in **Supplementary Table S1**. Several studies evaluated HEV seroprevalence in HIV-specific populations. In this sense, three studies evaluated the HEV IgG prevalence in pregnant women with HIV in Africa, reporting a seroprevalence of 7.1% in Gabon (Caron et al., 2012), 7.4% in Malawi (Mancinelli et al., 2017), and 33.3% in Ethiopia (Abebe et al., 2017). Finally, the prevalence of HIV-infected patients who are candidates for liver and kidney transplants in the United States was 19.2% (Sherman et al., 2014). Nevertheless, when interpreting and comparing HEV seroprevalence data, it should be considered that the immune assays used for HEV antibody identification demonstrate different sensitivities (Aggarwal, 2013). These differences were noted in a recent meta-analysis that included studies conducted in Europe, with the main aim to evaluate the seroprevalence in different subsets of patients according to the serological assays employed (Hartl et al., 2016). In this study, the seroprevalence rates found in HIV-infected patients were 1.8, 3.75, 5.9, 9.26, 11.55, and 15.69%, depending on the assay used. Similarly, a study conducted in Germany that included 246 HIV-infected individuals reported that the seroprevalence strongly varied from 1.6 to 25.6%, depending on the anti-HEV assays used (Pischke et al., 2015).

Few studies have reported HEV seroincidence in HIV-infected patients. Two studies conducted in Spain showed HEV seroincidence rates of 2.4 and 6.5% in 1 year, respectively (Pineda et al., 2014; Rivero-Juarez et al., 2017a). In a study that enrolled HIV-infected patients from China, the annual HEV seroincidence was 15.4% (Zeng et al., 2017). Finally, another study that included HIV-infected pregnant women from Tanzania reported an annual seroincidence of 1% (Harritshøj et al., 2018).

Transmission and Risk Groups

The HEV transmission route varies depending on the viral genotype. HEV genotypes 1 and 2, which exclusively affect humans, are mainly transmitted by the consumption of fecal-contaminated water during the rainy season and are associated with flooding, as noted by the WHO (World Health Organization [Who], 2014). Similarly, a lack of hygienic measures, such as a lack of hand washing or the absence of proper sanitation, is an important risk factor for the acquisition of HEV in the general population (World Health Organization [Who], 2014). In a study conducted in Nigeria that enrolled HIV-infected patients, an inadequate toilet system (pit and bush) and water supply source (well and stream) were identified as major risk factors for HEV infection (Junaid et al., 2014). In contrast, HEV genotypes 3 and 4 as well as the less-common genotype 7 can infect both humans and a wide range of animals; thus, they constitute a zoonotic infectious disease (EFSA, 2017). In the general population, the main routes of transmission of these genotypes are the consumption of raw or undercooked meat (overall pork) and contact with infected animals (EFSA, 2017). In this sense, the HEV seroprevalence in HIV-infected individuals who eat raw/undercooked pork was 33% compared with 9% in those who not reported consumption in a study conducted in southwest England (Keane et al., 2012). In addition, one outbreak, which was linked to the consumption of wild boar meat in Spain, that involved HIV-infected patients has been recently reported (Rivero-Juarez et al., 2017c). Other factors associated with HEV seroprevalence in the general population, such as older age (Feldt et al., 2013; Rapicetta et al., 2013; Pineda et al., 2014; Rivero-Juarez et al., 2015b; Shrestha et al., 2017; Zeng et al., 2017;

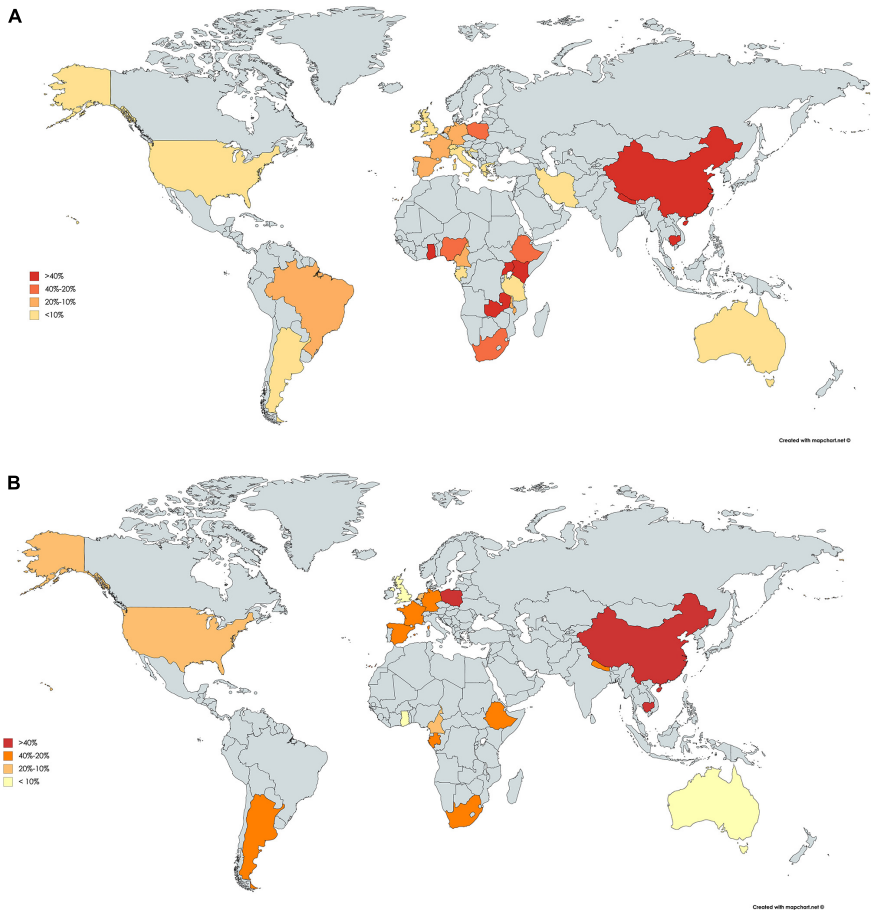

FIGURE 1 | Worldwide seroprevalence of anti-IgG hepatitis E virus in HIV-infected patients **(A)**. Schematic representation of the distribution of hepatitis E IgG antibody seroprevalence. Worldwide seroprevalence of anti-IgG hepatitis E virus in HIV-infected patients using Wantai Diagnostics assay **(B)**. Maps created based on the data obtained in **Supplementary Table S1**.

Zhou et al., 2018), geographical location or habitat (Renou et al., 2010; Feldt et al., 2013; Rapicetta et al., 2013; Junaid et al., 2014; Scotto et al., 2014; Yong et al., 2014; Rivero-Juarez et al., 2017a; Shrestha et al., 2017; Zeng et al., 2017), and male sex (Pineda et al., 2014; Zeng et al., 2017; Boon et al., 2018), have also been associated with HIV-infected patients. Consequently, the main route of HEV, including all viral genotypes, does not differ between the general and HIV populations.

Several studies have evaluated whether HIV infection constitutes a risk factor for HEV infection (**Table 1**) (Fainboim et al., 1999; Keane et al., 2012; Maylin et al., 2012; Rapicetta et al., 2013; Payne et al., 2013; Junaid et al., 2014; Riveiro-Barciela et al., 2014; Scotto et al., 2014; Taha et al., 2015; Madden et al., 2016; Abebe et al., 2017; Abravanel et al., 2017; Bura et al., 2017a,b; Shrestha et al., 2017; Boon et al., 2018). Of these studies, only three matched the controls by age, sex and geographical area (Abravanel et al., 2017; Bura et al., 2017a; Boon et al., 2018). Consequently, HIV *per se* seems not to be a risk factor for HEV infection. Other not matched studies found differences in favor to healthy donors or HIV infected patients. A study conducted in Uganda that included 491 healthy blood donors and 494 HIV-infected patients showed no differences in terms of HEV IgG seroprevalence between both groups (47.7 and 46.4%) (Boon et al., 2018). Another study performed in Poland showed a relatively higher HEV seroprevalence in healthy donors (3.8%) than that in subjects infected with HIV (0.95%) (Bura et al., 2017a). Similarly, in a French study, the HEV IgG seroprevalence in healthy donors was higher than that in HIV-infected patients (47.3 and 38.7%) (Abravanel et al., 2017).

Specific conditions related to HIV infection have been evaluated as potential factors associated with HEV infection. First, HIV viremia seems to have no association with a higher risk for HEV infection (Jardi et al., 2012; Keane et al., 2012; Pineda et al., 2014; Rivero-Juarez et al., 2015b; Abravanel et al., 2017; Ferreira et al., 2018). Therefore, the rate of IgG HEV antibodies is similar among patients with detectable and undetectable HIV viral loads (Jardi et al., 2012; Keane et al., 2012; Pineda et al., 2014; Rivero-Juarez et al., 2015b; Abravanel et al., 2017; Ferreira et al., 2018). In the same way, the use and duration of antiretroviral therapy has not been associated with a higher HEV seroprevalence in different studies that

have reported a comparable percentage between patients on antiretroviral therapy and those not using this type of therapy (Jardi et al., 2012; Keane et al., 2012; Feldt et al., 2013; Pineda et al., 2014; Rivero-Juarez et al., 2015b; Abravanel et al., 2017; Ferreira et al., 2018). Furthermore, one study did not find differences in HEV seroprevalence between HIV-1- and HIV-2-infected patients (Kaba et al., 2011). Finally, studies evaluating the association between CD4+ cell count and HEV seroprevalence present controversial results. The majority of these studies found a similar HEV seroprevalence between patients with a CD4+ cell count that was either higher or lower than 200 cells/mL (Kaba et al., 2011; Kenfak-Foguena et al., 2011; Jardi et al., 2012; Pineda et al., 2014; Bura et al., 2017b; Ferreira et al., 2018), or when a cut-off of 250 cells/mm^3 was applied (Zeng et al., 2017). Nevertheless, there are studies that found a higher HEV seroprevalence in patients with a total CD4+ cell count higher than 200 cells/mL (Kenfak-Foguena et al., 2011), while other studies found the opposite effect, in that patients with a CD4+ count below 200 cells/mL showed a higher seroprevalence of IgG antibodies (Renou et al., 2010; Debes et al., 2016; Zhou et al., 2018).

Several observations have suggested that men who have sex with men (MSM) may be exposed to an increased risk for HEV transmission. In a study conducted in the United Kingdom (UK), the seroprevalence of HEV was compared among 146 HIV-infected MSM, 135 HIV-non-infected MSM, and 141 HIV-non-infected heterosexual men. The seroprevalence was similar between both MSM groups (7.5% vs. 10.4%; $p = 0.4$) but was higher than that found in heterosexual men (3.5%; $p = 0.025$) (Payne et al., 2013). Similarly, in an Italian study in which the HEV seroprevalence was evaluated in a large cohort of individuals that included HIV-infected patients, the seroprevalence found in MSM was higher than the seroprevalence reported in non-MSM (7.5% vs. 4.7%; $p = 0.04$)

TABLE 1 | Studies evaluating HIV infection as a risk factor for hepatitis E virus infection, including comparator groups.

Study references	HIV population (n)	Comparator group (n)	HEV seroprevalence (%) HIV group	HEV seroprevalence (%) Comparator group	HIV infection identified as risk factor
Payne et al., 2013	146 MSM	135 MSM 141 Htx	7.5%	10.4% MSM 3.5% Htx	No
Keane et al., 2012	138	464 patients without history of CLD	9.4%	13.8%	No
Rapicetta et al., 2013 Scotto et al., 2014	72	896 HD	19.4%	11.1%	Yes
Abebe et al., 2017	18 pregnant women	368 pregnant women	33.3%	31.5%	No
Bura et al., 2017b	244	246 HD	50.8%	49.6%	No
Madden et al., 2016	60	896 HD	23.3%	29.1%	No
Fainboim et al., 1999	484	1500 HD	6.6%	1.8%	Yes
Maylin et al., 2012	261	46 kidney Tx	1.5%	6.5%	No
Bura et al., 2017a	105	105 HD	0.95%	3.8%	No
Abravanel et al., 2017	300	600 HD	IgG: 38.7% IgM: 3.6%	IgG: 47.3% IgM: 3.8%	No
Boon et al., 2018	494	491 HD	46.4%	47.7%	No
Shrestha et al., 2017	459	581 HD	IgG: 39.4% IgM: 15.3%	IgG: 9.5% IgM: 4.4%	Yes
Junaid et al., 2014	80	190 HD 108 pregnant women 48 animal handlers	30%	44.7% healthy donors 41.6% pregnant women 58.3% animal handler	No
Taha et al., 2015	403	397 HD	12.9%	20.2%	No
Riveiro-Barciela et al., 2014	238	301 CLD patients 338 Liver Tx 296 Kidney Tx 200 HD	9.2%	4.9% CLD patients 9.4% Liver Tx 3.7% Kidney Tx 3.5% HD	Yes

HIV, human immunodeficiency virus; n, number of subjects; HEV, hepatitis E virus; MSM, men who have sex with men; Htx, heterosexual; HD, healthy donors; HCV, hepatitis C virus; CLD, chronic liver disease; Liver Tx, liver transplant; Kidney Tx, kidney transplant.

(Lanini et al., 2015). Finally, another Italian study analyzed the seroprevalence of HEV and hepatitis A virus (HAV) in a cohort of 636 MSM and compared the seroprevalence with that of a control group of 288 non-MSM (Greco et al., 2018). This study reported a higher seroprevalence for both HAV and HEV (42.8 and 10.2%) in the MSM group than that in the non-MSM group (29.2 and 5.2%). In contrast, a recent study conducted in Taiwan during an HAV outbreak including 3,293 HIV-infected patients, majoritarian MSM, the seroprevalence and seroincidence of HEV infection did not differ between sexual risk practice or underlying infection (HAV, HCV, or Syphilis) (Lin et al., 2019). Interestingly, in only 1 of the 23 HEV seroconversion documented in the study also showed seroconversion for HAV. For this reason, the fact that HIV-infected MSM might be a population that is at higher risk for HEV acquisition need to be clarify.

Diagnosis of HEV Infection in HIV-Infected Patients

The virological markers for the diagnosis of HEV infection comprise viral components (HEV RNA and HEV Antigen [HEV Ag]) and products related to the host immune response (anti-HEV specific antibodies of IgA, IgG, and IgM classes) (Aggarwal, 2013). Typically, in the general population, after an incubation period between 2 and 6 weeks, viral RNA and HEV Ag are detectable in the blood, urine, and feces (Zhao and Wang, 2016). After 4–6 weeks of infection, HEV-RNA is usually undetectable in the blood but can remain detectable in the feces for several weeks (Aggarwal, 2013). The immune response follows a typical pattern of seroconversion with an initial and transient increase in IgM that leads to a sustained IgG response. Anti-HEV IgM antibodies are detected only during the acute phase and remain detectable up to the 5th month after infection, making these antibodies the best serological markers for the diagnosis of acute HEV infection. Furthermore, IgG antibodies can be detected very close in time to the detection of IgM antibodies, and they remain detectable for more than 10 years; thus, they may be used to establish past exposure to HEV (Aggarwal, 2013). Finally, IgA antibodies can also be detected during the acute phase of HEV infection, but their use in the diagnosis of HEV infection is controversial (Aggarwal, 2013).

Studies have evaluated the prevalence of HEV IgM antibodies in HIV-infected patients, and they have found a good value for the diagnosis of acute/recent HEV infection in this population (Abravanel et al., 2017; Shrestha et al., 2017). Furthermore, several studies have shown that the absence of HEV-IgM antibodies is correlated with the absence of HEV RNA in HIV-infected patients (Ramezani et al., 2013; Harritshøj et al., 2018), even in patients with severe immunosuppression (Nouhin et al., 2015; Rivero-Juarez et al., 2015a). In addition, several studies of HEV infection show that HEV-IgM positivity coincides with the first detectable HEV viral load (Renou et al., 2010; Sellier et al., 2011; Bouamra et al., 2013; Robbins et al., 2014; Abravanel et al., 2017). In contrast, there is evidence that the use of HEV IgM to diagnose acute HEV infection in this population has limited value because studies have shown that HIV-infected patients may have a delayed immune response or may lack an immune response against acute HEV infection. One of these studies, in which three cases of acute HEV infection were described, showed that all cases lacked HEV IgM positivity and seroconversion over time; additionally, they all showed a CD4+ count higher than 200 cells/mm^3 (Kuniholm et al., 2016). Similarly, another study that included 5 cases of acute HEV infection diagnosed by HEV RNA found that HEV IgM could only be detected at diagnosis in two subjects (Rivero-Juarez et al., 2015b), and three of these patients lacked HEV IgM positivity, with a CD4+ cell count higher than 200 cells/mm^3. Similarly, in a study of 10 cases of acute HEV infection in Brazil, none of the patients presented with HEV HEV IgM, or HEV IgG antibodies (Salvio et al., 2018). Of the 9 patients with an available CD4+ count, only one showed a CD4+ cell count lower than 200 cells/mL (Salvio et al., 2018). These data show that the lack of HEV IgM during the acute phase may not be limited to those patients with severe immunosuppression. For these reasons, in HIV-infected patients, the application of HEV IgM alone may not be sufficient to exclude the diagnosis of acute HEV infection, and it is mandatory to include a direct diagnosis procedure such as HEV RNA (European Association for the Study of the Liver [EASL], 2018; Rivero-Juarez et al., 2018).

After the occurrence of the acute phase in HIV-infected patients, HEV can persist and may develop into a chronic infection, which is defined as the persistence of HEV RNA for more than 3 months (European Association for the Study of the Liver [EASL], 2018; Rivero-Juarez et al., 2018). In these patients, IgM and IgG antibody seroconversion is usually absent or can be detected intermittently (Colson et al., 2009; Dalton et al., 2009; Kaba et al., 2011; Kenfak-Foguena et al., 2011; Andersson et al., 2013; Jagjit, Singh et al., 2013; Neukam et al., 2013; Ingiliz et al., 2016; Kuniholm et al., 2016; Todesco et al., 2017). On the other hand, it has been described that in HIV infected patients HEV reinfection may occur (Rivero-Juarez et al., 2017b). The serological pattern of HEV reinfection is characterized by the presence of HEV RNA with positivity to HEV IgG antibodies and the persistent absence of HEV IgM (European Association for the Study of the Liver [EASL], 2018; Rivero-Juarez et al., 2018). Thus, the only marker that is indicative of these two virological situations is the use of methods evaluating HEV RNA.

Clinical Impact of HEV Infection in HIV-Infected Patients

Acute HEV infection usually occurs as mild-severity acute hepatitis in both the general and HIV-infected populations. In several patients, hospitalization is necessary (Kamar et al., 2012), with an associated mortality of up to 8.7%, which varies depending on the comorbidities of the patients affected (Péron et al., 2006; Dalton et al., 2008). In patients with underlying chronic liver disease, acute HEV infection is associated with a high overall mortality rate in patients from low-income countries who are infected with HEV genotypes 1 and 2 (Kumar Acharya et al., 2007). Several studies have evaluated the prevalence of acute HEV infection in HIV-infected patients with acute increases in transaminases. In a study conducted in Scotland of 99 HIV-infected patients, the prevalence of acute

HEV was 1.06% (Bradley-Stewart et al., 2015). Another study performed in France found that acute HEV infection was a cause of unexplained elevated transaminases in one patient of the 108 HIV-infected subjects evaluated (0.9%) (Sellier et al., 2011). In the United States, among 458 HIV-infected patients who were United States military beneficiaries, evidence of acute HEV infection was detected in 4% of 194 HIV-infected persons with an episode of increased transaminase levels (Crum-Cianflone et al., 2012). In contrast, a study that included 256 HIV-infected patients at follow-up in the Netherlands did not find any cases of acute HEV (Hassing et al., 2014). For this reason, clinical guidelines recommend excluding HEV in cases of acute hepatitis (European Association for the Study of the Liver [EASL], 2018; Rivero-Juarez et al., 2018). Until today, no hepatic decompensation among HIV cirrhotic patients has been reported.

Furthermore, there is increasing knowledge regarding extrahepatic manifestations that are linked to acute HEV infection, highlighting neurological injury, renal injury, cryoglobulinemia, pancreatitis, and hematological disorders (Dalton et al., 2016; Pischke et al., 2017). Although there are no specific studies that describe the course of HEV infection with extrahepatic manifestations in HIV-infected patients, the cases reported in this population suggest a similar clinical pattern to that reported in a non-HIV-infected population during the acute phase (Bouamra et al., 2013; Robbins et al., 2014; Kuniholm et al., 2016). Nevertheless, in a series of cases that included both immunocompetent and immunocompromised patients (with only three HIV-infected patients in this group), it was suggested that acute hepatitis-related symptoms and neurological manifestations may occur at lower frequencies in immunocompromised patients (Abravanel et al., 2018).

Furthermore, 4–6 weeks after the appearance of clinical symptoms, the infection is usually self-limited and does not need therapy. However, in immunosuppressed patients with various causes, the infection can evolve into a chronic infection (European Association for the Study of the Liver [EASL], 2018; Rivero-Juarez et al., 2018). This development usually occurs with HEV genotypes 3 and 4 and is characterized by a rapid progression to liver cirrhosis and the persistence of changes at the transaminase level (Neukam et al., 2013). The prevalence of chronic HEV infection among HIV-infected patients is rare, with an estimated prevalence between 0 and 0.5% (Madejón et al., 2009; Pischke et al., 2010; Renou et al., 2010; Sellier et al., 2011; Maylin et al., 2012; Scotto et al., 2014; Sherman et al., 2014; Nouhin et al., 2015; Rivero-Juarez et al., 2015b; Abravanel et al., 2017; Ferreira et al., 2018). In a Spanish study, the prevalence of chronic HEV infection among HIV-infected patients with unexplained increases in liver stiffness was 0.5% (Rivero-Juárez et al., 2013). In two cohorts of HIV-infected patients from France and the United States, the prevalence of chronic HEV infection was 0.5 and 0.05%, respectively (Kaba et al., 2011; Kuniholm et al., 2016). Finally, in a study conducted in Switzerland that included 735 HIV-infected patients with persistent ALT levels, the prevalence of chronic HEV infection was 0.13% (Kenfak-Foguena et al., 2011). Currently, 12 cases of chronic HEV infection have been reported in HIV-infected patients (**Table 2**)

TABLE 2 | Cases of chronic hepatitis E virus infection in HIV-infected patients.

Case references	Gender	Age	CD4+ cells count	HEV anti IgM/IgG	HEV genotype	Duration of HEV viremia (months)	Outcome	Comment
Dalton et al., 2009	Male	48	<100	Pos/Pos	3a	24	Treatment initiation	
Colson et al., 2009	Male	50	<100	Neg/Neg	3	12	Not reported	
Jagjit, Singh et al., 2013	Male	45	54	Neg/Neg	3a	132	Treatment initiation	
Kaba et al., 2011	Male	44	40	Pos/Neg	3f	10	Deceased	Diagnosis of chronic infection coincides with treatment time for non-Hodgkin's lymphoma therapy
Kenfak-Foguena et al., 2011	Male	46	34	NT/Pos	3b	36	Spontaneous viral clearance	Viral clearance after CD4+ count increases
Kenfak-Foguena et al., 2011	Male	59	50	NT/Neg	3c	At least 6	Spontaneous viral clearance	
Andersson et al., 2013	Male	42	37	Neg/Neg	3	36	Spontaneous viral clearance	Clearance after HAART initiation and CD4+ count restoration
Neukam et al., 2013	Male	47	<200	Pos/Neg	3	36	Treatment initiation	
Neukam et al., 2013	Male	53	<200	Neg/Pos	3	60	Treatment initiation	
Kuniholm et al., 2016	Female	–	<200	Neg/Neg	3a	36	Not reported	
Ingiliz et al., 2016	Male	47	<200	Pos/Pos	3	48	Treatment initiation	
Todesco et al., 2017	Male	59	<100	NT/NT	3i	48	Treatment initiation	

CD4+, lymphocytes CD4+; HEV, hepatitis E virus; IgM, immunoglobulin M; IgG, immunoglobulin G; Pos, positive; Neg, negative; NT, non-tested.

(Colson et al., 2009; Dalton et al., 2009; Kaba et al., 2011; Kenfak-Foguena et al., 2011; Andersson et al., 2013; Jagjit, Singh et al., 2013; Neukam et al., 2013; Ingiliz et al., 2016; Kuniholm et al., 2016; Todesco et al., 2017). All HIV cases have a CD4+ cell count lower than 200 cells/mm^3, which is the only risk factor associated with the development of chronic HEV infection in this population.

Treatment of Acute and Chronic HEV Infection in HIV-Infected Patients

Most cases of acute HEV infection are self-limited without the need for the implementation of therapy in both the general and HIV-infected populations. However, in several subsets of patients, such as patients with underlying chronic liver disease or acute liver failure, acute HEV infection is associated with a higher risk of complications and a worse prognosis (Kumar Acharya et al., 2007). Currently, there are no specific therapies available against acute HEV infection (European Association for the Study of the Liver [EASL], 2018; Rivero-Juarez et al., 2018). The evidence available regarding the use of ribavirin (RBV) in the HIV-infected population for the treatment of acute HEV infection is limited to three cases of infection with genotype 3 (**Table 3**), all of which were successfully treated with RBV monotherapy for 12 or 24 weeks (Bouamra et al., 2013; Robbins et al., 2014; Abravanel et al., 2017).

The development of chronic HEV infection is associated with immunosuppression; thus, measures aimed at restoring the immune response may induce the clearance of HEV (European Association for the Study of the Liver [EASL], 2018; Rivero-Juarez et al., 2018). In this sense, two cases of chronic HEV infection in HIV-infected patients were self-limiting after the initiation of antiretroviral therapy and the suppression of HIV viral load (**Table 2**) (Kenfak-Foguena et al., 2011; Andersson et al., 2013). Relatively large amounts of evidence regarding the therapeutic options for chronic HEV infection are available for liver transplant patients (McPherson et al., 2018). In contrast, evidence in HIV-infected patients is scarce and limited to the description of 6 cases (**Table 3**). Three cases have reported treatment with RBV monotherapy (Neukam et al., 2013; Ingiliz et al., 2016), and two of these patients experienced viral relapse after the cessation of therapy [63]. Another three cases reported the combination of pegylated interferon with RBV for 12 and 24 weeks of treatment (Dalton et al., 2011; Jagjit, Singh et al., 2013; Todesco et al., 2017), and two of these patients attained sustained viral clearance (Dalton et al., 2011; Jagjit, Singh et al., 2013). Finally, the *in vitro* antiviral activity of sofosbuvir against HEV has been demonstrated (Dao Thi et al., 2016). Nevertheless, the use of this drug in the transplant population for the treatment of chronic HEV has not been demonstrated to have efficacy in eliminating the virus (van der Valk et al., 2017; Todesco et al., 2018). In the HIV-infected population, the use of sofosbuvir in combination with RBV for 12 weeks has been evaluated in only one case (Todesco et al., 2017). This patient experienced viral relapse after the completion of therapy.

Prevention of HEV in HIV-Infected Patients

The most effective preventative measure for HEV infection is to avoid contact with the source of infection, such as avoiding the consumption of raw/undercooked food where HEV has been isolated or the consumption of unchlorinated contaminated water in low-income countries (European Association for the Study of the Liver [EASL], 2018; Rivero-Juarez et al., 2018). Treating meat at a temperature of 70°C for 30 min has been shown to strongly inhibit HEV activity (Johne et al., 2016). For contaminated milk, thermal treatment at 100°C has shown complete inactivation of the virus (Huang et al., 2016). For contaminated water, a chlorine dose of 5 mg/L for 15 min appears to be sufficient to reduce the HEV viral load; nevertheless, it may be necessary to increase the

TABLE 3 | Treatment of acute and chronic HEV in HIV-infected patients.

Phase	References	HEV genotype	Regimen	Outcome
Acute	Abravanel et al., 2017	3f	RBV 24 weeks	Treatment induced viral clearance
Acute	Robbins et al., 2014	3c	RBV 24 weeks 1,200 mg	Treatment induced viral clearance
Acute	Bouamra et al., 2013	3c	RBV 24 weeks 1,200 mg	Treatment induced viral clearance
Chronic	Dalton et al., 2011	3a	Peg-IFN 24 and 6 weeks of Peg-IFN/RBV	Treatment induced viral clearance
Chronic	Jagjit, Singh et al., 2013	3a	Peg-IFN 24 weeks	Treatment induced viral clearance
Chronic	Neukam et al., 2013	3	RBV 24 weeks 1,200 mg	Viral relapse
Chronic	Neukam et al., 2013	3	RBV 24 weeks 1,000 mg	Viral relapse
Chronic	Ingiliz et al., 2016	3	RBV 20 weeks 800 mg	Treatment induced viral clearance
Chronic	Todesco et al., 2017	3i	1° Peg-IFN/RBV 12 weeks 2° SOF/RBV 12 weeks	1° Viral relapse 2° Viral relapse

HEV, hepatitis E virus; RBV, ribavirin; Peg-IFN, pegylated interferon; SOF, sofosbuvir.

chlorine dose if water contains solid material (World Health Organization [Who], 2014; EFSA, 2017). Clinical guidelines recommend that immunocompromised patients, such HIV-infected patients with a CD4+ cell count below 200 cells/mm^3 and those with chronic liver disease including HIV-infected patients with coinfection with HCV or HBV, should avoid the consumption of raw or undercooked meat and shellfish due to the risk of developing a serious or even fatal course of HEV infection (European Association for the Study of the Liver [EASL], 2018; Rivero-Juarez et al., 2018).

Currently, there is only one vaccine available for the prevention of HEV infection, which is a recombinant vaccine against genotype 1 that has demonstrated high protection of up to 5 years for people over 16 years of age, with potential protection of up to 30 years (Zhang et al., 2015; Su et al., 2017). Nevertheless, the application of this vaccine is currently limited to China, following the position of the WHO (World Health Organization [Who], 2015). This position is supported by the fact that there are no clinical trials evaluating the safety and efficacy of this vaccine in populations susceptible to a worsened prognosis of the disease (HIV-infected patients, transplant recipients, pregnant women, and patients with underlying chronic liver disease) and because of only demonstrated efficacy in preventing HEV genotype 1 infection. For this reason, the WHO recommends that vaccination should be considered individually in people who plan to travel to an area where an epidemic is occurring (e.g., aid workers and health workers).

Finally, in immunosuppressed patients, such as transplant recipients and HIV-infected patients (Abravanel et al., 2014; Rivero-Juarez et al., 2017b), HEV reinfection has been described. Furthermore, in immunocompetent patients, an IgG antibody concentration of 2.5 WHO units/mL could protect against reinfection (Zhang et al., 2015); in immunocompromised patients, this titre can be increased up to 7 WHO units/mL (Abravanel et al., 2014). Nevertheless, there are no data regarding the minimum protective titre of IgG antibodies in HIV-infected patients. This fact reinforces the recommendation that preventive measures should be applied in immunosuppressed HIV-infected patients, even in the presence of IgG antibodies. Furthermore, due to the risk of reinfection and the lack of diagnostic value of IgM antibodies at this point, annual testing for HEV-RNA in HIV immunocompromised patients is currently recommended in clinical guidelines (European Association for the Study of the Liver [EASL], 2018; Rivero-Juarez et al., 2018).

CONCLUSION

Despite the increasing knowledge about the pathogenesis, epidemiology and clinical impact of HEV infection, several major factors are faced by HIV-infected patients. First, HIV infection seems not to be a risk factor for HEV acquisition. Nevertheless, the risk population for HIV infection, such as MSM, may also have a higher risk for HEV infection. A better understanding of this risk significantly increases the awareness of prevention in this population. Second, in recent years, HEV-associated extrahepatic manifestations have been described, with special emphasis on acute neurological injury. However, there is a lack of clinical data on these extrahepatic manifestations in HIV-infected patients; thus, there is no evidence on the frequency, clinical course and management that can support any special recommendations. Third, therapy for both acute and chronic HEV infections is suboptimal, with very limited evidence for combination and posology therapies in the HIV-infected population. The use of direct-acting viral drugs, such as sofosbuvir, against other viral infections has shown a lack of efficacy in clinical practice in terms of chronic HEV infection in both the general and HIV populations. Therefore, evaluating alternative therapeutic options is a priority. Finally, the only approved vaccine for preventing HEV infection has a lack of data regarding safety and efficacy in HIV-infected patients. Thus, before vaccination of these patients, there is a need for data as well as the overall evaluation of the persistence of protected antibodies over time in severely immunocompromised HIV patients.

AUTHOR CONTRIBUTIONS

AR-J drafted the manuscript and performed the research strategy with the support of all authors. All authors approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.01425/full#supplementary-material

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Protein Interactions Network of Hepatitis E Virus RNA and Polymerase With Host Proteins

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Host-pathogen interactions are crucial for the successful propagation of pathogens inside the host cell. Knowledge of interactions between host proteins and viral proteins or viral RNA may provide clues for developing novel antiviral strategies. Hepatitis E virus (HEV), a water-borne pathogen that causes acute hepatitis in humans, is responsible for epidemics in developing countries. HEV pathology and molecular biology have been poorly explored due to the lack of efficient culture systems. A contemporary approach, to better understand the viral infection cycle at the molecular level, is the use of system biology tools depicting virus-host interactions. To determine the host proteins which participate in the regulation of HEV replication, we indentified liver cell proteins interacting with HEV RNA at its putative promoter region and those interacting with HEV polymerase (RdRp) protein. We employed affinity chromatography followed by liquid chromatography quadrupole time-of-flight mass spectrometry (LC-QTOF/MS) to identify the interacting host proteins. Protein-protein interaction networks (PPI) were plotted and analyzed using web-based tools. Topological analysis of the network revealed that the constructed network is potentially significant and relevant for viral replication. Gene ontology and pathway enrichment analysis revealed that HEV RNA promoter- and polymerase-interacting host proteins belong to different cellular pathways such as RNA splicing, RNA metabolism, protein processing in endoplasmic reticulum, unfolded protein response, innate immune pathways, secretory vesicle pathway, and glucose metabolism. We showed that hnRNPK and hnRNPA2B1 interact with both HEV putative promoters and HEV RdRp, which suggest that they may have crucial roles in HEV replication. We demonstrated *in vitro* binding of hnRNPK and hnRNPA2B1 proteins with the HEV targets in the study, assuring the authenticity of the interactions obtained through mass spectrometry. Thus, our study highlights the ability of viruses, such as HEV, to maneuver host systems to create favorable cellular environments for virus propagation. Studying the host-virus interactions can facilitate the identification of antiviral therapeutic strategies and novel targets.

Keywords: host-protein interactions, protein interactions network, system biology, gene ontology analysis, hepatitis E virus, viral RNA, viral polymerase

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INTRODUCTION

Hepatitis E virus (HEV) is a hepevirus that is transmitted via contaminated drinking water to cause acute hepatitis in humans. Although prevalence of HEV has been mainly observed in developing countries, its spread has been reported in many industrialized countries across the globe in recent years. In infected adults, mortality rate due to HEV is up to ∼2% while in infected pregnant women, it increases up to 30% (Meng, 2010; Nan and Zhang, 2016). Due to the lack of efficient culture systems and robust animal models for HEV propagation, molecular mechanisms underlying the HEV lifecycle are not known (Himmelsbach et al., 2018). Non-specific treatment with pegylated interferons along with ribavirin is recommended in rare instances for severe cases because specific antiviral drugs or vaccines against HEV are still not available worldwide (van de Garde et al., 2017).

Hepatitis E virus belongs to the Orthohepevirus genus, and its genome consists of positive sense, single-stranded RNA comprised of three open reading frames (ORFs) (Kenney and Meng, 2018). First step of HEV replication cycle is the translation of ORF1 present on the positive sense genomic RNA to form non-structural polyprotein, which consists of functional domains of methyltransferase, protease, helicase and RNA-dependent RNA polymerase (RdRp). Two additional ORFs present in the sub-genomic intermediate RNA, ORF2 and ORF3, encode capsid protein and a small multifunctional phosphoprotein, respectively (Kenney and Meng, 2018). HEV replication cycle involves the formation of a negative sense RNA complementary to the positive sense genomic RNA. HEV RdRp recognizes and starts the transcription at the promoter present at the 3′ end of the positive sense strand to form a complementary negative strand RNA. Negative strand RNA bears two putative promoters: one is the genomic promoter (3′ end of negative sense RNA) for the synthesis of positive sense RNA and the other is the sub-genomic promoter for the synthesis of sub-genomic RNA. Transcription at different viral promoters has to be regulated to maintain the correct stoichiometry of positive sense, negative sense and sub-genomic intermediate RNA. Host proteins, binding at viral promoters as components of the viral replicase complex, help in the regulation of molecular switches responsible for maintaining viral RNA stoichiometry, and their temporal synthesis.

Viruses bear relatively compact genomes, encoding a limited number of proteins and, therefore, rely on host factors to establish replication in the infected cell. Being obligatory intracellular parasites, viruses have to subvert the biosynthetic pathways of the host cell. Constant interactions between the virus and its host during the process of co-evolution have shaped the anti-viral immune system of the host and, in turn, the capability of viruses to manipulate host control mechanisms to facilitate their propagation (Stebbing and Gazzard, 2003; Fermin and Tennant, 2018). Classical scientific approaches to understanding the molecular basis of such virus-host interactions involve analysis of individual gene or protein targets and study of their functional significance. However, these approaches have not been sufficient to address the challenges of the host-pathogen interface. System biology tools provide a multidimensional approach for a comprehensive view of the biological system at molecular network levels. High throughput genomic and proteomic studies, such as siRNA and microRNA screens, and microarrays have greatly expanded our understanding of virus-host networks. Advances in several tools for data acquisition, processing, integration and computation provide rapid, and promising strategies for the development of new therapies for infectious diseases (Peng et al., 2009; Aderem et al., 2011; Xue and Miller-Jensen, 2012).

Studies on several viruses reveal that viral proteins or viral RNA interact with host proteins to regulate viral replication. Previous studies on the HEV ORF1 and ORF2 interactome showed the involvement of factors associated with different biological processes, such as ubiquitin proteasome system, innate immunity and RNA metabolism (Ojha and Lole, 2016; Subramani et al., 2018). In another study by Paingankar et al. host factors have been found to interact with the untranslated region on HEV genomic RNA (Paingankar and Arankalle, 2015). However, no conclusive studies have been carried out to analyze host factors present in HEV replicase complex. Also, host factors interacting with promoter sequences on HEV negative sense RNA have not been explored so far. We hypothesized that the set of host proteins interacting with HEV polymerase protein and HEV RNA at its promoter region must play crucial roles in tightly regulating the synthesis of viral RNAs. The host factors may form differential replicase complexes along with HEV RNA and polymerase protein at the promoter region. Therefore it was interesting to find out the proteins which bind at both the genomic and sub-genomic promoters on negative sense RNA of HEV and those which bind to only one specific promoter along with the RdRp. Proteins binding at both the promoters may act as primary transcription factors while, the differential proteins may guide RdRp for where to bind and which strand to synthesize at a given time. We thus believe that host factors interacting with HEV polymerase and promoters play crucial roles in regulating the molecular switches in HEV replication.

In order to better understand the HEV-host interface, we identified liver cell proteins interacting with the HEV polymerase and HEV putative promoters and generated a protein-protein interaction network. We further utilized a bioinformatics approach to analyze these interaction networks and assess their significance. Our study identified host proteins related to cellular processes like RNA metabolism, unfolded protein response, stress granules, secretory vesicles, endoplasmic reticulum protein processing, and innate immune pathways. HNRNPK and HNRNPA2B1 proteins were found to be interacting with both HEV promoters and HEV RdRp. We demonstrated the *in vitro* binding of HEV promoters and HEV RdRp with HNRNPK and HNRNPA2B1, confirming the validity of interactions obtained by mass spectrometry.

MATERIALS AND METHODS

Virus Replicon and Cells

Infectious replicon of Sar55 strain of genotype 1 of HEV (pSK-HEV2) and a subclone of a human hepatoma cell line Huh7 S10-3

TABLE 1 | List of primers used in the study.

Sr. No.	Primer name	Sequence (5′-3′)	Amplified construct
1	pTandem-F	GCTAGCCAAGCGCTTGGTTAAC	pTandem
2	pTandem-R	CCATGGTGGCATATCTCC	
3	RdRpFLAG_pTF	TTAAGAAGGAGATATGCCACCATGGCGCCACCATGGACTACAAAG	RdRp
4	RdRpFLAG_pTR	TGGTGATGGTGTGTCATTCCACCCGACACAG	
5	RdRpFLAG_ pTJncF	TCGGGTGGAATGACACACCATCACCACCATC	Junction region
6	RdRpFLAG_ pTJncR	TTTGTTCCATGTTGTTTAAACTTTCAAAGGAAAACCAC	
7	HNRNPK_F	TTTCCTTTGAAAGTTTAAACATGGAAACTGAACAGCCAG	hnRNPK
8	HNRNPK_R	TAACCAAGCGCTTGGCTAGCTTACAGATCCTCTTCTGAGATG	
9	HNRNPA2B1_F	TTTCCTTTGAAAGTTTAAACATGGAGAGAGAAAAGGAACAGTTC	hnRNPA2B1
10	HNRNPA2B1_R	TCTGAGATGAGTTTTTGTTCGTATCGGCTCCTCCCACC	
11	RdRp_F	ATCCGAATTCATGGACTACAAAGACGATGACGACAAGGGTGGCGAAATTGGCCACCA	pcDNA_FLAG-RdRp
12	RdRp_R	CGGAGGGATCCTCATTCCACCCGACACAGAATTGA	
13	G promoter_F	GCAGACCACATATGTGGTCGATGCCATGGA	G promoter
14	G promoter_R	GACTACTAATACGACTCACTATAGGGAGAAAAGGCCTAACTACC	
15	Sg promoter_F	AGTCAGTGAAGCCAGTGCTTGACCTGACAAATTCAATTCTGTGT	Sg promoter
16	Sg promoter_R	GACTACTAATACGACTCACTATAGGGCGGGCAGCATAGGCAGAA	

which is permissive for the replication of HEV infectious clone was obtained from Dr. Suzanne U. Emerson, NIH, Bethesda, MD, United States. Cells were maintained in DMEM GlutaMAX (Invitrogen) medium supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and 100 U/ml penicillin and 100 mg/ml streptomycin (Sigma).

Construction of Recombinant Plasmids

Coding sequence of HEV RNA dependent RNA polymerase (RdRp) was amplified from pSK-HEV2 replicon. RdRp coding sequence was cloned in pcDNA 3.1/myc-His (-) mammalian expression vector in such a way that it will be expressed as FLAG tagged RdRp at its N terminal. This clone has been designated as pcDNA_FLAG-RdRp. Primers used for the amplification have been listed in **Table 1**.

To confirm interaction of HEV RdRp with hnRNPK/hnRNPA2B1, FLAG tagged RdRp and c-Myc tagged hnRNPK or hnRNPA2B1 encoding sequence was cloned in pTandem vector (Clontech) under CMV promoter and IRES, respectively. The constructs have been designated as pTandem_FLAG-RdRp_Myc-hnRNPK or pTandem_FLAG-RdRp_Myc-hnRNPA2B1. Primers used for the cloning are mentioned in the **Table 1**.

Preparation of HEV Promoter RNA Baits

Sequences coding for the putative genomic promoter (G promoter: nt 1 to 139 on positive sense RNA) and putative sub-genomic promoter (Sg promoter: nt 5051 to 5200 on positive sense RNA) of HEV genotype 1 were PCR amplified from pSK-HEV2 replicon. The primers for the amplification of template were designed with T7 promoter sequence in such a way that the RNA of anti-sense orientation is generated. Primers used for the amplification have been listed in **Table 1**. PCR products were used as templates for the synthesis of RNAs bearing respective promoter sequences. *In vitro* RNA was synthesized by using MEGAscript kit (Ambion) following the manufacturer's instructions. Biotinylated *in vitro* transcribed RNAs were prepared using 5 mM rATP, 5 mM rGTP, 5 mM rUTP, 4.5 mM rCTP, and 0.5 mM of biotin-14 CTP (Invitrogen) in the rNTP mix for the *in vitro* transcription reaction. For synthesizing non-biotinylated RNAs of respective regions, total 5 mM rCTP was added instead of biotin-14-CTP. Unincorporated nucleotides were removed by purifying the RNA using phenol-chloroform precipitation method. Purified RNAs were visualized on 2% agarose gel.

RNA Affinity Chromatography

A total of 2 μg of each of biotinylated RNA corresponding to either HEV putative genomic or sub-genomic promoter were coupled with M280 streptavidin dynabeads (Invitrogen) in the presence of nucleic acid binding and washing buffer (B&W buffer: 10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 2M NaCl) for 15 min at room temperature on a rotator. Before RNA binding step, beads were washed with solution A (DEPC-treated 0.1 M NaOH, 0.05M NaCl) followed by solution B (DEPC treated 0.1 M NaCl) to remove RNase. Huh7 S10-3 cells were harvested at ∼80% confluency in the lysis buffer (10 mM Tris–Cl, pH 7.4, 10 mM KCl, 2 mM $MgCl_2$, 0.5% Tritin X-100 with protease inhibitor cocktail). The lysate was prepared by centrifugation at 12000 rpm at 4°C for 20 min. The bound RNA-beads complexes were incubated with Huh7 S10-3 cell lysate pre-cleared with 20 μl beads for 1 h at 4°C. Cell lysate and RNA-beads complexes were mixed and incubated together at 4°C on a rotator for 2 h. Bound complexes were washed with B&W buffer and proteins bound to RNA were eluted in 100 μl elution buffer (50 mM Tris–Cl, pH 7.4, 0.2% SDS, 0.1% Tween 20). Eluted proteins were loaded on 12% SDS PAGE followed by silver staining for visualization of protein bands using ProteoSilver staining kit (Sigma). Eluates from three independent RNA affinity chromatography experiments were

pooled together and subjected to protein identification by mass spectrometry.

Immunoprecipitation

pcDNA_FLAG-RdRp construct was transfected into Huh7 S10-3 cells using Lipofectamine 3000 (Invitrogen) transfection reagent. After 48 h post transfection, cells were harvested and lysed in IP lysis buffer (50 mM Tris–Cl pH 7.4, 150 mM NaCl, 1% IGEPAL and protease inhibitor cocktail). Protein G dynabeads (30 μl; Invitrogen) were used for each immunoprecipitation experiment. Next, 4 μg of either rabbit anti FLAG antibody or a non-specific isotype IgG was incubated with washed dynabeads for 30 min at room temperature on a rotator. The cell lysate was incubated with antibody plus dynabeads complex for 2 h at 4°C on a rotator. Three washes with IP lysis buffer were given, followed by a final wash with PBS. Interacting proteins were eluted in 50 μl of elution buffer (50 Mm Tris–Cl pH 7.4, 0.2% SDS, and 0.1% Tween 20). Eluted proteins were loaded on 12% SDS PAGE followed by silver staining for visualization of protein bands using ProteoSilver staining kit (Sigma). Eluates from three independent immunoprecipitation experiments were pooled together and subjected to protein identification by mass spectrometry.

Liquid Chromatography Quadrupole Time-of-Flight Mass Spectrometry (LC-QTOF/MS)

Hepatitis E virus RNA-dependent RNA polymerase and RNA interacting proteins isolated by immunoprecipitation and RNA affinity chromatography, respectively, were subjected to liquid chromatography-mass spectrometry (LC-MS) analysis. Total 30 μg of each of the protein samples was acetone precipitated, and the protein pellets were dissolved by adding 10 μl of 8 M urea, and the volume was brought to 15 μl with water. Samples were then reduced by the addition of 1.5 μl of 100 mM DTT and heated at 90°C for 15 min. The sample was cooled and alkylated by adding 1.5 μl of 200 mM IAA and incubated in the dark at RT for 15 min. 82 μl of ABC was added, and proteins were digested by adding 1 μl of 1 mg/ml trypsin protease and incubating at 37°C for 16 h. The reaction was stopped by the addition of 1–2 μl of concentrated TFA. Then peptides were dissolved in 0.1% TFA, 5% ACN in water for MS-analysis. Agilent 1260 infinity HPLC-Chip/MS system is a microfluidic chip-based technology was used for peptide enrichment and separation. Charged peptides from HPLC-Chip system were directly infused into mass-spectrometer for detection. Agilent Mass Hunter software was used for data acquisition and analysis of total ion chromatograms. Protein searches were carried out using Morpheus software. Protein identification was performed with the following criteria: (a) Trypsin digested peptides with 2 missed cleavages allowed, (b) peptide tolerance <10 ppm, (c) >2 unique peptides, (d) FDR <1%. Fasta files for Human Proteome database were downloaded from the UniProt was used for protein searches. Proteins found in respective negative control sample were eliminated from the dataset to remove non-specific interactions.

Construction of the Molecular Interaction Network

All the experimentally derived data sets were used to generate HEV-host proteins interaction network by using "Cytoscape version 3.6.1" (Shannon et al., 2003). To analyze the interaction among host proteins, IntAct protein interaction database was used. Only interactions confirmed by direct physical binding were considered for plotting inter protein interaction map. Topological parameters and central measures of the network were calculated by using a network analyzer tool in Cytoscape. Human protein-protein interaction analysis was also performed by using STRING database. In all the networks and throughout the study, we have used NCBI gene names to represent the proteins in order to have a consensus in protein accession. Corresponding gene names, protein names, and Uniprot protein identifiers have been listed separately (**Supplementary Tables 1a–c**).

Gene Ontology Analysis

Gene ontology "GO" analysis was carried out by using web-based tools like Panther (Gene Ontology Consortium's web tool), Gprofiler, STRING, and Enricher. To analyze the enrichment of specific pathway, KEGG annotation database was used. Gene set and pathway enrichment analysis was validated by Fisher's exact *t*-test. To control false discovery rate (FDR) Benjamini and Hochberg multiple test correction was used; p-value ≤ 0.05 was considered significant. The corrected p-value for each GO term has been given in the section "Results."

Co-immunoprecipitation for Validation of Interactions *in vitro*

Huh7 S10-3 cells expressing FLAG-RdRp and Myc-hnRNPK/hnRNPA2B1 were harvested at 48 h post transfection and resuspended in IP lysis buffer (50 mM Tris–Cl, pH 7.5, 150 mM NaCl and 1% IGEPAL detergent) for half an hour at 4°C. Cell lysate was prepared by centrifugation at 12000 rpm for 30 min. Clear supernatant was mixed with anti Myc antibody or isotype IgG antibody attached to protein G dynabeads (Invitrogen) for 2 h. Three washes of IP lysis buffer were given to remove non-specific interactions. Interacting RdRp-host cellular protein complexes were eluted by using elution buffer (50 mM Tris–Cl, pH 7.4, 0.2% SDS, 0.1% Tween 20). Eluted proteins were subjected to western blot to confirm RdRp-host protein interaction using anti-FLAG antibody.

RNA Immunoprecipitation and RT PCR

Huh7 S10-3 cells were harvested at ∼80% confluency in cold DPBS. Cells were lysed in lysis buffer (10 mM Tris–Cl, pH 7.4, 10 mM KCl, 2 mM $MgCl_2$, 0.5% Tritin X-100 with protease inhibitor cocktail) for 20 min at 4°C followed by centrifugation at 12000 rpm at 4°C for 10 min. Clear supernatant was incubated with *in vitro* transcribed RNAs of either putative genomic or sub-genomic promoters of HEV for 2 h at 4°C on rotator. 25 μl of protein G dynabeads were incubated with 4 μg of anti-hnRNPK antibody produced in rabbit (Gene Tex) or anti-hnRNPA2B1 antibody produced in rabbit (Gene Tex) or a non-specific isotype

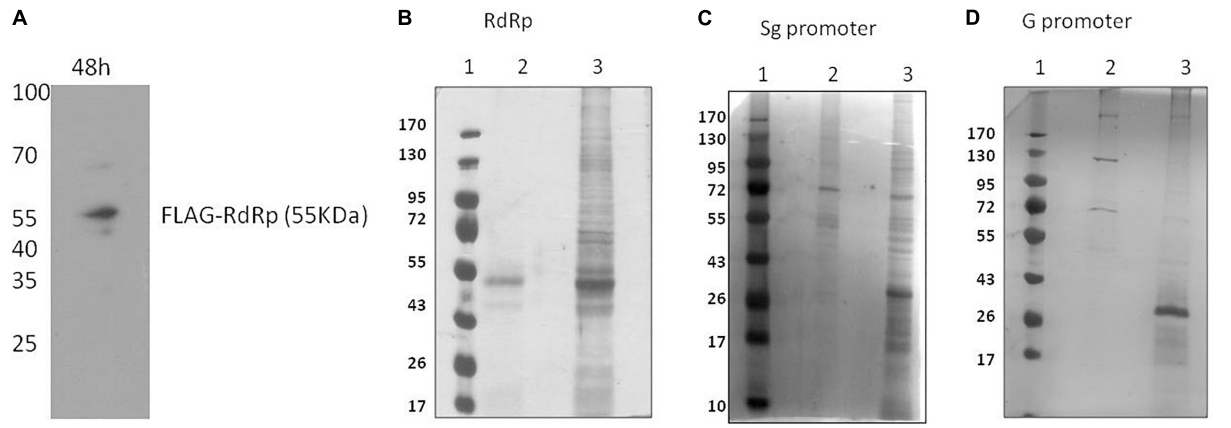

FIGURE 1 | Identification of HEV interacting host proteins. **(A)** pcDNA_FLAG-RdRp construct was transfected into Huh7 S10-3 cells. Post 48 h of transfection cells were harvested and checked for expression of RdRp by western blot using anti-FLAG antibody. **(B)** Mock-transfected Huh 7 S10-3 cells or cells transfected with pcDNA_FLAG_RdRp (expressing FLAG-tagged RdRp) were harvested after 48 h of transfection. Immunoprecipetation was performed by anti-FLAG antibody or an isotype control antibody. RdRp interacting host proteins were eluted with protein G dynabeads and analyzed on SDS PAGE followed by silver staining. **(C)** HEV sub-genomic promoter (Sg) RNA interacting cellular proteins were pull down by using RNA affinity chromatography. Biotinylated HEV sub-genomic promoter RNA were immobilized on M280 streptavidin dynabeads. RNA immobilized beads were incubated with cell lysate of Huh7 S10-3. Interacting host proteins were eluted and checked on SDS PAGE followed by silver staining for visualization. Non-biotin RNA of the sub-genomic promoter was taken as control. **(D)** HEV genomic promoter (G) RNA interacting cellular proteins were pull down by using RNA affinity chromatography. HEV genomic promoter RNA interacting host proteins were eluted and checked on SDS PAGE followed by silver staining. For **(B–D)** lane 1, protein molecular weight ladder; lane 2, negative control pull-down; lane 3, experimental test pull down.

IgG antibody in antibody binding buffer (PBS + 0.0.2% Tween 20) for 35 min at room temperature. Beads bound antibody complexes were added to previously incubated lysate plus RNA complex for 1 h at 4°C on rotator. Three washes of lysis buffer were given followed by a final wash of PBS. Complexes were eluted with 80 μl of elution buffer. Elutes were processed for total RNA isolation using phenol-chloroform method. The RNA was transcribed into cDNA using reverse transcriptase polymerase chain reaction (RT PCR) with Superscript III first strand synthesis kit followed by PCR amplification using Platinum taq DNA polymerase (Invitrogen) using specific primers for the detection of promoter regions. The PCR product was visualized on 2% Agarose gel.

RESULTS AND DISCUSSION

Identification of HEV-Interacting Host Proteins

To pull down HEV RdRp-binding proteins, the HEV RdRp protein was expressed as a FLAG-tagged recombinant protein in Huh7 S10-3 cells. pcDNA_FLAG-RdRp construct was transfected in the cells, and the expression of RdRp was confirmed at 48 h post-transfection by western blotting using an anti-FLAG antibody (**Figure 1A**). Protein G magnetic beads were used to pull down specific interactions using the anti-FLAG antibody in a classical immunoprecipitation experiment from the RdRp-expressing cell lysate. The eluted complexes were subjected to 12% SDS-PAGE followed by silver staining with the ProteoSilver kit (Sigma) for visualizing protein bands (**Figure 1B**). Mock-transfected Huh7 S10-3 cell lysate was taken as control to detect the non-specific interactions. For the pull down of specific HEV RNA-interacting proteins, biotinylated HEV putative sub-genomic (Sg), or putative genomic promoter (G) RNA was synthesized *in vitro*. *In vitro* synthesized Sg RNA includes the recently mapped intragenomic promoter region regulating the Sg RNA transcription which is conserved across all HEV genotypes (Ding et al., 2018). Genomic promoter region has not been yet mapped functionally, however, based on the HEV RdRp binding studies reported previously with the 3′UTR of HEV anti-sense RNA, we have designed the putative G promoter RNA (Mahilkar et al., 2016). RNA affinity chromatography was performed using streptavidin magnetic beads with Huh7 S10-3 cell lysates incubated with HEV RNAs *in vitro*, and the elutes were subjected to protein identification. Non-biotin RNAs of sub-genomic and genomic promoters were taken as control. The eluted complexes were subjected to 12% SDS-PAGE followed by silver staining (**Figures 1C,D**). A distinct banding pattern could be observed in specific pull-down experiments as compared to the negative control. We further employed liquid chromatography quadrupole time-of-flight mass spectrometry (LC-QTOF/MS) to identify the interacting proteins. Proteins represented by at least two unique peptides and having less than 1% FDR were considered for analysis Proteins represented in respective negative control data were eliminated. The list of proteins identified to be interacting with HEV is shown in **Table 2** and **Supplementary Tables 1a–c**.

Construction and Analysis of HEV-Protein Interaction Network

A combined list of proteins interacting with HEV promoters and RdRp was generated, and a protein network named the HEV-host

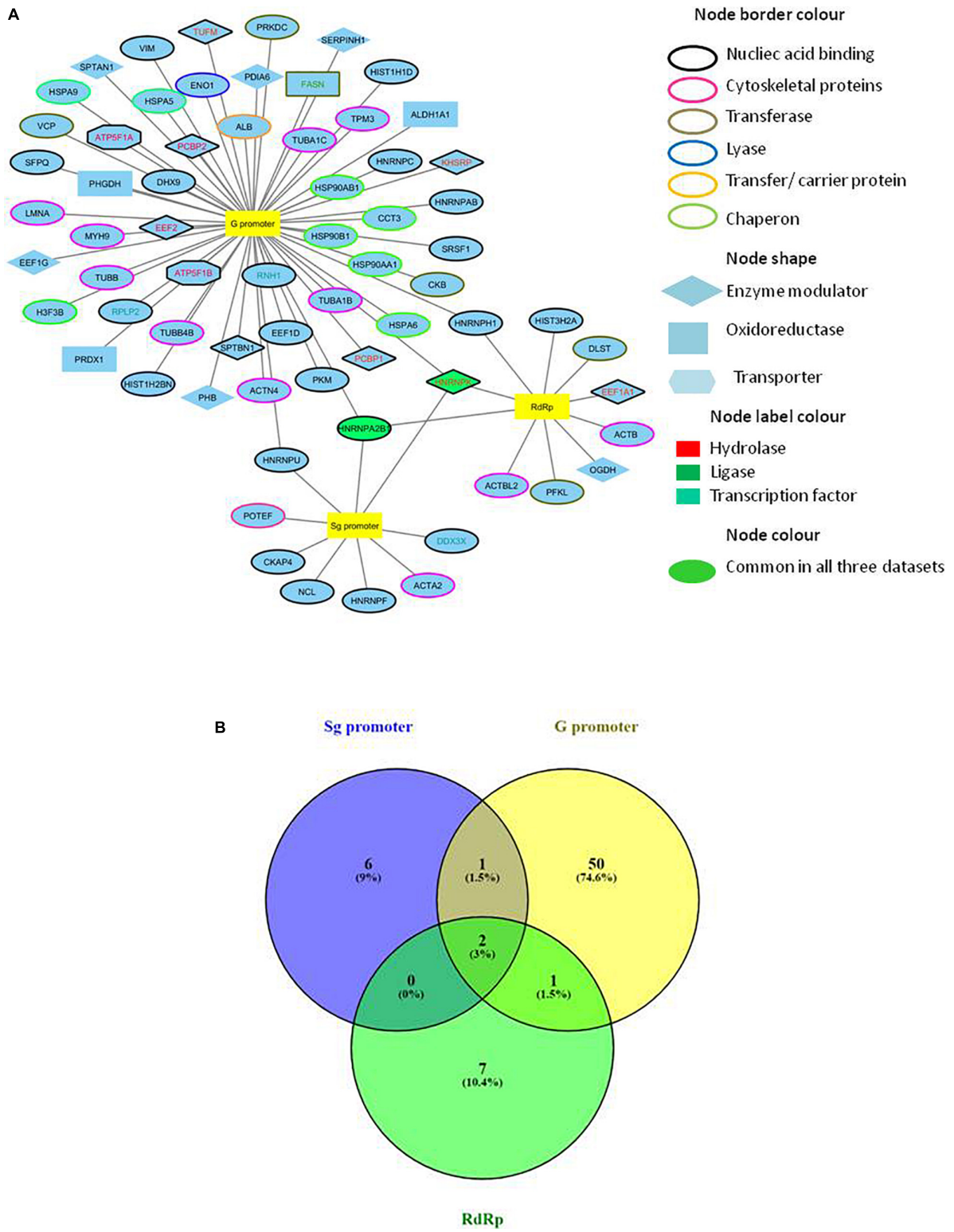

FIGURE 2 | Construction of HEV-host interaction network. **(A)** HEV-host interaction network: Interaction map of HEV sub-genomic promoter (Sg), genomic promoter (G) and RdRp with interacting host proteins constructed in Cytoscape 3.6.1. Proteins were classified on the basis of their protein class by Panther gene ontology tool. The corresponding symbols indicating different protein classes have been mentioned on the figure. **(B)** Venn diagram comparing HEV interacting host proteins with different HEV components. Blue, yellow and green colors indicate proteins interacting with the sub-genomic promoter, genomic promoter, and RdRp, respectively. Common proteins within the data sets have been indicated in the colored intersections. Proteins have been represented as the respective NCBI gene names.

TABLE 2 | List of HEV genomic promoter, sub-genomic promoter, and RdRp interacting host proteins.

HEV component	Gene symbol of interacting protein
Sub-genomic promoter	HNRNPU, HNRNPA2B1, HNRNPF, NCL, CKAP4, HNRNPK, DDX3X, ACTA2, and POTEF
Genomic promoter	TUBA1B, TUBA1C, HSP90AA1, HSP90AB1, TUBB, PKM, CKB, FASN, ENO1, PRDX1, HSP90B1, PDIA6, RPLP2, HSPA5, PHGDH, KHSRP, PCBP2, CCT3, ALB, MYH9, VCP, SERPINH1, SPTBN1, ALDH1A1, VIM, SPTAN1, ATP5F1A, PRKDC, LMNA, HSPA9, PHB, SRSF1, DHX9, TUBB4B, HNRNPH1, HNRNPK, HNRNPA2B1, ACTN4, TUFM, HSPA6, HNRNPU, HIST1H1D, EEF1D, TPM3, H3F3B, HNRNPC, EEF2, RNH1, SFPQ, EEF1G, PCBP1, HNRNPAB, ATP5F1B, and HIST1H2BN
RNA dependent RNA polymerase	HNRNPA2B1, HNRNPK, DLST, HNRNPH1, OGDH, HIST3H2A, ACTBL2, EEF1A1, ACTB, and PFKL

Proteins have been represented as NCBI gene names.

interaction network was constructed using "Cytoscape version 3.6.1" (**Figure 2A**). A list of total 70 HEV-interacting proteins was generated, amongst which two proteins, HNRNPA2B1 and HNRNPK, were found to be present in all the three data sets. One more protein (HNRNPH) was found to be shared between RdRp and G promoter data, while another protein (HNRNPU) was common between the Sg promoter and G promoter besides HNRNPA2B1 and HNRNPK. However, about 94% of the proteins were found to be specifically interacting with only one of the HEV partners in the study (**Figure 2B**).

We then searched for the proteins interacting with other host proteins within our data to plot the protein-protein interaction network. We used advanced search options of IntAct database by providing "direct interactions" filter to find experimentally proven protein-protein interactions within our data and plotted a second network named HEV-host PPI network using Cytoscape 3.6.1 (Orchard et al., 2013). IntAct database reports evidences like pull down, X-ray crystallography, functional assays, etc under experimentally proven criteria of interaction prediction. IntAct tool sources the molecular interaction data from several curated databases like MINT, Uniprot, molecular connections, EMBL-EBI, and DIP. Primary interactions revealed through mass spectrometry in this study have been shown with black edges, while the secondary protein-protein interactions revealed through IntAct have been shown in red (**Figure 3**). We then calculated the topological parameters of the generated PPI network to access its modularity using the network analyzer tool of Cytoscape. It is revealed that 70 nodes representing HEV-host interactions were connected via a total of 141 edges. The average degree centrality, average path length distribution, and the clustering coefficient of the network were observed to be 0.748, 2.365, and 0.348, respectively. The topological parameters were compared with HEV-human protein interaction networks previously reported in literature which validated the significance of HEV-host protein interaction network constructed in our study (**Supplementary Table 2**). Amongst the proteins present in our data, PFKL, SERPIN, HNRNPH, HSP90AB1, and TUBB have been previously reported to interact with HEV macrodomain (Ojha and Lole, 2016). Besides, PCBP1 and EEF1A1 have been reported to interact with HEV ORF2, while DHX9, HNRNPC, and HNRNPK have been reported to interact with HEV non-coding regions on the sense strand (Paingankar and Arankalle, 2015; Subramani et al., 2018). This confirms the authenticity of HEV-specific interactome found in our study.

We also used STRING database to generate the inter-protein interaction network from our data (**Figure 4** and **Supplementary Table 3**) (Szklarczyk et al., 2015). This analysis gave us a more intense multidimensional network as, along with the experimentally determined interactions, those predicted from gene fusion; co-expression, homology, and text mining were also considered. The observed number of edges for the network (487) was significantly higher than the expected number of edges (132) for the given number of nodes (67), implying that there are more interactions than expected. The result suggested that the proteins in our data dispaly more interactions than expected for a random set of proteins. Such enrichment indicates that the proteins are at least partially biologically connected as a group, highlighting the significance of the HEV-host network reported in this study. This also indicates the probability of isolating HEV-specific protein complexes gathered at the HEV replication site.

Gene Ontology Annotation

To investigate the cellular components or pathways that have been enriched in the HEV-host protein interaction network, we performed gene ontology analysis (GO annotation) of the data using various web-based tools. The GO annotation resulted in classification of the proteins based on their functional clusters or GO categories. The enrichment analysis performed using Panther (Gene Ontology Consortium's web tool) (**Supplementary Figures 1A–C, 2**), Gprofiler (**Supplementary Figure 3**), STRING (**Supplementary Figure 4**), and Enricher was in agreement and showed a significant enrichment of similar GO terms. FDR <0.05 was set as a statistical threshold for GO analysis. Enrichment of proteins in three different categories, namely biological processes, molecular function, and cellular component, was performed using Enricher (Chen et al., 2013; Kuleshov et al., 2016). Proteins were arranged according to the combined enrichment score, which is a combination of the *p*-value and z-score calculated by multiplying the two scores. The *p*-value is the probability of any gene belonging to any set and is calculated by using exact Fisher's test. The Z-score is calculated by using a modified Fisher's exact test and assesses the deviation from the expected rank. The combined score provides a compromise between the available methods for multiple test corrections to control the FDRs (Kuleshov et al., 2016).

Enrichment of GO terms in biological process category revealed the enrichment of proteins related to mRNA splicing (GO: 0000380; *p*-value: 3.5×10^{-3}), response to unfolded proteins (GO: 0006986; *p*-value: 3.6×10^{-6}), nucleic acid metabolic process (GO: 0090304, *p*-value: 1.3×10^{-6}), and regulation of protein processing (GO: 0010954, *p*-value:

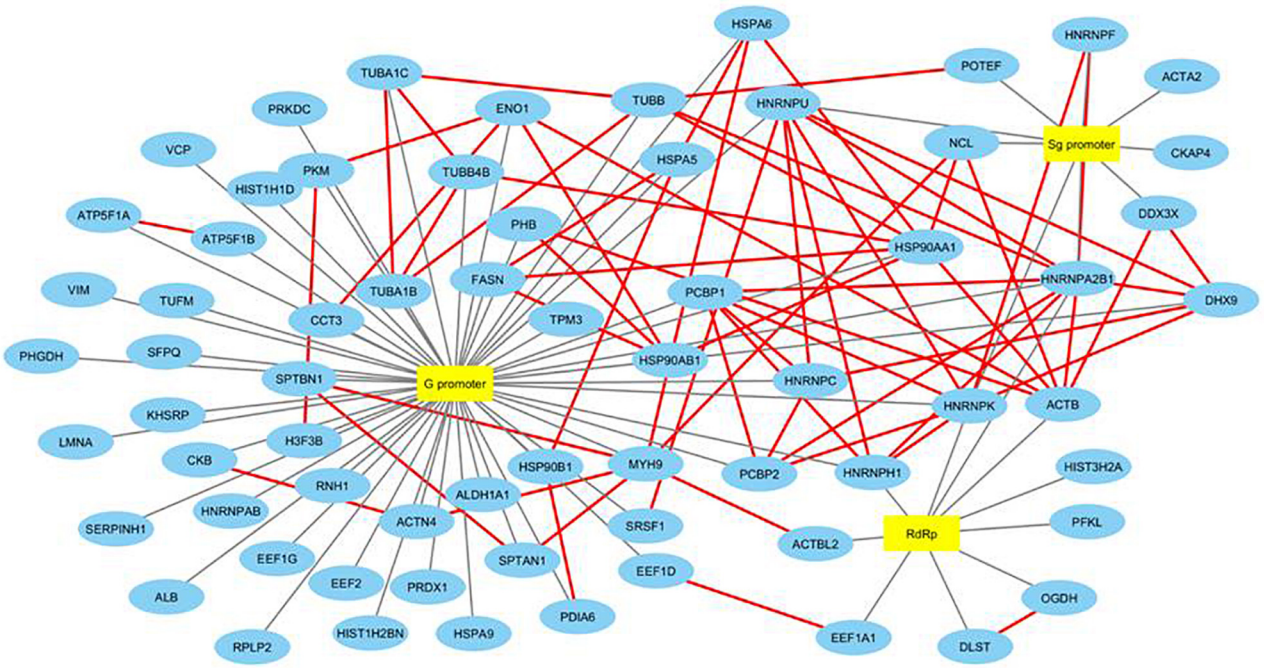

FIGURE 3 | Construction and analysis of HEV-host PPI network. Interaction map of HEV interacting host proteins further interacting with the other proteins of our data. Black edges represent interactions revealed through mass spectrometry reported in this study. Secondary protein-protein interactions among host proteins revealed through literature mining have been indicated in red colored edges. Proteins have been represented as the respective NCBI gene names.

8×10^{-3}) (**Figure 5A** and **Supplementary Table 4A**). A vital characteristic of any virus-host interaction is the manipulation of cellular gene expression in order to establish favorable cellular conditions for efficient viral replication. Eukaryotic genes are highly regulated at post-transcriptional stages, such as splicing, export, regulation of translation, subcellular localization and mRNA turnover. Viruses have often been observed to target these RNA processing stages to hijack the host RNA metabolism which is evident from the enrichment of host RNA splicing and nucleic acid metabolism related protein in our data. Such enhancement could also hint at the reliance of HEV on host systems for the regulation of viral RNA translation, transcription, and stability within the host cell (Ahlquist et al., 2003). We observed the highest number of proteins having nucleic acid-binding activity in our data, which further confirms the dependence of HEV on the host for viral RNA metabolism. We have incorporated the data of classification of proteins on the basis of protein class in the HEV-host interaction network using different color codes as indicated on the figure (**Figure 2A** and **Supplementary Figure 2**).

We also observed enrichment of stress granule proteins (GO:1903608; p-value: 3.3×10^{-2}) in our data. Stress granules are dynamic structures that are formed when cellular translational rates decline after external stresses are applied to cells. Viral infection into the host cell has been shown to induce cytoplasmic stress granule formation in the host cells due to the upregulation of stress granule proteins, such as helicase DDX3X and DHX9 (Ariumi et al., 2011; Pène et al., 2015). As stress granules regulate the cycle of mRNA turnover and gene expression, they happen to be another vital point for viruses to manipulate cellular systems. Stress granules have also been reported to play a role in promoting innate immune responses (Reineke and Lloyd, 2013). This is an additional reason why viruses must counteract the effects of such granules for efficient replication by interacting with proteins of stress granules.

Virus replication alters the normal metabolic processes of the host by interacting with components of different molecular pathways. Enrichment of GO terms in molecular function category also resulted in enrichment of proteins involved in RNA metabolism processes, which again highlights the need of the virus to interact with proteins of host RNA turnover. The enriched proteins in molecular function category belong to Translation elongation factor activity (GO: 0003746, p-value: 1×10^{-3}), RNA binding (GO: 0003723, p-value: 3.9×10^{-25}) and RNA stem loop binding (GO: 0035613, p-value: 3.4×10^{-2}).

MHC protein complex-binding proteins (GO: 0023023, p-value: 2.5×10^{-3}) have also been observed to be enriched in the molecular function category (**Figure 5B** and **Supplementary Table 4B**). The anti-viral responses of the host and the invading strategies of the pathogens have evolved concurrently for millions of years. Infecting pathogens have developed several escape strategies to cripple the immune system. Several viruses have evolved with proteins that interfere with antigen presentation and which target both MHC-I and MHC-II antigen processing pathways in order to distort the anti-viral immune response of the host. Several viruses such as HSV, Epstein-Barr virus, Bovine herpes virus and cytomegalovirus have been shown to

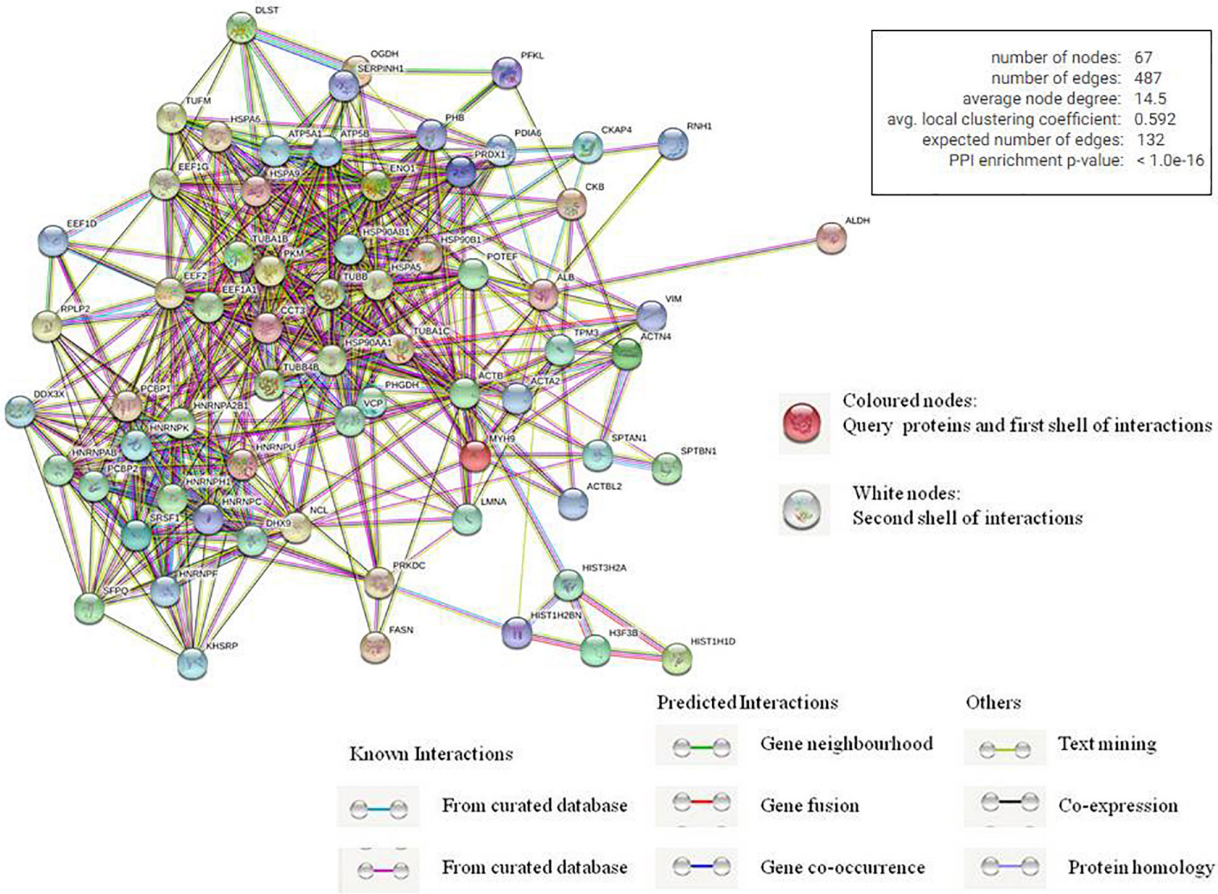

FIGURE 4 | Analysis of inter protein interaction network using STRING database. Each edge color indicates a different method of protein-protein interaction prediction as indicated below the figure.

have evolved strategies to combat MHC-mediated host immunity (Yewdell and Bennink, 1999; Røder et al., 2008). However, there are no literature reports of HEV interactions with MHC molecules. In the HEV interactome reported in this study, we observed the presence of MHC-interacting proteins, such as HSP90AA1 and PKM, which could be explored further for their ability to alter host immune response in the context of HEV infection.

Enrichment analysis of GO terms according to cellular component enhanced the representation of GO terms, such as ribonucleoprotein granules (GO: 0035770, p-value: 4×10^{-8}), secretory granule lumen (GO: 0034774, p-value: 1×10^{-9}) and endocytic vesicle lumen (GO: 0071682, p-value: 3.8×10^{-2}) (**Figure 5C** and **Supplementary Table 4C**). HEV has been reported to enter liver cells through receptor-mediated endocytosis (Kapur et al., 2012). In agreement with this, our data also suggest enrichment of proteins belonging to endocytic vesicle lumen. HEV replication takes place in the cytoplasm. Along with the replicase complex, HEV RdRp has been shown to localize to the endoplasmic reticulum (ER) membrane (Rehman et al., 2008). A recent report also concluded that ORF1 polyprotein co-localizes with the markers of ER-Golgi intermediate compartment, suggesting the involvement of secretory pathway during replication (Szkolnicka et al., 2019). Previous studies have also indicated that HEV forms membrane-associated particles in the cytoplasm by means of budding into intracellular vesicles. HEV exploits the multivesicular body pathway to release infectious virion particles outside the cell through the cellular exosomal pathway (Nagashima et al., 2014).

Pathway Enrichment Analysis

To understand the cellular pathways targeted by HEV, we performed pathway enrichment analysis by using the KEGG functional annotation pathway database through Enricher. The results revealed enrichment of different pathways involving pathogenic *Escherichia coli* infection, protein processing in endoplasmic reticulum, legionellosis, spliceosome, antigen presentation and processing, gap junction, citrate cycle, IL17 signaling pathways, apoptosis, and phagosome (**Figure 6A** and **Supplementary Table 5**). Furthermore, the schematic of the entire pathway network for endoplasmic reticulum protein processing and spliceosome pathway was obtained from the KEGG pathway database (Kanehisa and Goto, 2000; Kanehisa et al., 2019).

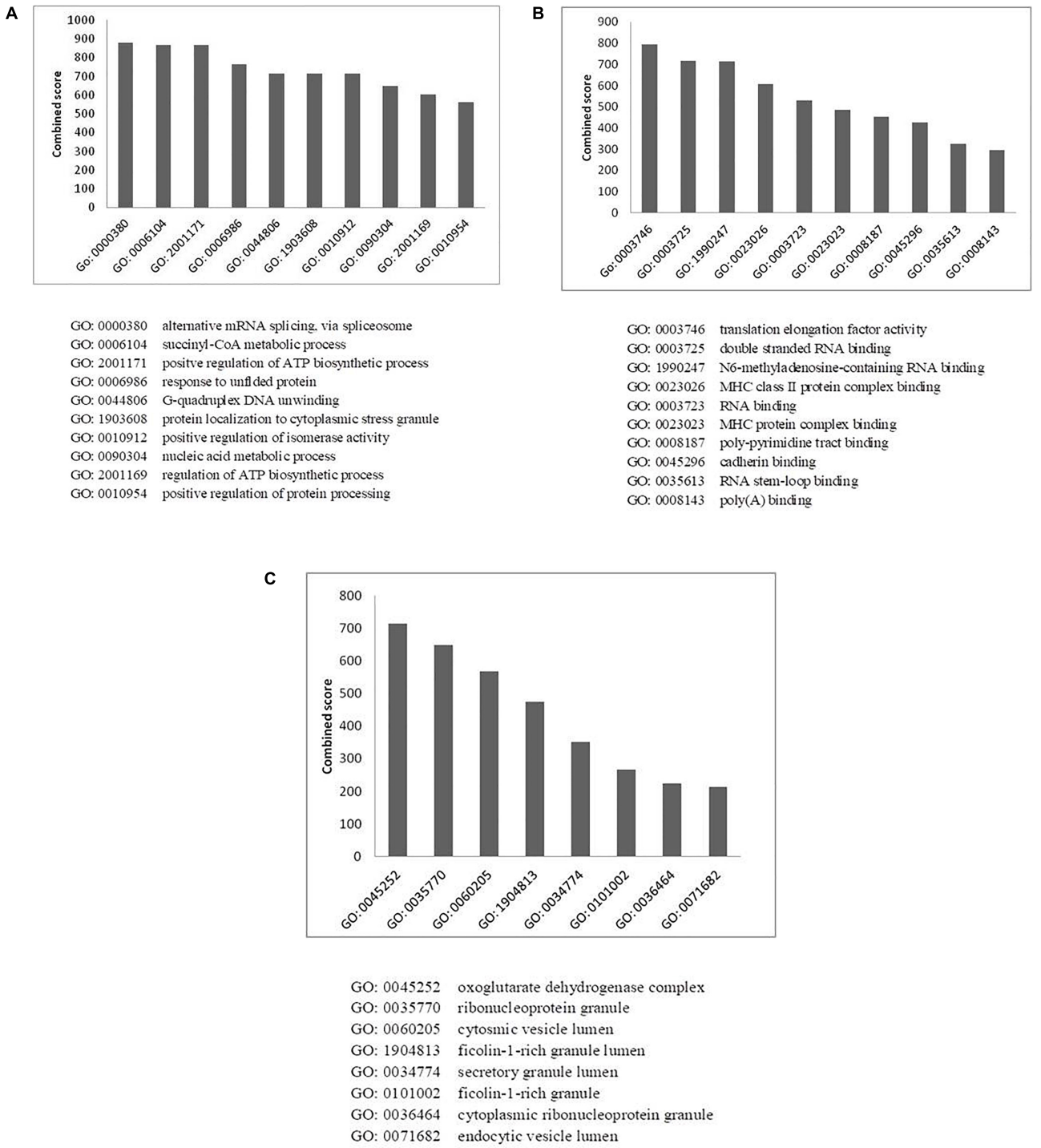

FIGURE 5 | Gene ontology analysis of HEV- host interactions based on **(A)** biological process, **(B)** molecular function, and **(C)** cellular component category. *Y*-axis represents the combined enrichment score computed using Enricher.

Protein processing in the endoplasmic reticulum pathway was enriched (p-value 1.2×10^{-5}). Seven proteins of this pathway, namely, CKAP4, HSPA5, HSP90B1, PDIs, HSP70, HSP90, and VCP were found to interact with HEV RNA and polymerase (**Figure 6B**). These factors are involved in proper folding and processing of newly synthesized proteins in the endoplasmic reticulum. As HEV virus replicates on the ER membrane and its polymerase localizes onto the ER, its interaction with

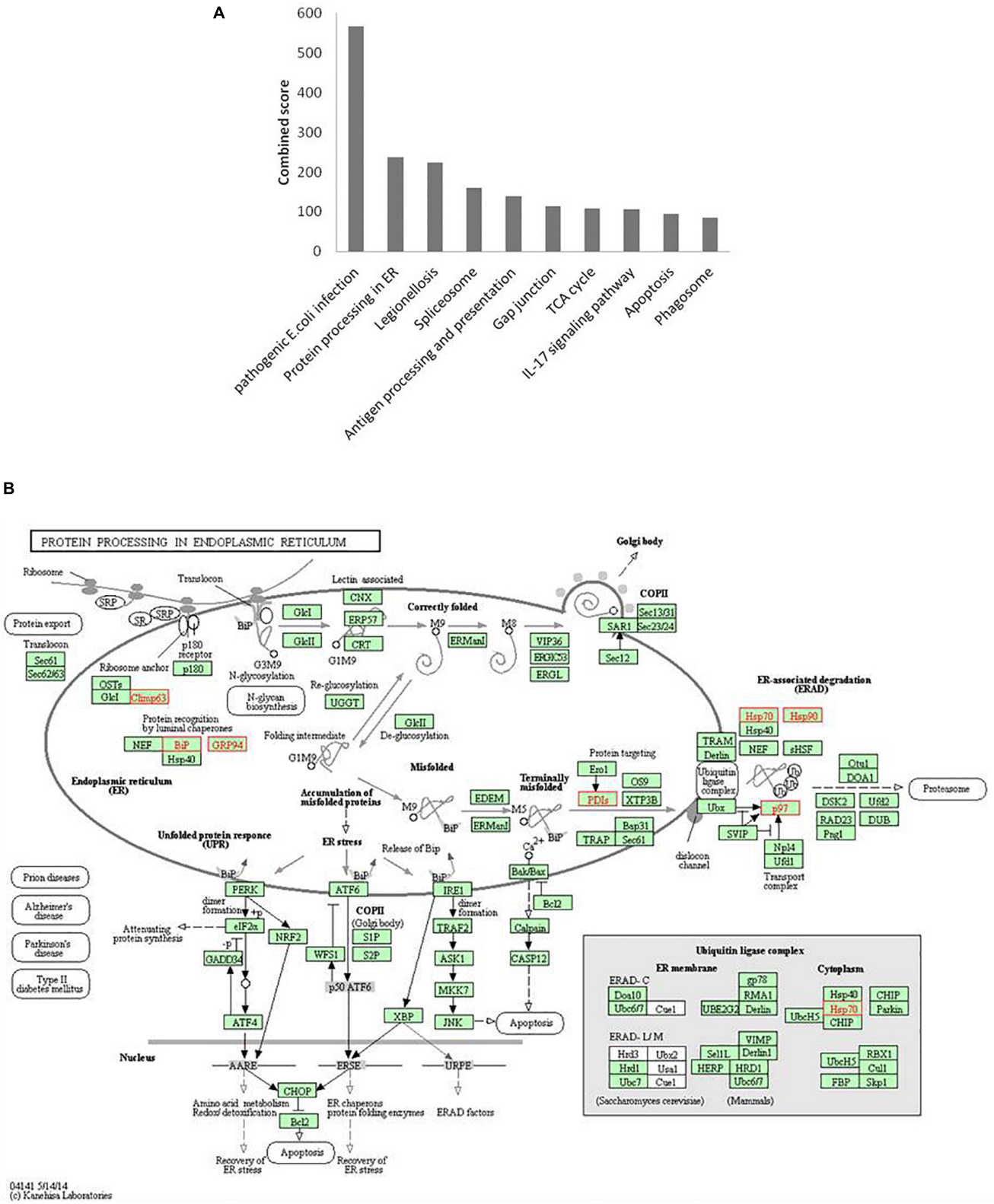

FIGURE 6 | Continued

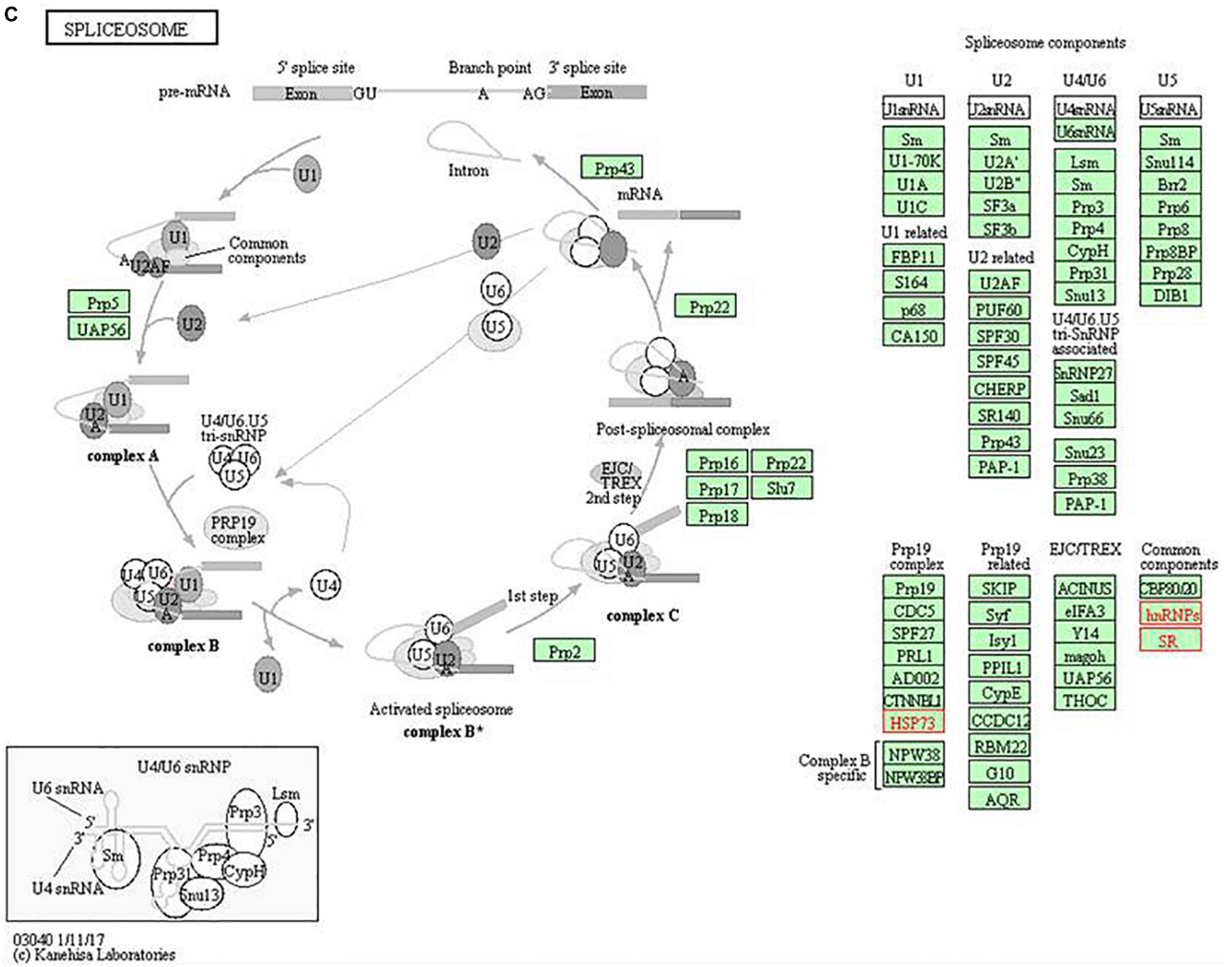

FIGURE 6 | Pathway enrichment analysis. **(A)** Graph shows the enriched pathways targeted by HEV, analyzed by KEGG functional annotation pathway database. *Y*-axis represents the combined score computed using Enricher. **(B)** Schematic representation of "protein processing in endoplasmic reticulum" pathway (imported from KEGG: map04141). **(C)** Schematic representation of the spliceosome pathway targeted by HEV host interacting proteins (imported from KEGG: map03040). Proteins interacting with HEV RNA promoter (G or Sg) or polymerase within the entire pathway are shown in red color.

proteins residing at the endoplasmic reticulum is apparent during its replication.

Proteins belonging to the heat shock protein (HSP) family are well known for their roles as chaperons in protein folding. These proteins have been observed to play similar roles in the maintenance of proper viral protein folding and stabilization (Seo et al., 2018). In many viral infections, viruses commandeer vital cellular components, leading to cellular stress. Cellular stress is often represented as unfolded protein response (UPR) in cells (Geller et al., 2012; Wang et al., 2018). Previous studies have reported that ORF3 and ORF2 of the virus induce UPR and ER stress (Surjit et al., 2007; John et al., 2011; Xu et al., 2014). In our study, we found enrichment of proteins related to processing in the endoplasmic reticulum, and UPR further confirms the dependence of HEV on the host ER machinery.

In our analyses, the spliceosome pathway was found to be enriched by p-value of 5.9×10^{-4}). Different heterogeneous nuclear ribonucleoproteins, such as HNRNPK, HNRNPA2B1, HNRNPH, HNRNPE1, HNRNPE2, HNRNPC and HNRNPU, and other spliceosomal accessory complex proteins, such as HSP73 and SR, interact with HEV (**Figure 6C**). Spliceosomal complex proteins help in the generation of stable RNA structures, while ribonucleoproteins play roles in RNA stability and transport (Will and Lührmann, 2011). Previous studies have reported the binding of HNRNPA2B1 to Influenza virus and Dengue virus RNA and explained its role in regulation of viral transcription (Paranjape and Harris, 2007; Wang et al., 2014). HNRNPK binds to the core protein of Dengue virus, whereas in Sindbis virus infection, it binds to its non-structural protein and sub-genomic RNA to regulate viral replication (Chang et al., 2001; Burnham et al., 2007; LaPointe et al., 2018). HNRNPK binds to hepatitis C virus RNA near the miR-122 binding site to facilitate its replication (Fan et al., 2015). In another study, the role played by HNRNPK in HCV virion

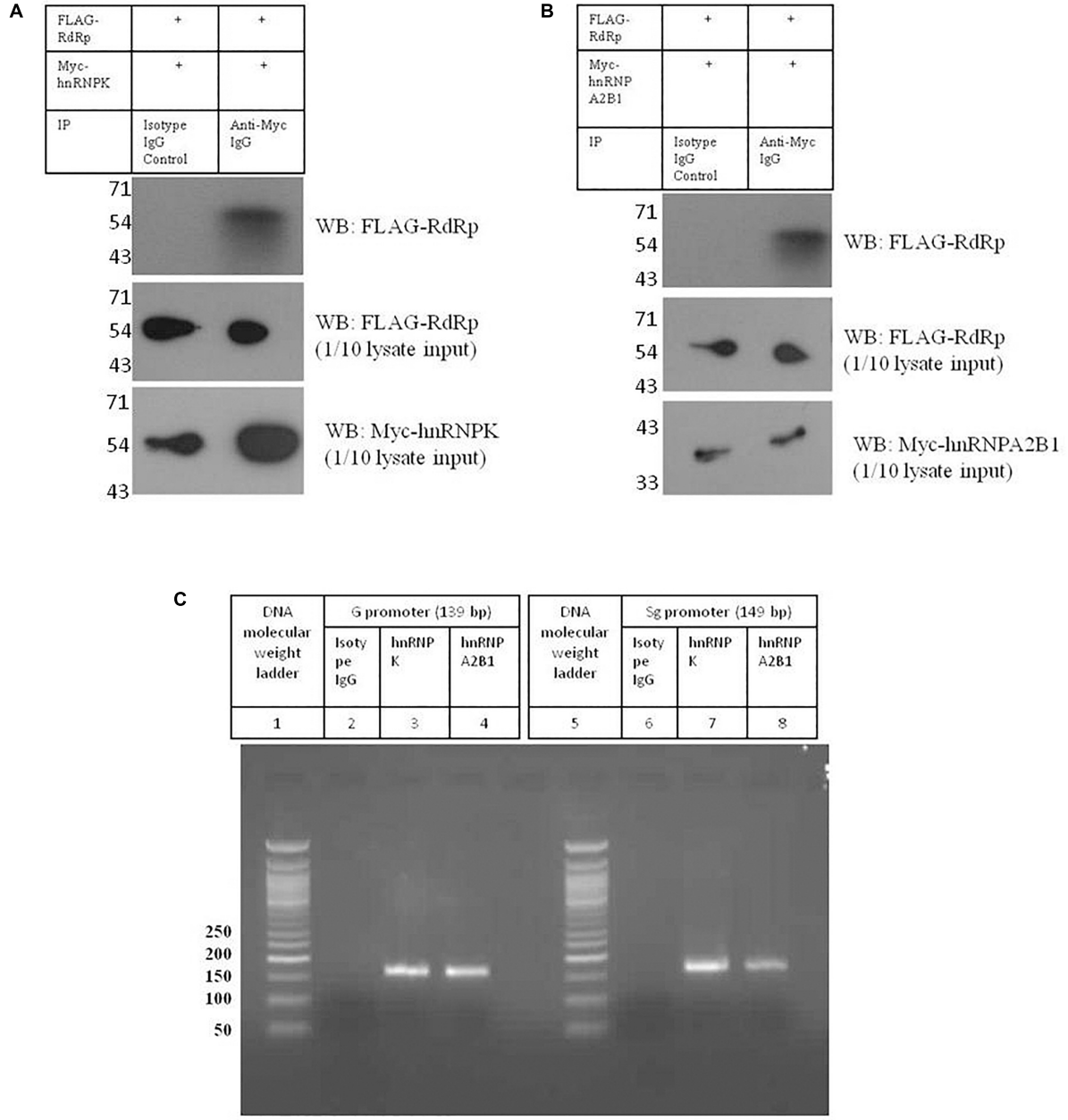

FIGURE 7 | Validation of interactions between HNRNPK and HNRNPA2B1 with HEV promoters and RdRp. **(A)** pTandem_FLAG-RdRp_Myc-HNRNPK plasmid was transfected in Huh 7 S10-3 cells. 48 h post transfection co-IP was performed with anti c-Myc antibody. Interaction of c-Myc tagged HNRNPK with that of FLAG tagged RdRp was checked with western blot by using anti c-Myc antibody. **(B)** pTandem_FLAG-RdRp_Myc-HNRNPA2B1 plasmid was transfected in Huh 7 S10-3 cells. 48 h post transfection co-IP was performed with anti c-Myc antibody. Interaction of c-Myc tagged HNRNPA2B1 with that of FLAG tagged RdRp was checked with western blot by using anti c-Myc antibody. **(C)** Huh 7 S10-3 cell lysate was incubated with HEV G and Sg promoter RNA followed by immunoprecipitation with anti HNRNPK or anti HNRNPA2B1 antibody. RT-PCR was performed to detect HNRNP-bound HEV RNAs in the elutes. Figure shows amplified PCR products on 2% agarose gel.

production is reported to be mediated by viral RNA binding (Poenisch et al., 2015). HNRNPK binds to Influenza M1 RNA and regulates its splicing while maintaining appropriate ratio of M2/M1 protein (Thompson et al., 2018). HNRNPH binds to the negative regulator of splicing elements in Rous sarcoma virus to regulate the splicing and polyadenylation machinery (Fogel and McNally, 2000). A quantitative proteomics study by Rogée et al. (2015) evaluating the alteration of host factors during HEV infection in swine revealed an upregulation of HNRNPK in infected livers (Rogée et al., 2015). In another study of modulation of host factors during HEV infection in A549 cells, a significant increase in the expression of HNRNPH was observed

in response to virus infection (Shen et al., 2014). Our study is consistent with these findings as it underlines a putative role of hnRNP proteins in modulating HEV RNA transcription by binding at the promoter site and HEV polymerase.

Validation of Interactions Between HNRNPK and HNRNPA2B1 With HEV Promoters and RdRp

Analysis of HEV-host protein interactions suggested that HNRNPK and HNRNPA2B1 are common factors associating with HEV RNA promoters and polymerase (**Figures 2A,B**). To demonstrate the validity of the interactions obtained through mass spectrometry, we further *in vitro* validated the interactions between HNRNPK and HNRNPA2B1 with that of HEV promoters and RdRp. In order to confirm the interaction of RdRp with host proteins, coimmunoprecipitation technique was employed. HEV RdRp encoding sequence and the coding sequence of selected host protein were cloned in pTandem vector. pTandem vector was chosen for its additional feature which enables cloning of two different genes in the same construct to increase the co-transfection efficiency. Huh7 S10-3 cells transfected with pTandem_Flag-RdRp_Myc-HNRNPK or pTandem_Flag-RdRp_Myc-HNRNPA2B1 plasmids. Post 48 h of transfection co-immunoprecipitation was performed by using anti myc antibody or isotype IgG antibody. After co-immunoprecipitation, interaction of host factors with RdRp was confirmed by western blot using anti FLAG antibody. We observed that both HNRNPK and HNRPA2B1 specific immunoprecipitation could pull down HEV RdRp (**Figures 7A,B**). Our results confirmed the binding of both of these proteins with HEV RdRp as obtained in the mass spectrometry data.

To further confirm the binding of HNRNPK and HNRNPA2B1 with HEV putative promoters; we employed RNA immunoprecipitation followed by RT PCR. Huh7 S10-3 cell lysate was incubated with G or Sg promoter RNA followed by pull down by anti-HNRNPK or anti-HNRNPA2B1 or isotype IgG antibody. From the eluted complexes, total RNA was isolated and presence of promoter region RNA sequence was checked by RT PCR. We observed that both HNRNPK and HNRNPA2B1 could successfully pull down HEV genomic and sub-genomic promoter RNA as evident by the specific band on agarose gel (**Figure 7C**). Therefore we could successfully demonstrate the *in vitro* binding of HNRNPK and HNRNPA2B1 with HEV RdRp and HEV promoters as obtained from the mass spectrometry data, further increasing the confidence level of the obtained interactions. Thus, we believe that HNRNPK and HNRNPA2B1 play crucial roles in HEV replication and their functional significance in the context of HEV replication can be further assessed. Taken as a whole, our study reveals the importance of host cellular machinery in HEV lifecycle regulation. Studying host pathways targeted by HEV can facilitate the hunt for putative anti-viral candidates for therapeutic purposes. In conclusion, our study shows that analyzing host-virus interactions through system biology approach can be beneficial in understanding the molecular regulation of viral lifecycle and can put forth set testable hypotheses for future experimental validation.

DATA AVAILABILITY STATEMENT

All datasets generated/analyzed for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

GK, KP, and YK conceived and designed the experiments, analyzed the data, and wrote the manuscript. GK and KP performed the experiments.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.02501/full#supplementary-material

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A Novel In-House Enzyme-Linked Immunosorbent Assay for Genotype 3 Hepatitis E Virus Reveals High Seroprevalence in Blood Donors in Northern Argentina

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The Hepatitis E virus (HEV) is an emergent virus that causes acute hepatitis in immunocompetent hosts and chronic hepatitis in immunocompromised hosts. In Latin America, the main circulating genotype HEV-3 is usually of zoonotic origin. Diagnosis and seroprevalence studies mainly rely on the detection of specific antibodies. There are scarce data on the seroprevalence of HEV infection in Latin America mainly due to the lack of awareness of HEV circulation. Furthermore, in some countries, like Argentina, HEV testing is not included in routine assays. In order to provide tools to deepen the knowledge on HEV epidemiology in South America, we designed a new in-house ELISA based on the native recombinant protein ORF2 aa112–608 and demonstrated its potential for detecting anti-HEV immunoglobulin G (IgG) in human serum samples. The following conditions were determined: an optimal antigen concentration of 0.25 μg/ml, a serum dilution of 1:80, gelatin as a blocking agent, and a secondary antibody dilution of 1:2000. A relative sensitivity of 93.33% (95% CI: 77.9–99.2%) and a relative specificity of 99.4% (95% CI: 96.7–100%) were determined using a panel of previously characterized sera and a gold standard (HEV IgG ELISA, DIA.PRO, Italy). Further, we obtained a very good agreement (κ index = 0.94, 95% CI: 0.87–1.00) with the gold standard. We screened 813 blood donor samples with this newly developed ELISA and found a seroprevalence of 9.23% (95% confidence interval, 7.33–11.43%). We show for the first time evidence of past HEV infection in Tucuman, the most populated city in northern Argentina. We expect that this study will raise the interest of health decision makers who should intercede to include indirect testing of HEV in regular diagnostic protocols. In conclusion, the in-house ELISA developed in this work shows a very good agreement with an already licensed commercial HEV IgG ELISA (DIA.PRO, ITALY), which can be used as an epidemiologic tool for HEV surveillance.

Keywords: hepatitis E virus, enzyme-linked immunosorbent assay, seroprevalence, blood donors, recombinant protein

INTRODUCTION

The Hepatitis E virus (HEV) is an emergent virus that is causing hepatitis worldwide. The clinical presentation of HEV infection varies from mild and self-limiting to severe cases with typical features of hepatitis: malaise, abdominal, muscle and joint pain, anorexia, and jaundice (Purcell and Emerson, 2008; Lewis et al., 2010), which can end up as fulminant hepatitis. Furthermore, chronic hepatitis has recently been reported in patients with immune disorders and that is hepatically compromised (Bricks et al., 2019; Yang et al., 2019). For unclear reasons, the incidence of fulminant hepatitis in pregnant women is up to 20% (Purcell and Emerson, 2008; Lewis et al., 2010). Several extra-hepatic manifestations such as arthralgia, Guillain-Barré syndrome, meningitis, and others have been attributed to HEV infection (Krain et al., 2014; Dalton et al., 2016; Pasha et al., 2018). These aspects of the disease underline the need to further investigate HEV, improve diagnostics, and increase the awareness of its circulation.

HEV-3 circulates in several countries in Europe and America and is transmitted zoonotically mainly by wild boars, domestic pigs, and deers. HEV genomic RNA and replication intermediates have also been detected in donkeys, goats, horses, macaques, mongoose, rabbits, rats, and sheep (Pavio et al., 2010; Kenney, 2019). The main infection mechanism seems to be the consumption of contaminated raw or undercooked meat (Cai et al., 2017; Cook et al., 2017) or contact with infected animals (Vonesch et al., 2019). Certain concern has arisen regarding the need to control blood transfusion units for the presence of HEV nucleic acids (Kraef et al., 2018; Harvala et al., 2019; Rivero-Juarez et al., 2019). Still, there is no clear consensus on whether HEV detection should be included in routine blood product screenings.

Hepatitis E can be diagnosed by the detection of viral RNA in blood and feces by end-point RT-PCR or qRT-PCR, but more accessible diagnostic assays are ELISA or immunoblotting to detect specific antibodies. Direct-to-consumer-testing laboratories in developing countries have limited access to HEV diagnostic tests. In South America, there is scarce data on the epidemiology of HEV. HEV-1 has been detected only in Venezuela and Uruguay in isolated cases (Mirazo et al., 2014). In the rest of the continent, HEV-3 has been isolated from patients and environmental samples; the most frequent subtypes reported were: −3a, −3b, −3c and −3i, which were related to European, American, and Japanese strains (Pisano et al., 2018b). In the 1990s, a 1.8% seroprevalence of anti-HEV antibodies was found in blood donors (n = 2,157 samples) in Buenos Aires (Rey et al., 1997). The next epidemiological study looking for specific anti-HEV antibodies in blood donors was carried out also in Buenos Aires in 2012 by Munne et al. who found a seroprevalence of 10.6% in 123 adults voluntarily screened on the World Hepatitis Day (Munne et al., 2014).

Further evidence of past infections was found in epidemiological studies of specific patient groups such as immunocompromised individuals (HIV positive and transplant recipients) and patients undergoing dialysis in other regions of Argentina. No differences with a control group (4.3%) were found in transplant recipients (5.8%; Pisano et al., 2017), while a higher seroprevalence of antibodies to HEV (7.3%) was found in HIV-positive patients (Debes et al., 2016) and patients undergoing hemodialysis (10.2%; Pisano et al., 2017) in Argentina, similar to findings in other countries. In a serological survey conducted in 433 patients attending primary care centers in the central region of Argentina, the seroprevalence for antibodies to HEV as detected with a commercial kit (HEV IgG ELISA, DIA.PRO, Italy) was 4.4% in 2011 (Martinez Wassaf et al., 2014). In the central region of Argentina, the seroprevalence of HEV in blood donors was much lower with a value of 1.81% in 1997 and later, in 2012 the seroprevalence increased to 9% (Rey et al., 1997; Munne et al., 2014). Recently, a surprisingly high HEV seroprevalence of 40.25% was reported in Brazil using an in-house ELISA, suggesting that in this region of Brazil, HEV is endemic (Pandolfi et al., 2017).

In Argentina, only one HEV ELISA kit is available imported from Italy and distributed from Buenos Aires to the entire country. This kind of monopoly is associated with higher costs, longer delays, and diminished accessibility. A way to circumvent this caveat is the development of in-house assays.

Therefore, we aimed to develop an ELISA to detect anti-HEV IgG antibodies that can be used for surveillance purposes and as a tool to gain knowledge on HEV epidemiology.

MATERIALS AND METHODS

Recombinant Cloning of Hepatitis E Virus-3 ORF2

The viral antigen used in the development of the in-house ELISA was 66 kDa recombinant polypeptide comprising $aa_{112–608}$ of the capsid protein of HEV-3. A pMK plasmid containing the coding sequence for ORF2 flanked by attB sites was obtained by synthesis at GeneArt Gene (TermoFisher Scientific) based on the ORF2 available sequence in GenBank BAG15899.1 (Takahashi et al., 2008) and further subcloned into pETG-A-His-N-[rfB] using an LR clonase (Gateway® recombinatorial cloning) as described by Vizoso Pinto et al. (2010). Briefly, the LR reaction was set using 1 µl entry vector pMK-HEV3ORF2$_{aa112–608}$, 1 µl destination vector pETG-A-His-N-[rfB], 1 µl LR clonase, and 2 µl extra pure water; the reaction was incubated 2 h at 37°C and transformed in *E. coli* DH10B by heat shock. After this, bacterial cells were plated onto LB agar added with ampicillin (100 µg/ml) and grown o.n. At least two colonies were selected, grown o.n. in LB added with ampicillin after which the plasmid was purified using a High Pure Plasmid Isolation Kit (Roche). Plasmids were checked by enzyme restriction with *HindIII* and *XbaI* (New England Biolabs) followed by agarose electrophoresis.

Expression and Purification of RGS-His_5-Tagged Hepatitis E Virus-3 ORF2

Chemically competent *E. coli* Rosetta (DE3) was transformed with pETG-A-His-N-ORF2 by heat shock and selected on LB plates supplemented with 100 µg/ml ampicillin and 17 µg/ml

chloramphenicol. Several transformants were selected and kept in LB medium supplemented with 25% glycerol at −20°C. Overnight cultures were inoculated in fresh medium and grown for 2 h, after which protein expression was induced with different concentrations of IPTG (0.25, 0.5, 1.0, or 2.0 mM) during 1–5 h, or overnight, and at 30°C or 37°C. After centrifugation, bacterial pellets were resuspended with ice-cold lysis buffer (10% glycerol, 20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, pH 7.9, supplemented with 0.02 mg/ml DNAse, 0.1% Triton, 0.2 mM PMSF, 1 mM DTT, and 1 mg/ml lysozyme) and incubated on ice for 1 h. Cells were lysed by 3 cycles of freeze-thawing. The supernatant was separated by centrifugation and kept as the soluble fraction. Then, inclusion bodies were solubilized in a buffer containing 0.5 M NaCl, 5 mM imidazole, 20 mM Tris–HCl, and 8 M urea pH 7.9. The ORF2 protein was purified under native and denaturing conditions using NiNTA chromatography (Thermo Fisher Scientific) following the manufacturer's instructions. Expression and purity of recombinant proteins were analyzed by SDS/PAGE followed by staining and verified by Western blotting using a mouse monoclonal anti-RGS-His antibody (Qiagen, Germany). Purified proteins were stored at −70°C. Protein concentration was determined with Bradford's reagent (BioRad) following the manufacturer's instructions.

Serum Samples

To determine the cut-off value and performance of the in-house ELISA (specificity, sensitivity, ROC curve, and κ index), we used a panel of 197 serum samples (30 HEV IgG positive and 167 HEV IgG negative sera) obtained as follows: 24 serum samples belonged to patients with signs and symptoms of hepatitis and elevated transaminases of unknown origin, which were previously searched for total antibodies to HEV using the HEV Ab ELISA (DIA.PRO, Italy) for diagnostic purposes at CEMIC. Only two of the sera were also RT-PCR positive (LightMix®, Modular Hepatitis E Virus, Roche SAP), and the amplified product was sequenced and corresponded to genotype 3. Further, only five of these samples presented specific IgM antibodies to HEV as determined with the HEV IgM ELISA (DIA.PRO, Italy). Two of the samples were anti-HEV IgG positive samples from Inst. Malbran. A further, three anti-HEV IgG positive and 121 negative anti-HEV IgG samples belonging to our blood donor panel were also screened with HEV IgG (DIA.PRO) and therefore included in the characterized panel. All 197 samples were retested in duplicate using the HEV IgG (DIA.PRO) to confirm the results provided before.

Enzyme-Linked Immunosorbent Assay In-House

To develop an assay to detect specific anti-HEV IgG antibodies, we optimized the antigen (recombinant ORF2) concentration, the dilution of serum, and the dilution of secondary antibody; we chose among three different blocking agents and tested the optimal TMB concentration. High-protein binding 96-well plates (JetBiofil® and Nunc®) were coated with the purified antigen and diluted in carbonate buffer at different concentrations (0.1–20 μg/well). Plates were incubated overnight at 4°C. The wells were washed with PBS, added with 0.5% Tween-20 (PBST), and then blocked with 1% (v/v) gelatin (Sigma), 5% skim milk or 1% BSA and diluted in PBST. Human sera were serially diluted to find the optimal dilution, added to the plates, and incubated at 37°C for 60 min. Then, a HRP-secondary antibody (Dako) was tested at different dilutions, added to the wells, and incubated at 37°C for 60 min. The plates were washed with PBST and revealed with 0.1 mg/ml substrate (3,3′,5,5′-tetramethylbenzidine, Sigma), and the reaction was stopped with 1 M phosphoric acid. Plates were read on an ELISA reader (Allshen) at 450 nm.

Evaluation of the In-House Enzyme-Linked Immunosorbent Assay Performance

To find the most appropriate cut-off value and to describe the test thoroughly, receiver-operated characteristic (ROC) analyses and calculated area under the curves (AUCs) were performed to achieve minimum target values for both sensitivity and specificity along with corresponding estimates and a Wilson binomial confidence interval. Agreement between the commercial and the in-house assays was assessed by pairwise comparisons using the κ coefficient.

To determine the detection limit of the assay, we serially diluted a WHO HEV serum standard (NIBSC 95/584) and tested the dilutions on the in-house ELISA plate.

The intra-assay variability was calculated as an average from the individual coefficients of variation (CV) from well to well of high, low, and negative anti-HEV-IgG samples within the same plate (10 replicates each). The inter-assay precision was calculated from the individual coefficients of variation (CV) from well to well of high, low, and negative anti-HEV-IgG samples from different plates. Acceptable criteria for intra- and inter-assay variability were defined as coefficient of variation (CV) <10 and <15%, respectively. Acceptable criteria for functional sensitivity were CV <20% (Pisanic et al., 2017).

Commercial Enzyme-Linked Immunosorbent Assay

We determined the presence of anti-HEV IgG antibodies in the 197 serum samples of the panel using the only commercially available kit in Argentina (HEV IgG ELISA, DIA.PRO, Italy) according to the manufacturer's instructions. The HEV IgG ELISA (DIA.PRO) is a qualitative test. Its microplates are coated with HEV-specific synthetic antigens encoding for conservative and immunodominant determinants derived from Mexican and Burmese virus strains. Serum is diluted 1:100 before testing. We used the cut-off value 0.3515 and calculated as $A_{450\ nm\ (negative\ control)} + 0.350$, as suggested by the test's leaflet.

Seroprevalence Study

The minimal sample size necessary to determine the seroprevalence in blood donors of Tucumán was 126 serum samples, as calculated using the InfoStat software and the EpiTool considering an estimated seroprevalence of 7% (a mean value of the seroprevalence reported for other regions in

Argentina) with a confidence level of 95%, a desired precision of 5%, and the sensitivity and specificity values obtained for the in-house ELISA (93.3 and 99.4%, respectively). Nevertheless, a larger number with a total of 813 blood bank serum samples collected in 2017 were included in the present study. Briefly, the median age was 35. Most of the participants (73.05%) were male, and 34.22% were between 26 and 35 years old. The protocol was approved by the Committee on Research Ethics of the SI.PRO.SA. (Sistema Provincial de Salud, Tucumán, Argentina, case file 849,709). The in-house HEV ELISA was used to determine anti-HEV IgG seroprevalence in 813 blood donors of Tucuman, Argentina.

Statistical Analysis

The receiver operating characteristic (ROC) curve was used to assess the optimal cut-off values for interpretation of the results obtained with the in-house ELISA. Sensitivity and specificity were calculated.

The agreement between the in-house ELISA and the commercial ELISA (HEV IgG, DIA.PRO, Italy) was assessed using Cohen's κ coefficient. Confidence intervals were calculated according to the binomial (Clopper-Pearson) "exact method" bases on the β distribution. We used χ^2 at a 95% CI to compare differences between categorical variables. The values of p <0.05 were considered statistically significant. Confidence intervals were calculated according to the binomial (Clopper-Pearson) "exact method" bases on the β distribution. All analyses were conducted using EpiTools[1].

CV% in the intra- and inter-assay variability was calculated with Excel® (Windows®).

RESULTS

Recombinatorial Cloning of Hepatitis E Virus-3 ORF2

After subcloning, the recombinant plasmids were confirmed by double restriction enzyme digestion (*HindIII* and *XbaI*) followed by electrophoresis on 1% agarose (data not shown).

Expression and Purification of RGS-His_5-Tagged Hepatitis E Virus-3 ORF2

We selected and worked with the best clone among several different *E. coli* Rosetta ORF2-expressing ones. Protein expression was induced optimally with 1 mM IPTG when A_{595nm} reached a value of 0.6 after shaking at 37°C for 3 h. The RGS-His_5-tagged ORF2 protein was purified by NiNTA chromatography (Thermo Fischer Scientific) at higher yields under native conditions than under denaturing conditions, suggesting that most of the protein was present in the bacterial cytoplasm. The expression of the truncated ORF2 protein was checked by SDS-PAGE and Western blotting using an anti-RGS-His_5 antibody and a HEV positive serum (data not shown). Under native conditions, ORF2 protein forms dimers and trimers; its yield was approximately 4.93 mg/L.

Optimal Conditions for Developing an In-House Enzyme-Linked Immunosorbent Assay

The ELISA was optimized by comparing native and denatured antigens, different antigen concentrations, serum dilutions, and blocking agents, and two different plate brands. We first tried native and denatured antigens and selected the former for further development because of its better performance and yield. Then, we found the best dilution of the secondary antibody to be 10 μg/ml coating antigen with 5% milk as blocking agent (**Figure 1A**). Afterward, we selected the optimal serum dilution according to reactivities observed by coating plates with three different antigen concentrations (1, 10, and 20 μg/ml) plus 5% milk as blocking agent (**Figure 1B**). The optimal serum dilution tested was 1:80 (**Figure 1C**). For optimizing the relation between the positive and the negative samples (P/N), we diluted the coating antigen and tested in three blocking agents (**Figure 1D**); when plates were blocked with milk, negative samples exhibited a much higher background than with gelatin or BSA (p < 0.05). Thus, we selected a coating antigen concentration of 0.25 μg/ml, a serum dilution of 1:80, a secondary antibody dilution of 1:2000, and 1% gelatin as blocking agent. Finally, we tested two different brands of high-protein binding 96-well plates (Nunc Polysorb and JetBiofil), but they did not differ significantly from each other (data not shown). JetBiofil plates were chosen because they gave a better cost-benefit ratio.

Relative Specificity Sensitivity of the In-House Enzyme-Linked Immunosorbent Assay

To calculate a proper cut-off value, receiver-operated characteristic (ROC) and two graph ROC curve analyses were performed using a panel of 197 characterized serum samples resulting in a cut-off value of A_{450} = 0.498 (**Figures 2A,B**). The high area under curve (AUC) value of 0.988 (95% CI: 0.976–1.0) reflects the high accuracy of the assay (**Figure 2A**). Further, we compared our in-house ELISA with a commercial assay (HEV IgG ELISA, DIA.PRO; **Table 1**). We calculated a relative sensitivity of 93.33% (95% confidence interval, 77.93–99.18%) and a specificity of 99.4% (95% confidence interval, 96.71–99.98%; **Table 1**) at the selected cut-off value of A_{450nm} = 0.498. The κ agreement test yielded a high score of 0.9402 (0.8731–1.0073), reflecting a strong to very good agreement with the DIA.PRO HEV ELISA test (**Table 1**).

Detection Limit and Variability Intra- and Inter-Assay

We tested serially diluted samples of the HEV WHO standard on to the in-house ELISA plate. As a result, the lowest concentration detected by the assay was 0.25 IU/ml, while the

[1]Sergeant, ESG, 2019. Epitools epidemiological calculators. Ausvet Pty Ltd. Available at: http://epitools.ausvet.com.au

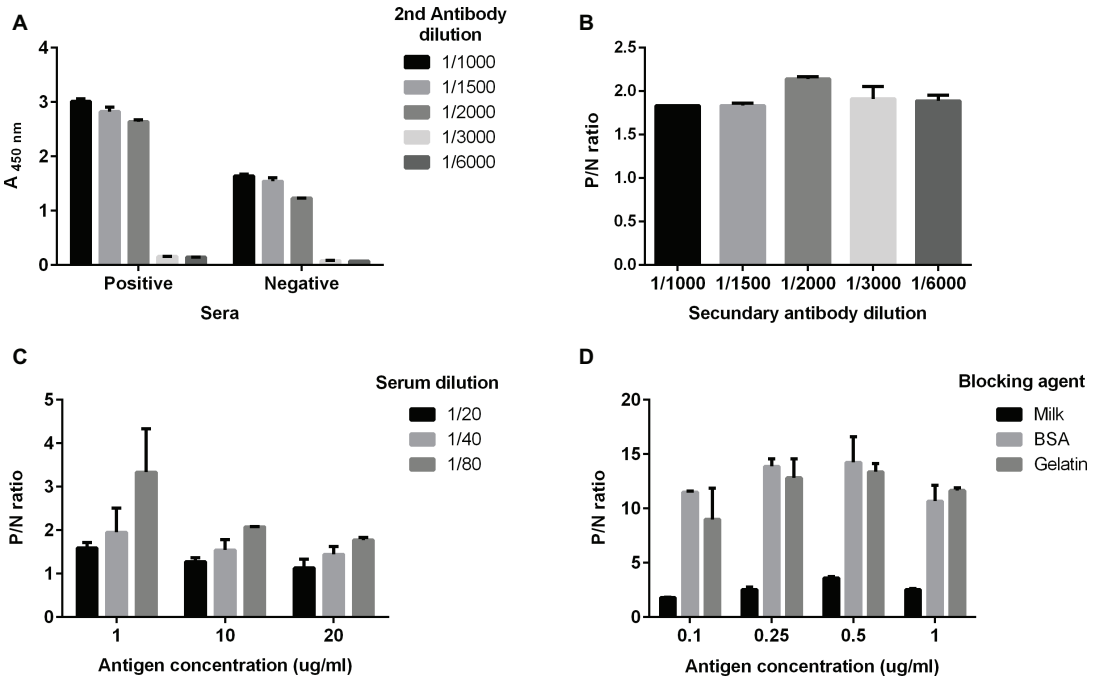

FIGURE 1 | Optimal conditions for the in-house ELISA. Serum samples previously determined as positive or negative for anti-HEV IgG with the commercial kit (DIA.PRO) were used. The optimal dilutions of serum samples **(A)** and of the secondary antibody were determined **(B)** using an antigen concentration of 10 μg/ml. Serum samples positive for anti-HEV IgG or negative for anti-HEV IgG, as previously determined with the commercial assay, were used. **(C)** Optimal antigen concentration at three serum dilutions 1:20 (black balks), 1:40 (light gray balks) and 1:80 (dark gray balks) and **(D)** different blocking agents. P/N ratio in **(B–D)** is calculated as the relation $A_{(\lambda\ 450\ nm)}$ positive sample/$A_{(\lambda\ 450\ nm)}$ negative control.

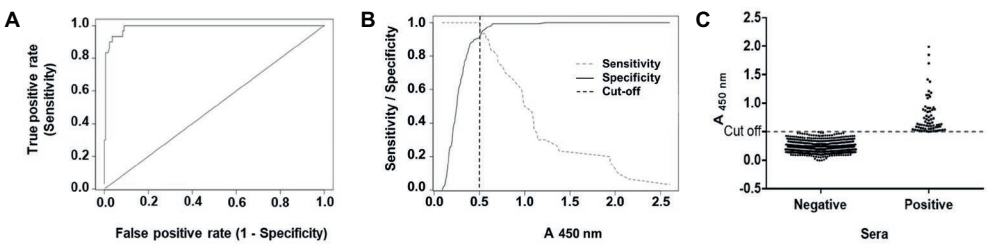

FIGURE 2 | ROC curve, sensitivity and specificity graph of the in-house ELISA, and seroprevalence of anti-HEV IgG antibodies in blood donors. **(A)** ROC curve obtained with 197 characterized sera for their reactivity to HEV. **(B)** The cut-off value of the assay (A_{450nm} values = 0.498) as determined by the optimal values of the sensitivity (dashed gray line) and specificity (full black line) curves. **(C)** Prevalence of anti-HEV antibodies in 813 blood donors. Results are depicted as absorbance values at λ = 450 nm. Samples with A_{450nm} values > 0.498 were considered as positive using the optimal parameters determined before for the ELISA.

TABLE 1 | Performance of the in-house ELISA compared to the commercial ELISA (DIA.PRO) in detecting anti-HEV IgG.

		Commercial ELISA		
		Positive	**Negative**	**Total**
In-house ELISA	**Positive**	28	1	29
	Negative	2	166	168
	Total	30	167	**197**
Relative sensitivity	93.33% (95%CI, 77.93–99.18%)			
Relative specificity	99.4% (95%, 96.71–99.98%)			
κ	0.9402 (95%CI, 0.8731–1.007)			

detection limit of the DIA.PRO ELISA is 0.2 IU/ml according to reports in a comparative study done with five different assays (Norder et al., 2016). We found that the intra-assay variability of negative, low, and high positive HEV-IgG samples (10 replicates each) was 8, 4, and 4%, respectively, whereas the inter/assay variability was 6, 8, and 4%, respectively (data not shown).

Seroprevalence of Anti-Hepatitis E Virus Immunoglobulin G in Blood Donors

We screened 813 serum samples from blood donors collected in Tucumán in 2017. We found 75 positive samples for anti-HEV

IgG using the in-house ELISA (**Figure 2C**). Thus, the overall seroprevalence for anti-HEV IgG was of 9.23% (Clopper-Pearson exact 95% confidence interval, 7.33–11.43%). HEV seropositivity was independent of sex (p = 0.3015) and age (p = 0.8376).

DISCUSSION

We developed a HEV-3 ELISA based on the ORF2 recombinant protein produced in *E. coli* under native conditions. The protein ORF2 is the structural component of the capsid, the most immunogenic HEV protein, and the antigen of choice for serological diagnostics (**Table 2**). The C-terminal region is exposed on the surface of the capsid and harbors neutralizing epitopes, whereas the N-terminal region is hidden within the particle (Mori and Matsuura, 2011; Tang et al., 2011; Shata et al., 2012). Most of the assays reviewed in **Table 2** are based on denatured ORF2 protein, which exposes the linear epitopes but not the conformational ones. Our assay differs from others (**Table 2**) in the nature of the antigen, which is obtained in *E. coli* under native conditions. Under the conditions used in this study, the protein forms dimers and trimers like it was previously seen by Zheng et al. (2018). Some of the assays presented in **Table 2** obtained the antigen under denaturing conditions and refolded it after purification. The size of our antigen is like most of the antigens used in commercial assays. Most of the ELISAs in **Table 2** use ORF2 or combinations with ORF3 from HEV-1. Only three of the assays (Mikrogen, DIA.PRO and Biomedical) include ORF2 or parts thereof belonging to HEV-1 and HEV-3. The ELISA from DIA.PRO is based on synthetic peptides covering ORF2 and ORF3 from different genotypes. We showed that the recombinant HEV-3 ORF2, under the conditions tested in this study, is enough to detect anti-HEV IgG with a high agreement with the commercial assay [K = 0.9402 (0.8731–1.0073)].

About 2.54 μg of the purified protein was used to coat each ELISA plate. We estimate that 1972 plates can be prepared from 1 L bacterial culture (yield = 4.93 mg/L), which allows testing approximately 178,000 serum samples. Results obtained with our

TABLE 2 | Comparison of commercial ELISA tests for Hepatitis E and the in-house ELISA developed in this study.

Anti-HEV ELISA (IgG)	Antigen type	Genotype (GT)	Antigen size	Se[1] (%)	Sp[2] (%)	Reference
Wantai	Recombinant antigen ORF-2C-terminal	GT 1	211 aa	97.96	99.6	(Bendall et al., 2010; Shrestha et al., 2016; Abravanel et al., 2017)
	Recombinant antigen ORF-2C-terminal	GT 4	210 aa	93.2	97.8	(Park et al., 2012; Abravanel et al., 2013)
Axiom	Recombinant antigen ORF-2C-terminal (Burmese strain)	GT 1	n.s	95	98	(Norder et al., 2016)
Mikrogen	Recombinant antigen ORF-2C-terminal	GT 1 and 3	n.s	62	99	(Avellon et al., 2015; Norder et al., 2016)
Abbot	Recombinant antigen ORF-2 and ORF-3 (Burmese strain)	GT 1	ORF2 123 aa ORF3 full length	60	96	(Psichogiou et al., 1996; Myint et al., 2006)
Adaltis	Synthetic peptides encoded by the ORF-2 and ORF-3	GT 1 and 2	n.s	80	62.9	(Abravanel et al., 2013; Wu et al., 2014)
MP Biomedical	3 recombinant antigens, ORF-2 and ORF-3 (Burmese, Mexican strains)	GT 1 and US type 2	n.s	n.s	n.s	(Vollmer et al., 2016)
	3 recombinant proteins from ORF-2 GT2, ORF-3 GT3 and ORF-3 GT1	GT 1, 2 and 3	ORF2 GT2 42 aa ORF3 GT3 33 aa ORF3 GT1	73.3	65.3	(Schnegg et al., 2013; Wu et al., 2014)
Euroimmun	Recombinant antigen ORF-2 (USA strain)	GT 3	n.s	42	99	(Avellon et al., 2015; Norder et al., 2016; Vollmer et al., 2016)
DIA.PRO	4 synthetic peptides with conservative epitopes of ORF-2 and ORF-3	GT 1, 2, 3, and 4	n.s	98	96	(Avellon et al., 2015; Norder et al., 2016)
Genelab	Recombinant peptides ORF-2 and ORF-3C- terminal (Burmese, Mexican strains)	GT 1 and 2	n.s	50–90	93–100	(Bendall et al., 2010; Park et al., 2012)
DSI	Recombinant peptides ORF-2 and ORF-3	GT 1, 2 and 3	n.s	72	99	(Norder et al., 2016)
This study	Recombinant antigen ORF-2C-terminal	GT3	496 aa	77.93–99.18*	96.71–99.98*	

*n.s.: not specified; [1]Sensitivity; [2]Specificity; *with a 95% confidence interval.*

in-house ELISA highly agree with the ones furnished by the commercial assay DIA.PRO HEV IgG ELISA kit. We established a cut-off value of A_{450nm} = 0.498 for the in-house ELISA based on the ROC curve analysis (**Figure 2B**). **Figure 2C** depicts a common problem that several epidemiological studies have faced, including HEV seroprevalence studies, because small changes in the cut-off value may have a considerable influence on the seroprevalence rates.

In Argentina, diagnostic tests are scarcely developed and produced, in part because of regulations which prevent patenting diagnostic assays. We aim to offer an alternative to the only available assay: a test to be used in epidemiological studies with a similar sensitivity and specificity, but that is able to be produced locally at a lower cost. In order to use the in-house ELISA for diagnostic purposes, detection of anti-HEV IgM should be evaluated and validated.

The HEV seroprevalence of 9.23% (95% confidence interval, 7.33–11.43%) is within the range (4.4, 15.8%) found in central Argentina (Munne et al., 2011; Martinez Wassaf et al., 2014), and 9% found in Buenos Aires in 2012 (Munne et al., 2014) Although the IgG seropositivity does not represent active infection or carrier state but a past infection, it suggests that infection can be spread during periods of infectivity. Therefore, standardized screening provides an opportunity for public health services to address this concern. Indirect tests better suit the equipment available in routine laboratories in Latin America, where regular molecular testing is still uncommon.

Recently, 1–4% of chronic HEV infections were acquired by blood transfusion and developed persistent liver graft damage (Pawlotsky, 2014). Hewitt et al. evidenced the presence of HEV RNA in blood donations and the transmission of HEV through different blood components and described the morbidity of infected recipients (Hewitt et al., 2014). Chronic hepatitis E infection in immunocompromised patients is a serious issue, which may cause cirrhosis leading to liver failure. This underscores the new threat that HEV represents to blood transfusion safety. Some experts from industrialized countries recommend that systematic HEV testing by qRT-PCR should be implemented in blood banks to reduce the existent risk of serious complications and death (Hewitt et al., 2014). Systematic testing implies practical, economic, and logistic issues not currently solvable in Argentina. As seroprevalence of HEV changes over time, suggesting that some generations have been more exposed than others, it seems necessary to implement at least the epidemiological surveillance of HEV – with serological methods like the in-house ELISA presented here – in order to take public health decisions timely. Despite HEV circulation in northern Argentina (this study; Martinez Wassaf et al., 2014; Debes et al., 2016; Pisano et al., 2017, 2018a), differential diagnoses are barely done at the most important hospitals of Tucuman, indicating that HEV is not considered a possible etiologic agent. As HEV prevalence worldwide increases in parallel with the physicians' awareness of the disease and the higher availability of diagnostic assays, we expect that this study will also raise the interest of health decision makers who should intercede to include indirect testing of HEV in regular diagnostic protocols.

In conclusion, the in-house ELISA developed in this work shows a very good agreement with an already licensed commercial HEV IgG ELISA (DIA.PRO, ITALY). We provide an accessible tool for studies to deepen the knowledge on HEV epidemiology in Argentina and neighboring countries. Using this in-house ELISA, we determined a seroprevalence of 9.23% (95% confidence interval, 7.33–11.43%) in northern Argentina.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Committee on Research Ethics of the SI.PRO. SA (Sistema Provincial de Salud, Tucumán, Argentina, case file 849709). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LA expressed and purified the proteins, performed all the experiments, analyzed the data, and helped draft the manuscript. MM performed some experiments. AM contributed to sample preparation and characterized sera *via* commercial ELISA and RT-PCR. GM collected serum samples, analyzed sera for blood transmitted diseases, and interviewed blood donors. FA contributed to sample preparation. AB participated in cloning and discussions of the results. MV-P conceived and designed the study, performed some experiments, analyzed the data, and wrote the manuscript with input from all the authors. All authors contributed to the final manuscript.

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Mutations in the Progesterone Receptor (PROGINS) May Reduce the Symptoms of Acute Hepatitis E and Protect Against Infection

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Background: Mutations in the progesterone receptor (PR) gene, PROGINS, have been studied in relation to hepatitis E virus (HEV) infection. Patients with the PROGINS gene may develop a worse clinical course of hepatitis E. The aim of our study was to evaluate the influence of PROGINS on the susceptibility to and the clinical course of HEV infection in HIV patients.

Methods: This study included patients with HIV who were evaluated in previous prospective studies for the prevalence and incidence of HEV. The following three groups of patients were studied: (i) never infected, (ii) past infections, and (iii) recently infected. We determined the PR genotype to evaluate the proportion of patients who were homozygous for PROGINS according to HEV infection. We also compared the proportion of PROGINS carriers with a recent HEV infection according to their symptomatology.

Results: In this study, 311 patients infected with HIV were included. Of those patients, 198 were homozygous wild type (63.7%), 91 were heterozygous (29.3%), and 22 were homozygous PROGINS (7.1%). We found that the homozygous PROGINS genotype in women was associated with a lower HEV seroprevalence. In addition, in patients with a recent HEV infection, none of those homozygous for PROGINS presented symptoms.

Conclusion: The PROGINS mutation plays a protective role against HEV infection and is associated with subclinical infection in HIV-infected patients, particularly women.

Keywords: PROGINS, progesterone-receptor, hepatitis E virus, HIV, susceptibility, symptoms, protect

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INTRODUCTION

Progesterone is a steroid hormone that downregulates immune system activity (Butts et al., 2007; Jones et al., 2010; Hall et al., 2017). *In vitro* studies have demonstrated that high levels of progesterone promote the downregulation of proinflammatory cytokines and chemokines (Arruvito et al., 2008; Devadas et al., 2018). Consequently, progesterone levels may promote susceptibility to different processes, as well as clinical features and evolution.

Mutations in the progesterone receptor (PR) can reduce the activity of the hormone progesterone (Romano et al., 2006, 2007). These mutations in the PR, called PROGINS (Rowe et al., 1995), consist of a 320-bp Alu insertion in intron G and two substitutions, one in exon 4 (V660L), and the other in exon 5 (H770H) (Romano et al., 2007). In the overall population, the frequencies of these mutations range from 0.07 to 0.26 (Modugno, 2004). Several studies have evaluated the role of PR gene polymorphisms and their associations in different pathologies including malignancies, where PROGINS could be a risk factor for uterine cancer and leiomyomas (Lee et al., 2010; Yuan et al., 2013; Gallegos-Arreola et al., 2015), or reproductive disorders in women that can cause infertility, where carrying the PROGINS gene is a risk factor for developing endometriosis (Costa et al., 2011; Silva and Moura, 2016).

PROGINS has also been reported to influence the activity of the immune system (Lhomme et al., 2016) and have an impact on the clinical features and evolution of viral infections. In this context, the PR has been studied in relation to hepatitis E virus (HEV) infection (Bose et al., 2011; Debes et al., 2018), where those with the PROGINS gene were observed to develop a worse clinical course of hepatitis E. The aim of our study was to evaluate the influence of PROGINS on the susceptibility to and the clinical course of HEV infection in HIV patients in an area with high prevalence and incidence of hepatitis E.

MATERIALS AND METHODS

Patients

This study retrospectively included HIV patients who were evaluated in previous prospective studies of HEV prevalence and incidence carried out in the Province of Cordoba (Southern Spain) between 2012 through 2014 (Rivero-Juarez et al., 2015, 2017). Patient selection was based on a diagnosis of HEV infection and blood sample availability. Three groups of patients were created: (i) never infected, defined as IgG- and IgM-seronegative and aviremic; (ii) past infection, defined as IgG positive but negative for both IgM and HEV RNA; and (iii) recently infected, defined as IgM positive and/or HEV RNA positive. Data concerning the presence of symptoms associated with HEV infection as well as the epidemiological and clinical information of each patient were also collected in the recently infected subgroup. We followed the criteria for HEV screening as specified in clinical guidelines (European Association for the Study of the Liver, 2018; Rivero-Juarez et al., 2018).

Variable Collection and Definition

The main outcome variable was infection with HEV, which was defined as past or recent infection (primary analysis). The secondary outcome variable was the presence of symptoms associated with HEV infection (secondary analysis).

Anti-HEV IgG/IgM Serology and RT-PCR for the Detection of HEV

ELISA was used for the detection of anti-HEV IgG (Wantai HEV-IgG ELISA®; Beijing Wantai Biological Pharmacy Enterprise© LTD., Beijing, China) and anti-HEV IgM (Wantai HEV-IgM ELISA®; Beijing Wantai Biological Pharmacy Enterprise© LTD., Beijing, China). The ELISAs were carried out in accordance with the instructions provided by the manufacturer using a cut-off value of >1.1. The specimens with an absorbance value to cut-off ratio between 0.9 and 1.1 were considered borderline. All the anti-HEV IgG/IgM positive and borderline samples were confirmed by Western blot analysis (recomBlot HEV IgG/IgM®; Mikrogen Diagnostik GmbH, Neuried, Germany). RT-PCR for HEV RNA was performed on all patient samples (amplicube HEV®; Mikrogen Diagnostik GmbH, Neuried, Germany).

Determination of PROGINS

The PR genotype was identified from retrospectively collected blood samples stored at −80°C until analysis. Genomic DNA was extracted from 200 μL of blood using the QIAamp DNA Blood Mini Kit (QIAgen, Hilden, Germany) and an automated procedure (QIAcube, QIAgen, Hilden, Germany). PCR was performed with MyTaq^{TM} DNA Polymerase (Bioline, Meridian Life Science, Memphis, TN, United States) together with the following primers (20 μM) used to detect intron G and identify the PR genotype: forward primer 5′-GCCTCTAAAATGAAAGGCAGAAAG-3′ and reverse primer 5′-GTATTTTCTTGCTAAATGTCTG-3′ (Agoulnik et al., 2004). The thermal profile was 95°C for 1 min followed by 35 cycles at 95°C for 15 s, 60°C for 15 s, and 72°C for 10 s. Electrophoresis was conducted with 10 μL of PCR products mixed with 1.6 μL of (6×) Gel Loading Dye, Blue (New England BioLabs) on a 2% agarose gel with 5 μL of ethidium bromide in a volume of 150 mL for 50 min at a constant voltage of 90 volts. The Tracklt 100-bp DNA ladder (Invitrogen, Burlington, ON, Canada) was used to identify the molecular weight of the bands in the agarose gel. The bands were visualized using the Molecular Imager Gel Doc XR System (BioRad, Hercules, CA, United States).

Genotypic Classification of the Progesterone Receptor

We classified the patient genotypes by visualizing the different molecular weight bands in the gel. The 174-bp band corresponds to the wild-type genotype and the 494-bp band corresponds to the PROGINS genotype (**Figure 1**). The patients were classified prospectively as (i) homozygous wild-type; (ii) homozygous PROGINS; or (iii) heterozygous (**Figure 1**).

Statistical Analysis

The prevalence of the PR genotypes in the study population was calculated. The categorical variables were expressed as the numbers of cases (percentages). The frequencies were compared using the $\chi 2$ test or Fisher's exact test, and significance was set at a two-tailed p-value of less than 0.05. We used the $\chi 2$ test when the expected values of at least 80% of the cells in a 2×2 contingency table to be greater than 5. When these conditions are not verified, we compared the qualitative variables via the Fisher's exact test. We have included this point in the section "Statistical Analysis." The following formula was used to calculate the allele frequencies of the PR gene: 2*N homozygous + N

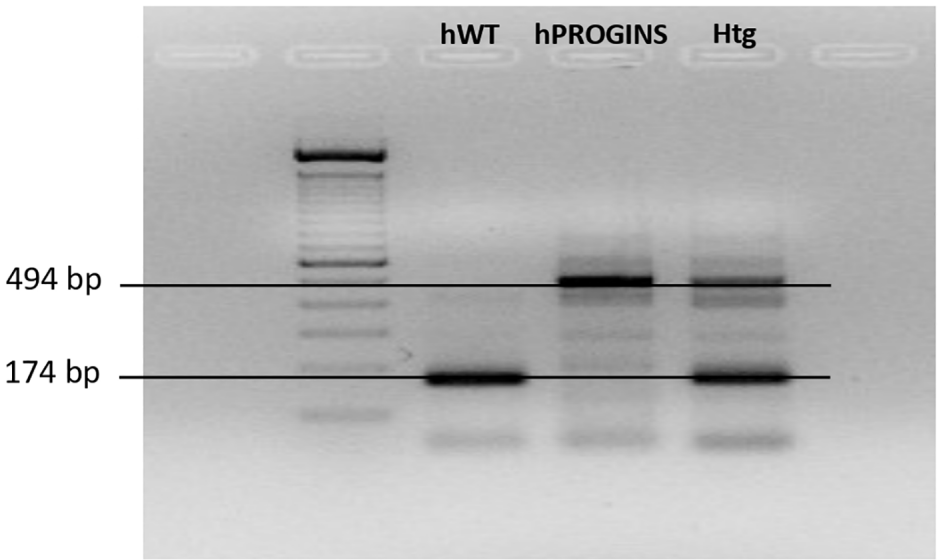

FIGURE 1 | Identification of the progesterone receptor genotypes. hWT, homozygous wild type; hPROGINS, homozygous PROGINS; Htg, heterozygous; bp, base pairs.

heterozygous/2*N total. We first evaluated the proportion of patients who were homozygous for PROGINS or not according to HEV infection [never infected (group i) vs. infected (groups ii and iii)]. For the patients with a recent HEV infection (group iii), we also compared the proportion of individuals who were PROGINS carriers or not according to the presence of symptoms (symptomatic vs. asymptomatic). The analyses were carried out using SPSS statistical software package version 18.0 (IBM Corporation, Somers, NY, United States).

Ethics Statement

This study was designed and performed according to the Helsinki Declaration. The local CEIC (Clinical Trial and Ethical Committee) approved the study protocol.

RESULTS

Study Population

A total of 311 HIV-infected patients were included in the study: 191 (61.4%) males and 120 (38.6%) females. All patients were on antiretroviral therapy with undetectable viral load The distribution of patients according to HEV infection was the following: (i) never infected, 141 (45.3%); (ii) past infection, 131 (42.1%); and (iii) recent infection, 39 (12.6%). In terms of PR genotype, 198 were homozygous wild type (63.7%); 91 were heterozygous (29.3%); and 22 were homozygous PROGINS (7.1%) (**Table 1**). The allele frequencies were 0.78 for the wild type and 0.22 for the PROGINS allele, which are similar to other studies (McKenna et al., 1995; Runnebaum et al., 2001). In addition, the allelic frequencies for the PROGINS genotype were 0.21 and 0.22 in patients infected and never infected by HEV, respectively.

The Association Between PROGINS and Risk for HEV Infection

The relationship between homozygous PROGINS and HEV infection in the total population was analyzed (**Table 2**). Among the homozygous PROGINS patients, 9 (40.9%) were never infected, while 132 of the non-homozygous PROGINS patients were never infected (45.7%) (p = 0.48) (**Table 2**). When the patients were classified by sex, an association was found between PROGINS and females in the never-infected group (**Table 2**).

The Association Between PROGINS and Symptomatic HEV Infection

Among the 39 patients with a recent HEV infection, 23 (59%) were asymptomatic and 16 (41%) showed symptomatic infection. The main symptoms identified in these patients were digestive alterations, nephropathies (chronic renal failure, and pyelonephritis), febrile syndrome, hepatic cytolysis, and

TABLE 1 | Prevalence of HEV infection according to progesterone receptor genotype.

Genotype	Never infected (*N* = 141)	Past infection (*N* = 131)	Recent infection (*N* = 39)	Total (*N* = 311)
Homozygous wild-type	91 (64.5%)	81 (61.8%)	26 (66.7%)	198 (63.7%)
Heterozygous	41 (29.1%)	42 (32.1%)	8 (20.5%)	91 (29.3%)
Homozygous PROGINS	9 (6.4%)	8 (6.1%)	5 (12.8%)	22 (7.1%)

HEV, hepatitis E virus; N, number of subjects.

TABLE 2 | Comparative analysis of never-infected and HEV-infected patients homozygous for PROGINS in the total population and according to sex.

	Homozygous PROGINS	Never infected (*N* = 141)	Infected (*N* = 170)	*p* value
Total	No	132 (45.7%)	157 (54.3%)	0.480
	Yes	9 (40.9%)	13 (59.1%)	
Males	No	50 (28.6%)	125 (71.4%)	0.102
	Yes	3 (18.7%)	13 (81.3%)	
Females	No	82 (71.9%)	32 (28.1%)	< 0.001
	Yes	6 (100%)	0 (0%)	

HEV, hepatitis E virus; N, number of subjects.

TABLE 3 | Patients with a recent HEV infection: analysis of the total population and according to sex.

	Homozygous PROGINS	Asymptomatic (*N* = 23)	Symptomatic (*N* = 16)	*p* value
Total	No	18 (52.9%)	16 (47.1%)	<0.001
	Yes	5 (100%)	0 (0%)	
Men	No	13 (48.1%)	14 (51.9%)	<0.001
	Yes	5 (100%)	0 (0%)	
Women	No	5 (71.4%)	2 (28.6%)	NC*
	Yes	0 (0%)	0 (0%)	

**Not calculable. HEV, hepatitis E virus; N, number of subjects.*

cholestasis. In the overall analysis, none of the homozygous PROGINS patients presented symptoms (**Table 3**).

DISCUSSION

The results obtained in the present study demonstrate that the presence of the homozygous PROGINS genotype in women is associated with a lower HEV seroprevalence in HIV-infected individuals. Our findings suggest that this genotype reduces the susceptibility to HEV infection and is associated with a better clinical course of infection.

The function of the PR is associated with its binding to progesterone, a steroid hormone involved in immune system modulation (Jones et al., 2010). Previous studies have suggested that high levels of progesterone may be related to increased susceptibility to infection. Byrne et al. (2016) observed that women who used injectable progestin-only contraception were more susceptible to HIV infection. Furthermore, *in vitro* studies have suggested that progestins could reduce the secretion of proinflammatory cytokines and chemokines, alter the attraction of inflammatory cells, such as neutrophils and macrophages, and affect the apoptosis of natural killer cells (Arruvito et al., 2008; Huijbregts et al., 2014; Devadas et al., 2018; Preciado-Martínez et al., 2018). The role of progesterone in the immune system is also influenced by its binding to the receptor. In this context, PROGINS has been shown to alter the function of the progesterone hormone (Romano et al., 2006, 2007). Two different PR exist. The wild-type receptor is assumed to bind normally to progesterone, which means that the progesterone levels modulate actions in the immune system naturally. The PROGINS receptor that presents mutations binds more weakly to progesterone (Romano et al., 2007), thereby reducing progesterone activity regardless of blood hormone levels. Consequently, the relationship between the PROGINS receptor and low progesterone activity could reduce susceptibility to HEV infection.

With respect to the symptomatology, the majority of cases of HEV infection (90%) are generally asymptomatic and self-limiting (Dalton and Seghatchian, 2016); however, certain risk groups, such as cirrhotic patients, pregnant women and patients with HIV infection, follow a worse clinical course (Krain et al., 2014; Frias et al., 2018). In the prospective studies from which the population included in this study were derived, we prospectively evaluated the presence or absence of signs or symptoms of HEV infection in 39 patients who presented acute infection (Rivero-Juarez et al., 2015, 2017). Our study found an association between the PR genotype and the development of symptoms during HEV infection in which none of the HEV-infected patients with the homozygous PROGINS genotype presented symptoms.

Two previous studies found the opposite situation, namely that PROGINS mutations could be a risk factor for HEV infection (Bose et al., 2011; Debes et al., 2018). Bose et al. (2011) analyzed a population of pregnant women, which is very different from our population of HIV-infected patients, and Debes et al. (2018) analyzed only the seroprevalence of HEV infection associated with the presence of the PROGINS gene and they did not differentiate between sexes, a variable in which we found differences. In addition, both of these studies focused their analyses on those with PROGINS versus those who were not carriers of this allele, without specifying homozygosity. Our group took into account the patient's genotype when analyzing the effect of PROGINS because heterozygous patients may have modulated responses through the presence of the wild-type allele. On this point, a meta-analysis performed by Pooley et al. (2006) suggested that the PROGINS gene has a codominant effect. Another study reported that the PROGINS allele has a gene dosage effect, whereby the expression of this gene is greater in individuals who present homozygosity (Wang-Gohrke et al., 2000). In addition, Alter et al. (2010) observed allelic dosage effects in transient tachypnea of the newborn, in which the PROGINS gene has a protective effect against this disease.

Our study also observed possible differences between males and females with respect to the effect of the homozygous PROGINS genotype on HEV infection. Prior studies have identified being male as a risk factor for HEV infection (Pineda et al., 2014; Zeng et al., 2017). The differences between the male and female sex hormone systems could also explain the differential immunological activity against HEV. According to Ghosh and Klein (2017), the disparity between men and women can be found in the development of immune responses to viral infections. Another study found that men have worse outcomes than women in infections such as hepatitis B and C associated with sex hormones (Ruggieri et al., 2018). In our study, the homozygous PROGINS genotype effect has more of an impact on women than men in terms of HEV infection or development

of symptoms, which may explain why being male was identified as a risk factor for HEV infection.

A limitation of our study is the number of patients included. Due to the low frequency of the PROGINS allele in our population, the number of patients homozygous for this allele was relatively low, and we therefore had to assume that the allele frequencies were constant in the population to perform the statistical analysis.

CONCLUSION

In conclusion, the PROGINS mutation in the PR gene plays a protective role against HEV infection and is associated with subclinical infection in HIV-infected patients, particularly women.

DATA AVAILABILITY STATEMENT

The raw datasets for this study can be found in the European Nucleotide Archive, PRJEB34891.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Andalusian Clinical Trial and Ethical Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AR-J: full access to all the data in the study, takes responsibility for the integrity of the data and the accuracy of the data analysis, study concept and design, and obtain funding. IM, AC, and AR: patient recruitment. PL-L, MR, JC-G, IG-B, MF, IO, and AR-J sample collection and procedures. PL-L, MF, and AR-J: analysis and interpretation of the data. PL-L and AR-J drafting of the manuscript and statistical analysis. All authors: critical revision of the manuscript.

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Occurrence of Hepatitis E Virus in Pigs and Pork Cuts and Organs at the Time of Slaughter, Spain, 2017

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Zoonotic hepatitis E, mainly caused by hepatitis E virus (HEV) genotype (gt) 3, is a foodborne disease that has emerged in Europe in recent decades. The main animal reservoir for genotype 3 is domestic pigs. Pig liver and liver derivates are considered the major risk products, and studies focused on the presence of HEV in pig muscles are scarce. The objective of the present study was to evaluate the presence of HEV in different organs and tissues of 45 apparently healthy pigs from nine Spanish slaughterhouses (50% national production) that could enter into the food supply chain. Anti-HEV antibodies were evaluated in serum by an ELISA test. Ten samples from each animal were analyzed for the presence of HEV RNA by reverse transcription real-time PCR (RT-qPCR). The overall seroprevalence obtained was 73.3% (33/45). From the 450 samples analyzed, a total of 26 RT-qPCR positive samples were identified in the liver (7/45), feces (6/45), kidney (5/45), heart (4/45), serum (3/45), and diaphragm (1/45). This is the first report on detection of HEV RNA in kidney and heart samples of naturally infected pigs. HEV RNA detection was negative for rib, bacon, lean ham, and loin samples. These findings indicate that pig meat could be considered as a low risk material for foodborne HEV infection.

Keywords: hepatitis E virus, prevalence, seroprevalence, pigs, pork cuttings, pig organs, food safety, slaughterhouse

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INTRODUCTION

Hepatitis E virus (HEV) is a small non-enveloped positive sense single-stranded RNA virus classified in Hepeviridae family (Emerson and Purcell, 2003) and is the main cause of viral acute hepatitis in humans worldwide (EFSA, 2017). In developing countries, the virus is mainly transmitted through contaminated water, whereas in industrialized countries, sporadic cases are

basically related to animals, and hepatitis E is currently considered an emerging zoonotic disease (EFSA, 2017). In Spain, anti-IgG-VHE prevalence ranges from 0.6 to 10% in the general population (Echevarria et al., 2015), whereas it could reach up to 19% in persons exposed to pigs (Galiana et al., 2008). Generally, hepatitis E is a self-limiting disease, but it can become chronic or cause a severe disease in immunocompromised patients or with previous liver or chronic diseases (Pavio et al., 2010).

Pigs are an important zoonotic source of HEV. The swine population is considered endemic for HEV-genotype 3 (gt3) in many European countries (Pavio et al., 2010). Human cases in which foodborne route was implicated have been increasing during the last decade (EFSA, 2017). Pigs are susceptible to infection, but they do not suffer clinical disease, so visual inspections are not valid to detect possible HEV infection in the necropsy or during slaughter (Meng et al., 1997; van der Poel et al., 2001). Thus, laboratory tests are necessary to determine the potential contamination of tissues, organs, muscles, and fluids, which could enter the food chain. The development of precise strategies for the prevention and control of HEV infection should be based on advances in the knowledge of source, epidemiology, and control methods. As pork products (including meat) are highly prevalent in European food markets, it is necessary to evaluate the potential risks they represent relatively to HEV infection of humans.

Pork meat is the most widely consumed meat type in the world, with 112. 472 thousand tons consumed in 2018 (United States Department of Agriculture Foreign Agricultural Service [USDA], 2019). In Europe, Spain is the second producer after Germany, with 19% of the total pork sector production (MAPAMA, 2018). Moreover, Spain exported 2,196,648 tons of pork products in 2018 (MAPAMA, 2018). HEV-gt3 is present in swine populations in different European countries and has been linked to cases of hepatitis E in several countries (Lapa et al., 2015). In Spain, HEV has been circulating in pig populations at least since the 1980s, reaching a farm seroprevalence up to 98% [95% confidence interval (CI) 96.1–99.9%] (de Deus et al., 2008; Seminati et al., 2008; Casas et al., 2009a; Jimenez de Oya et al., 2011). HEV prevalence in Spanish domestic pig serum samples was determined at 18.8% (64/341) (Jimenez de Oya et al., 2007).

The European Food Safety Authority (EFSA) has recommended integrated studies in the food chain to determine the potential risk of HEV in pork products (EFSA, 2017). Although some studies have evaluated HEV presence in pig liver, bile, feces, or serum in abattoirs, extensive data are lacking about the HEV presence in other organs or muscles that can enter the food chain. Consequently, the objective of the present work was to investigate the presence of HEV in pig products at the moment of slaughter, in an endemic country, to determine the potential risk of pork products, especially pork meat.

MATERIALS AND METHODS

Sampling Strategy

A cross-sectional study on pigs being slaughtered between November and December 2017 was undertaken through a sampling strategy with a national coverage. Nine Spanish slaughterhouses were selected according to their slaughter capacity, which represents 50% of national pig production, and were located in different regions within the country. In each slaughterhouse, five animals (between 5 and 6 months old) were randomly selected. From each animal, 10 different samples were obtained: 10 ml of blood, 100–125 g feces, and approximately 25 g of heart, kidney, liver, ribs, bacon, diaphragm, lean ham (femoral biceps), and loin head. To avoid cross contamination, sterile scalpel blades and disposable material were used for each sample, and samples were taken with the appropriate hygienic precautions. Samples were refrigerated and sent to the laboratory in less than 8 h and frozen at −80°C until processing.

Antibody Detection by ELISA

Once in the laboratory, blood was conserved at refrigeration temperature until the next day, when serum was obtained and stored at −20°C. Serum samples were tested for the presence of antibodies using the ID Screen Hepatitis E multi species indirect ELISA (IDvet, Montpellier, France) validated for swine based on recombinant gt3 capsid antigens. This ELISA kit detects IgG anti-HEV. Test procedures and interpretation of results were performed according to the manufacturer's instructions. ELISA tests were repeated three times when the sample tested negative.

Detection of HEV by Real-Time PCR

Sample Process Control Virus

A sample process control virus (SPCV) was added to each sample immediately before the start of the analysis. The SPCV was murine norovirus 1 (MNV-1) (Diez-Valcarce et al., 2011a), which had been propagated in RAW264.7 cells to a concentration of 10^7 $TCID_{50}$/ml, and a spike containing approximately 3×10^3 $TCID_{50}$ was added to each sample.

Virus Concentration and Nucleic Acid Extraction From Pork Organs and Cuttings

The meat samples (1 cm^3 from three different locations) were collected and stored in a sterile plastic bag. The extraction procedure was based on a mechanical disruption of the tissues followed by a silica-membrane-based RNA extraction (Di Bartolo et al., 2012; Rodriguez-Lazaro et al., 2012). Briefly, each sample (approximately 1 g) was finely chopped using a sterile razor blade, and then 100 mg of homogenate was transferred into a Fast Prep tube containing 200 µl phosphate-buffered saline (PBS) and 2 g of sterile 1-mm zirconia beads (BioSpec Products, Inc., Bartlesville, OK, United States). Twenty microliters of MNV-1 ($\sim 3 \times 10^3$ $TCID_{50}$) was added to each tube. The tube was then placed into a mechanical disruptor (FastPrep-24, MP Biomedicals, Santa Ana, CA, United States) and subjected to two cycles at a speed of 4 m $\times$ s^{-1} for 40 s. Afterward, 1 ml QIAzol (Qiagen, Hilden, Germany) was added to the tubes and vortexed 30 s, and the mixture was dispensed into a new Fast Prep tube and incubated 5 min at room temperature. At that point, 200 µl chloroform:isoamyl alcohol (24:1 Sigma Aldrich) was added, the mixture was vortexed 15 s and incubated 5 min at room temperature and then centrifuged at 10,000 $\times$ *g* for 15 min at 4°C. The resulting supernatant was used for immediate nucleic acid

extraction using RNeasy Lipid Tissue Mini kit (Qiagen, Hilden, Germany) following manufacturer instructions, and the final 100 µl RNA extract was assayed immediately or stored at −80°C.

Virus Concentration and Nucleic Acid Isolation From Pork Feces and Serum

Two hundred fifty milligrams of samples were transferred to a 15-ml centrifuge tube and suspended in 2.25 ml of PBS containing gentamycin (10 mg/ml), and 20 µl of the SPCV ($\sim 3 \times 10^3$ $TCID_{50}$) was added to the sample. The suspension was vortexed for 60–90 s and centrifuged at 3,000 × *g* for 15 min. For serum analysis, 20 µl of the SPCV ($\sim 3 \times 10^3$ $TCID_{50}$) was added to 1 ml of blood, and the blood sample was centrifuged at 2,500 × *g* for 10 min. The supernatants from fecal or serum samples were then immediately used for nucleic acid isolation or stored at −80°C. Nucleic acids were extracted using a QIAamp viral RNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The final elution was performed twice with 50 µl elution buffer, resulting in a 100-µl nucleic acid extract. The nucleic acid extract was assayed immediately or stored at −80°C until analysis.

Virus Detection by RT-qPCR

The presence of the target virus (HEV) and the SPCV (MNV-1) was evaluated using reverse transcription real-time PCR (RT-qPCR). All reaction mixes included an internal amplification control (IAC), which was constructed as described by Diez-Valcarce et al., 2011a,b.

One-step duplex RT-qPCRs were performed using the oligonucleotides, controls, and conditions previously described (Diez-Valcarce et al., 2011b, 2012; Martinez-Martinez et al., 2011; Di Bartolo et al., 2012; Rodriguez-Lazaro et al., 2015). The thermocycling conditions varied slightly: 15 min at 50°C, 2 min at 95°C, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C. All RT-qPCRs were conducted in a duplex format, targeting the specific viruses (HEV or MNV-1) with a FAM-labeled probe and the chimerical IAC using a VIC-labeled probe. All tests also included negative controls for viruses and for IACs.

Reporting and Interpretation of Data

For a proper interpretation of the results, four different signals were considered: (i) the target virus; (ii) the SPCV virus; (iii) the target IAC; (iv) the SPCV IAC (D'Agostino et al., 2011). When a PCR assay showed a Cq (quantification cycle) value $\leq$ 40, independently of the corresponding IAC Cq value, the result was interpreted as positive. When an assay showed a Cq value $\geq$ 40 with the corresponding IAC Cq value $\leq$ 40, the result was interpreted as negative. When both the target and its corresponding IAC showed Cq values $\geq$ 40, the reaction was considered to have failed. When at least one of the replicate HEV assays was positive, the sample was considered to be positive. In the absence of signals for SPCV and its IAC, the pre-amplification process (virus concentration and extraction steps) was concluded to have failed (D'Agostino et al., 2011). When signals for SPCV and its IAC and target IAC were present, the absence of target virus signal was conclusively considered a test negative result.

Extraction Efficiency

The extraction efficiency was calculated by comparing the Cq value of the sample containing the control (SPCV) with the Cq value of the SPCV alone, just spiked in the reagents used for concentration and extraction of the sample but without any food matrix, using the following formula: $2^{(CqTNPC - Cqsample)} \times 100$ (Diez-Valcarce et al., 2012). Efficiency results were classified as insufficient (extraction efficiency <5%), acceptable (5–25%), good (25–50%), and very good (>50%). Extraction efficiencies lower than 5% were not acceptable, and the pre-amplification process (virus concentration and extraction) of the given sample was repeated.

HEV Genotyping

Positive samples for HEV were subjected to sequence analysis, partially amplifying and sequencing ORF2, as described previously (Munoz-Chimeno et al., 2016). Phylogenetic analyses were performed with the Mega 7.0 using the method neighbor-joining with 1,000 bootstrap.

Statistical Analysis

Calculations for descriptive statistics were carried out using the WINPEPI (PEPI-for-Windows) computer programs for epidemiologist V.11.30 (Abramson, 2004). All data were compared using the χ^2 test with 95% CIs, and a p-value < 0.05 was considered statistically significant. In addition, a generalized linear regression model with mixed effects (GLM) was performed considering the type of sample collected (loin head, heart, ribs, diaphragm, liver, liver exudate, lean ham, bacon, kidney, feces, and blood) and slaughterhouse (nine establishments from A to I) as explanatory variables against the binary response variable (i.e., detection of HEV by using RT-qPCR method). A backward selection method was chosen, and mean estimated parameters together with goodness-of-fit indices were obtained. The latter corresponded to the log likelihood (logL), Akaike Information Criterion (AIC), and Bayesian Information Criterion (BIC). The model structure was defined as:

$$\log it(\pi) = \log\left(\frac{\pi}{1-\pi}\right) = y_i = \beta_o + \beta_1 \cdot x_{i,1} + \ldots + \beta_{p-1} \cdot x_{i,p-1} + \varepsilon_i \sim \text{Normal}(0, \sigma^2)$$

which models the log odds of probability of the presence of HEV RNA by RT-qPCR (y_i) as a function of a set of explanatory variables ($x_{i,\ 1} \ldots x_{i,\ p-1}$). β_0, β_1, $\ldots\beta_{p-1}$ are the unknown regression parameters, and σ^2 the unknown (constant) error variance. The logit link function (logit π) models the log odds of the mean (π), assuming a binomial distribution of y_i. The software R v.3.5.1[1] was used, taking as a level of significance a p-value < 0.05.

[1]www.cran.rproject.org

RESULTS

Detection of Anti-HEV Antibodies in Pig Sera

Thirty-three of the 45 pigs of the study showed IgG antibodies against HEV, which represents an overall seroprevalence of 73.3% (95% CI: 58.9–84.0). Seropositive animals were found in all of the nine slaughterhouses evaluated in this study.

Efficiencies of HEV Nucleic Acid Extraction

The mean virus extraction efficiency of the process was 50.3% with a standard error of 1.83%. Values ranged from 2.26 to 98.4%. Overall, 14.6% of the samples showed acceptable extraction efficiency (5–25%), and 42.2 and 42.6% showed good (25–50%) and very good (>50%) extraction efficiencies, respectively.

Detection of HEV RNA and Distribution Between the Type of Samples and Slaughterhouses

Table 1 summarizes the results obtained for the presence of HEV RNA in the 10 different types of samples tested in this study. Significant differences in the presence of HEV RNA were obtained ($p < 0.001$). Although only 26 out of 450 samples (5.78%; 95% CI: 3.97–8.33%) were positive by the HEV-specific RT-qPCR, those samples came from 20 pigs; that is, 20 pigs were positive for at least one of the 10 types of analyzed samples, which represents a 44.4% (95% CI: 30.9–58.8%) of the total of pigs tested (**Table 2**). However, only four pigs were HEV RNA-positive in two or more samples tested (one and three pigs with four and two HEV RNA-positive samples, respectively) (**Table 2**). Consequently, there were 16 animals (35.6%; 95% CI: 23.3–50.2%) with only one positive sample, three (6.67%; 95% CI: 2.29–17.9%) with two positive samples and one (2.22%; 95% CI: 0.39–11.6%) with four positive samples. In three of those four animals, one of the positive samples was feces (**Table 2**).

The mean Cq values were very low (37.3 ± 0.6 SE) regardless the type of sample analyzed, ranging from 27.8 to 39.9. Interestingly, the distribution of positive samples varied according to the type of samples analyzed; whereas the muscle type samples were all negative (except for a single positive sample in the case of the diaphragm), the number of positive samples was significantly higher in the case of samples from organs (liver: $n = 7$, 15.5%; kidney: $n = 5$, 11.1%; and heart: $n = 4$, 8.89%) or from stool samples ($n = 6$, 13.3%) and serum ($n = 3$, 5.57%) (**Table 1** and **Supplementary Table 1**). Regarding slaughterhouses (from A to I), all of them apart from E had HEV-positive samples. The highest prevalence corresponded to F (15.2%; 95% CI: 4.8–25.6%) with 7 out of 46 positive samples whereas for A, B, C, D, G, H, and I, average prevalence of HEV ranged from 3.9 to 7.8%. Although some variability was observed between slaughterhouses, differences were not statistically significant ($p = 0.186$).

HEV Genotyping

According to the mean Cq values, sequencing yield of the samples for HEV genotyping was low; however, two sequences were obtained (from liver samples of pigs 1 and 28). After phylogenetic analysis (**Figure 1**), Hi1 sequence was identified as genotype 3f, and Hi28 sequence showed a high identity with the KU513561 Spanish sequence, previously described in humans, which is pending of subtype assignment (Munoz-Chimeno et al., 2016).

Correlation Between ELISA and RT-qPCR Results

Serology by ELISA and RT-qPCR for detection of HEV RNA in blood indicated that positive results obtained by both methods were not significantly correlated (Pearson $\chi^2 = 0.073$; $p = 0.793$). Similarly, seven animals tested negative for HEV detection both in ELISA and in the RT-qPCR, which indicates that only a 15.5% (95% CI: 7.75–28.7%) of the pigs had not been in contact with the HEV. Similarly, 18 pigs (40.0%; 95% CI: 27.0–54.5%) tested seropositive but were negative by the HEV-specific RT-qPCR (**Table 2**), which highlights that although the animals were not infectious at the moment of slaughter, they had been in contact with the virus previously. Fifteen pigs were seropositive and also tested positive by RT-qPCR in at least one of the samples analyzed (33.3%; 95% CI 21.3–47.9%); HEV RNA was detected in four liver, four kidney, and four heart samples and in three feces (**Table 2**); 12 animals tested RNA-positive in one of the samples, and the three remaining pigs were positive in two samples (feces and liver; feces and kidney; serum and heart). Finally, five animals tested seronegative but were positive by RT-qPCR in at least one of the samples analyzed for each animal (11.1%; 95% CI: 4.84–23.5%). From those animals, two excreted HEV in feces (RNA-positive), although they tested negative by both RT-qPCR and ELISA in the rest of the samples, two tested RNA-positive only in liver or kidney, respectively, and finally one

TABLE 1 | Overall results of hepatitis E virus (HEV)-specific ELISA and RT-qPCR tests.

	ELISA	HEV qPCR										
	Serum	Serum	Feces	Liver	Kidney	Heart	Diaphragm	Bacon	Loin head	Rib	Lean	Total
Positive samples % (95% CI)	33 73.3% (58.9–84.0%)	3 6.7% (2.3–17.9%)	6 13.3% (6.3–26.2%)	7 15.6% (7.7–28.8%)	5 11.1% (4.9–23.9%)	4 8.9% (3.5–20.7%)	1 2.2% (0.4–11.6%)	0	0	0	0	26 5.8% (4.0–8.3%)
Cq values M ± ES	n.a.	36.0 ± 2.0	36.6 ± 1.9	36.6 ± 1.1	38.7 ± 0.6	38.7 ± 0.4	36.0	0	0	0	0	37.3 ± 0.6

TABLE 2 | Distribution of positive samples for hepatitis E virus (HEV)-specific ELISA and RT-qPCR tests according to the type of samples.

N. samples	ELISA	HEV qPCR									
	Serum	Serum	Feces	Liver	Kidney	Heart	Diaphragm	Bacon	Head loin	Rib	Lean
1	−	−	−	+	−	−	−	−	−	−	−
1	−	−	−	−	+	−	−	−	−	−	−
1	−	+	+	+	−	−	+	−	−	−	−
1	+	+	−	−	−	−	−	−	−	−	−
1	+	+	−	−	−	+	−	−	−	−	−
1	+	−	+	−	−	−	−	−	−	−	−
1	+	−	+	+	−	−	−	−	−	−	−
1	+	−	+	−	+	−	−	−	−	−	−
2	−	−	+	−	−	−	−	−	−	−	−
3	+	−	−	−	+	−	−	−	−	−	−
3	+	−	−	−	−	+	−	−	−	−	−
4	+	−	−	+	−	−	−	−	−	−	−
7	−	−	−	−	−	−	−	−	−	−	−
18	+	−	−	−	−	−	−	−	−	−	−
Positive samples	33	3	6	7	5	4	1	0	0	0	0

seronegative animal was RNA-positive in diaphragm, liver, fecal, and serum samples.

GLM Model for Detection of HEV RNA

The estimations obtained by the GLM model are represented in **Table 3**. Results from loin head samples together with those from slaughterhouse E were considered controls because all samples were negative. Considering the control group, the odds of having one HEV RNA-positive sample were calculated for the interactions between type of sample and slaughterhouse. Overall, all single factors were considered non-significant terms in the GLM model ($p > 0.05$). Furthermore, the odds ratio (OR) was estimated, quantifying the strength of the association between individual explanatory variables (type of sample and slaughterhouse) and the response variable (presence of HEV RNA). The strongest association was found for the kidney samples from slaughterhouse B (OR = 1.64, 95% CI: 1.11–2.44), meaning that the odds of finding one HEV RNA-positive sample was 64% higher than the control group for this combination. Other significant interactions were obtained for liver and kidney samples from slaughterhouses F, C, and G and for feces and blood samples from slaughterhouses A and F (**Table 3**). The odds of finding one HEV RNA-positive sample were 49% higher than the control group for these combinations.

The goodness of fit indices AIC, logL, and BIC were estimated as −14.31, 131.32, and 403.90, respectively. Regarding model predictions, considering a cutoff value of 0.10 for the probability of having one HEV RNA-positive sample in the collected samples, 84.06% of the cases observed as negative were correctly predicted by the model, whereas 15.94% of observed negative cases were misclassified as positive (fail-safe). However, all positive cases were correctly classified by the model as such, thus indicating the high discriminatory power of the GLM model according to the studied factors.

DISCUSSION

In this study, anti-HEV antibodies detection in serum and molecular analyses of 10 different samples (serum, feces, liver, diaphragm, kidney, heart, bacon, head of loin, rib, and lean) was performed in 45 apparently healthy pigs from different farms and collected from nine slaughterhouses geographically widespread in Spain. The overall seroprevalence obtained by an ELISA test, 73.3% (33/45), was not unexpected because a high anti-HEV antibody prevalence has been observed in an apparently healthy swine population since the 1980s in Spain (Casas et al., 2009b, 2011; Jimenez de Oya et al., 2011). This result is also in agreement with reports from a variety of European countries, as the seroprevalence described in farmed pigs ranged from 30 to 98% (Salines et al., 2017). Besides, in swine abattoirs, results are similar as for example a 59% (55.5–61.4%) of HEV seroprevalence was recently found in France (Feurer et al., 2018). Interestingly, in our study, a total of seven animals resulted negative for HEV detection by both ELISA and RT-qPCR tests, which indicates that only a maximum of 15.56% (95% CI: 7.75–28.7) was not previously in contact or infected by HEV. Not surprisingly, the presence of anti-HEV IgG in serum was higher (73.3%) than the presence of the virus detected by RT-qPCR (44.4%) in concordance with previous studies (Di Bartolo et al., 2011), and more than a half of the seropositive animals (18/33) were negative to the RT-qPCR (54.5%, 95% CI: 37.9–70.1), indicating a previous contact with HEV, but not current infection. This fact can be explained by the decrease in the prevalence of HEV RNA detection between 3 and 6 months described by previous studies (Casas et al., 2011). Five seronegative animals were positive for the RT-qPCR in different samples analyzed (41.7%, 95% CI: 19.3–68.1). Similar results were observed in the study of Di Bartolo et al. (2011) in which 4/6 seronegative animals were positive for the presence of viral RNA in bile, feces, and/or liver samples (Di Bartolo et al., 2011). Among them, two

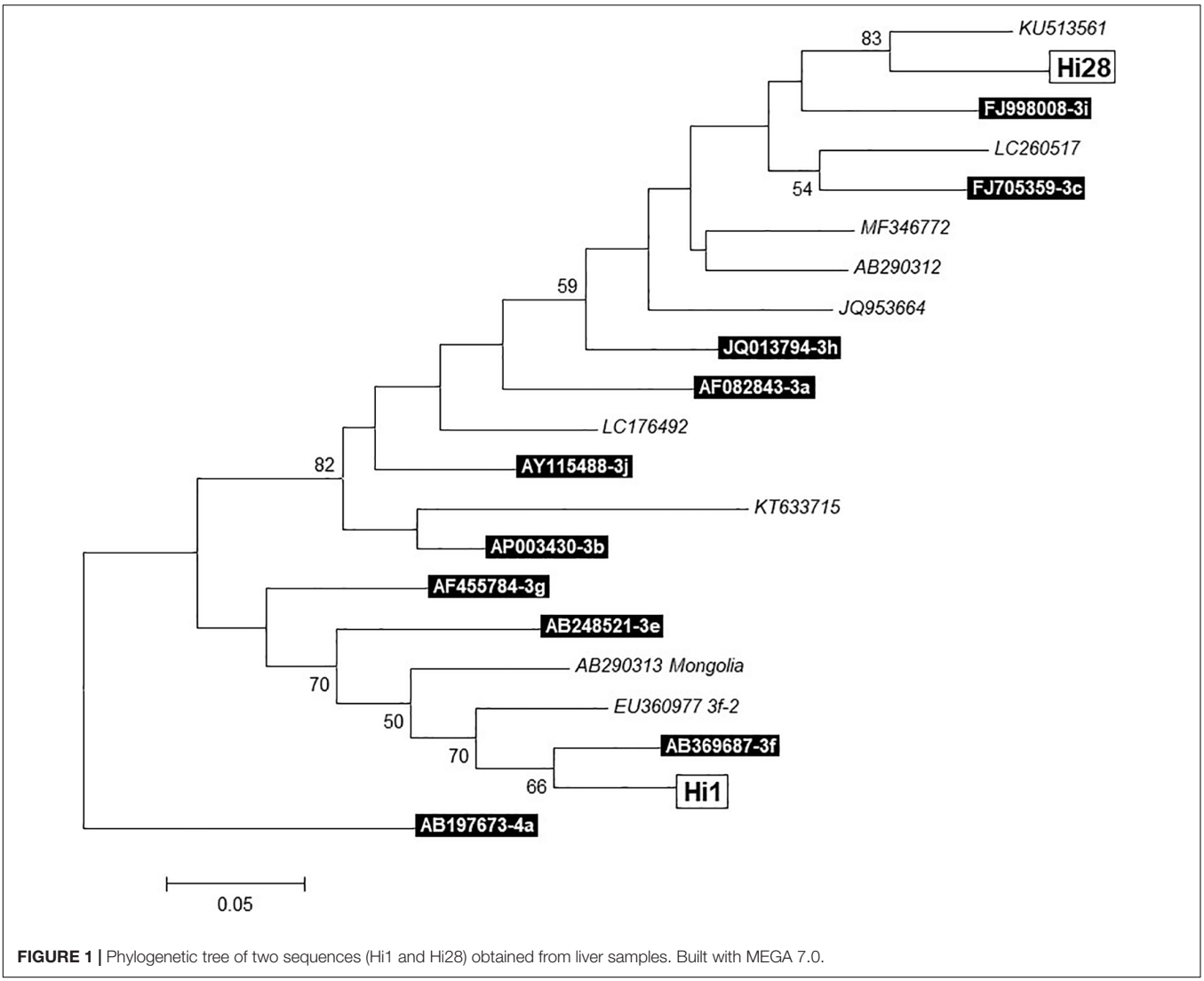

FIGURE 1 | Phylogenetic tree of two sequences (Hi1 and Hi28) obtained from liver samples. Built with MEGA 7.0.

TABLE 3 | Estimations of the generalized linear regression model with fixed effects for the presence of hepatitis E virus (HEV) RNA by RT-qPCR as a function of the type of sample and slaughterhouse.

Variable	Estimate	Std. Error	*t* value	*p*-value	Odds Ratio
Faces × Slaughterhouse A	0.400	0.195	2	0.0404*	1.49 (95% CI: 1.01–2.18)
Kidney × Slaughterhouse B	0.500	0.201	2	0.0130*	1.64 (95% CI: 1.11–2.44)
Kidney × Slaughterhouse C	0.400	0.195	2	0.0404*	1.49 (95% CI: 1.01–2.18)
Liver × Slaughterhouse F	0.400	0.195	2	0.0404*	1.49 (95% CI: 1.01–2.18)
Blood × Slaughterhouse F	0.400	0.195	2	0.0404*	1.49 (95% CI: 1.01–2.18)
Liver × Slaughterhouse G	0.400	0.195	2	0.0404*	1.49 (95% CI: 1.01–2.18)

** Non-significant in the GLM model ($p > 0.05$).*

animals excreted the HEV by feces, but they tested negative in other samples, which could indicate the pass of the virus through the intestinal system after oral ingestion without any replication of the virus, as other authors have suggested (Di Bartolo et al., 2011). Besides, one animal was positive only in liver and another one only positive in the kidney. Finally, one seronegative animal was HEV RNA-positive in diaphragm, feces, liver, and serum samples, although only IgG antibodies have been detected. Absence of anti-HEV antibodies (IgG, IgM, and IgA) in pigs with HEV RNA in muscle has been described before in an experimental study of coinfection with porcine reproductive and respiratory syndrome virus (PRRSV), hypothesizing that the cause could be HEV replication in muscle cells favored by PRRSV or an interaction between heparin

sulfate expressed at the surface of muscle cells with HEV particles during a long-term viremia (Salines et al., 2019a). This also could be explained by recent viral infection in which no immune response is detectable or due to a chronic infection in which antibodies may disappear because they do not persist for a long time, as other studies had demonstrated (Kanai et al., 2010).

Twenty animals tested HEV RNA-positive for at least one of the 10 samples analyzed, which indicated that in a high percentage of the pigs tested (44.4%, 95% CI: 30.9–58.8), HEV had disseminated through the organism, similar to the results previously obtained by Di Bartolo et al. (2012), with 38% (15/39) positive samples in a Spanish slaughterhouse. This is also in accordance with other authors who also reported the presence of HEV RNA in many different samples, such as lymph nodes, bladder, liver, bile, or tonsils collected from pigs in abattoirs (Leblanc et al., 2010; Raspor Lainscek et al., 2017; Feurer et al., 2018). Similarly, a total of 26 samples (5.78%) tested RT-qPCR-positive for HEV in our study: liver (n = 7; 15.5%), feces (n = 6; 13.3%), kidney (n = 5; 11.1%), heart (n = 4; 8.89%), serum (n = 3; 6.67), and diaphragm (n = 1; 2.22%) (**Table 1** and **Supplementary Table 1**). Cq values ranged from 27.8 to 39.5, indicating a different viral load, although no association was observed between the Cq value and the type of sample (**Table 2**). As expected, liver was the most frequently positive sample identified with 15.6% (95% CI: 7.75–28.8), as it is the target organ for HEV replication (Lee et al., 2009). In previous studies, liver, also with the bile, had been described as the highest infected tissue (de Deus et al., 2007; Leblanc et al., 2010). In contrast, other studies describe feces as the sample with the highest prevalence for HEV presence (Di Bartolo et al., 2012; Raspor Lainscek et al., 2017). The liver positivity rate evidenced in our study (15.6%) is similar to the one obtained in Italy (20.8%) (Di Bartolo et al., 2011). However, it is higher than results obtained by other researchers in different regions, including Europe, Africa, and South America, and summarized in 2017 by Salines et al. (2017) with a mean of 5.3% (from 0.8% in Cameroon to 10% in Canada). This must be explained by the fact that some risk factors have been associated with the presence of HEV RNA in pig liver such as coinfection with PRRSV (Salines et al., 2019b), age, genetic background, or lack of hygienic measures (Walachowski et al., 2014). Nevertheless, it must be taken into account that in the present study, as in most of the published works, the presence of the HEV genetic material (HEV RNA) had been demonstrated, but the potential infectivity was not evaluated due to the difficulty of systems, which determine the viability of the virus (reliable cell culture systems or animal experimental models). However, HEV-contaminated pig liver can enter the food chain with the consequent risks demonstrated by numerous studies. In the review of Salines et al. (2017), nine different studies conducted on market pork products (raw livers, sausages, paté, etc.) were analyzed, and contamination with HEV ranged from 1 to 50% depending on the product analyzed and on the country where the survey was conducted. The highest prevalence was in products made with raw liver such as figatelli from France (Pavio et al., 2014), confirming previous studies that indicated that liver is a risk product for HEV infection, especially if it is consumed raw or undercooked, not only from pigs but also from wild boars and deer (Yazaki et al., 2003; Mizuo et al., 2005; Feagins et al., 2007). Our findings highlight that liver could be contaminated with HEV and could represent a risk for the consumer if is not well-cooked and confirms that pig liver and liver-made products must be controlled.

Similarly, six of 45 animals were shedding HEV in feces at the time of slaughterhouse, reaching a positivity of 13.3% (95% CI: 6.26–26.2), similar (6/43) to that observed in France (Leblanc et al., 2010) but lower than those observed in other countries 20–32% in Portugal (Berto et al., 2012), 21.5% in the United Kingdom (McCreary et al., 2008), 33.3% in Italy (Di Bartolo et al., 2011), or 55% in Denmark (Breum et al., 2010). It is important to highlight that as pigs are excreting the HEV in the feces, it is indispensable to optimize hygienic measures in abattoirs to avoid cross contamination with materials that could enter the food chain.

A very interesting finding in our study was that only one muscle sample (1/225; 0.44%) was HEV RNA-positive. This animal was also RT-qPCR-positive in feces, liver, and serum but was negative to the ELISA test. HEV presence in pig muscle has been demonstrated in experimental studies (Williams et al., 2001; Bouwknegt et al., 2009; Salines et al., 2019a), but few studies have been performed to establish the presence of HEV in pig muscles in naturally infected animals. Our findings are in agreement with the studies conducted in French and Canadian slaughterhouses in which the HEV seroprevalence was high, but the virus was not found in muscle samples (Leblanc et al., 2010; Feurer et al., 2018) and with a longitudinal study performed in Denmark (Krog et al., 2019). Also, one study analyzing pork products at the market failed in the detection of HEV RNA in pork chops and fresh sausages (Boxman et al., 2019). Some studies have detected HEV RNA in meat samples, but the prevalence was very low; one study performed on lingual muscle revealed a prevalence of 3 and 6% in Czechia and in Italy, respectively, whereas no positive samples were found in Spain (Di Bartolo et al., 2012). Cross contamination with feces, bile, or utensils cannot be ruled out (Di Bartolo et al., 2012).

Studies linking boar meat (Matsuda et al., 2003; Tamada et al., 2004; Li et al., 2005; Masuda et al., 2005; Wichmann et al., 2008; Rivero-Juarez et al., 2017) and also deer meat (Tei et al., 2003, 2004) with hepatitis E cases have been described since many years ago. Reports were mainly from Asia probably due to consumption habits (eating raw or undercook food). However, cases directly associated with pork meat are limited to the study of Deest et al. (2007), who reported the case of two patients who had eaten undercooked pig meat 4 weeks before suffering hepatitis E (Deest et al., 2007). Besides, in a case control study in Germany, raw pig meat and sausage were not associated with HEV cases (Wichmann et al., 2008). Although pork meat is not usually consumed raw, it is more probably that pig meat is not frequently contaminated with HEV or the viral

load is too low to cause infection, confirmed by previous studies (Leblanc et al., 2010; Feurer et al., 2018) and the present study. The few studies previously performed on pig muscle have failed in the detection of HEV (Leblanc et al., 2010; Crossan et al., 2015; Krog et al., 2019).

This study is the first description of HEV RNA in kidney or heart in naturally infected pigs with five and four positive for heart and four kidney samples, respectively. These organs are not usually evaluated for the presence of HEV in pigs, and only two experimental studies have tested these organs (Williams et al., 2001; Bouwknegt et al., 2009). Extrahepatic dissemination in pigs was confirmed (Williams et al., 2001; Bouwknegt et al., 2009; Thiry et al., 2016), although the viral load was lower in those localizations (Krog et al., 2019). This fact is corroborated in the present study as the mean Cq values were higher in heart and kidney (**Table 1** and **Supplementary Table 1**). To explain the HEV distribution to other organs or tissues other than the liver, different causes could be invoked, such as other concomitant diseases (e.g., PRRSV), which could influence the infection dynamics (Salines et al., 2019b).

CONCLUSION

In conclusion, our findings highlight that the Spanish pigs were frequently in contact with HEV previously (high seropositivity rate). In addition, and at the moment of slaughter, HEV could be present in pig liver, the virus could be being actively excreted (HEV RNA found in feces), and even in some cases, pigs could display viremia (HEV found in serum). Unfortunately, we only obtained two HEV sequences that limit gaining a better understanding of HEV transmission between pigs and humans. However, our results demonstrate that HEV appears to be almost always absent in different pork muscles, so pork meat could be considered as a low risk material for HEV infections *via* foodborne route. To confirm this hypothesis, future studies that include a larger number of animals are needed.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding authors.

AUTHOR CONTRIBUTIONS

NG, JG, DR-L, LD, and MH designed the study. CB collected the samples at slaughterhouses. MG-B, FE, IM, AF-M, and AN performed the laboratory analysis. SG and AA designed the tables and figures. AA and MM-C performed the genotype analysis, including ORF amplification, sequencing, and phylogenetic analysis. AV, GF, and JE performed statistical analysis of the data obtained. NG and DR-L analyzed the data. NG drafted the first version of the manuscript. DR-L and SG modified and adapted the draft version, which was subsequently revised by MH and JG.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.02990/full#supplementary-material

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frontiers in Microbiology

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Spatial-Temporal Dynamics of Hepatitis E Virus Infection in Foxes (*Vulpes vulpes*) in Federal State of Brandenburg, Germany, 1993–2012

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Hepatitis E virus (HEV) is the main course for acute hepatitis in humans throughout the world. Human associated genotypes 1 and 2 as well as zoonotic genotypes 3 and 4 are grouped in the species *Orthohepevirus A*. In addition, a large variety of HEV-related viruses has been found in vertebrates including carnivores, rats, bats, and chickens, which were classified in species *Orthohepevirus B-D*. In 2015, partial genome sequences of a novel hepevirus were detected in feces of red foxes (*Vulpes vulpes*). However, no further information about virus circulation and the prevalence in foxes was available. We therefore assayed a unique panel of 880 transudates, which was collected from red foxes over 19 years (1993–2012) in Brandenburg, Germany, for HEV-related viral RNA and antibodies. Our results demonstrate a high antibody prevalence of HEV in red foxes, which oscillated annually between 40 and 100%. Molecular screening of the transudates revealed only a single RNA-positive sample, which was assigned to the carnivore species *Orthohepevirus C* based on the amplified partial sequence. These data indicate that the virus is circulating widely in the fox population and that foxes are carriers of this virus.

Keywords: hepatitis E virus, foxes, infection, transudates, *Orthohepevirus C*

INTRODUCTION

HEV is a member of the genus *Orthohepevirus* of the *Hepeviridae* family and causes acute liver diseases in humans. The virus consists of a single stranded RNA genome of positive polarity with a length of approximately 7.2 kb, which contains three open reading frames (ORF1-3). ORF1 encodes a nonstructural and further processed protein, which includes the RNA-dependent RNA polymerase (RdRp), ORF2 encodes the viral capsid protein and ORF3 for a small phosphoprotein, which is necessary for viral release (LeDesma et al., 2019).

The human associated as well as zoonotic genotypes are grouped into the *species Orthohepevirus A*, which includes a total of 8 genotypes, originating from pig, wild boar, rabbit, and camel species. *Orthohepevirus B* consists of avian hepatitis E virus species causing the "splenomegaly syndrome" as well as the "big liver and spleen disease" in poultry, whereas *Orthohepevirus C* viruses were isolated from rodents (rats voles and shrew) and carnivores (such as ferrets,

mink and foxes). HEV from bats are classed in the species *Orthohepevirus D*. Finally, fish-related HEV belongs to genus *Piscihepevirus* (Spahr et al., 2018).

To determine and quantify the circulation of HEV in the animal population, numerous monitoring studies have been performed in Europe in the past. Based on serological and molecular results, pigs and wild boars are considered as the main reservoirs of the virus and a potential source of zoonotic transmissions (Van der Poel, 2014). In Germany, activities mainly focused on zoonotic genotype 3 (species *Orthohepevirus A*) found in pig, wild boar, deer, and wild rabbits. The resulting seroprevalences ranged from 1% in deer (Neumann et al., 2016), 35–37% in rabbits (Hammerschmidt et al., 2017; Ryll et al., 2018), 41% in wild boar (Schielke et al., 2009) up to 100% in some pig holdings (Dremsek et al., 2013). Molecular studies revealed that especially rabbits, wild boar, and pigs frequently carried or excreted HEV (Kaci et al., 2008; Baechlein et al., 2013; Vina-Rodriguez et al., 2015; Anheyer-Behmenburg et al., 2017; Hammerschmidt et al., 2017; Ryll et al., 2018).

Epidemiological studies in other species including rats, voles, and small carnivores (e.g. mink and ferrets) assigned HEV sequences to the *Orthohepevirus C* group (Raj et al., 2012; Krog et al., 2013; Ryll et al., 2017, 2019). In Norway rats from Germany, the seroprevalence varied between 14.7 and 41.2% (Johne et al., 2012), while wild carnivores like raccoons and raccoon dogs were seropositive in the range of 37 to 54% (Dähnert et al., 2018). So far, only one red fox (*Vulpes vulpes*) from the Netherlands carried HEV-derived RNA in feces (Bodewes et al., 2013) and sequencing suggested that the virus clustered to the *Orthohepevirus C* group. However, no further serological or molecular data were available for foxes, although this species is considered to be a potential virus reservoir. We therefore undertook a comprehensive HEV surveillance study with a unique panel of fox transudate samples, which were collected over 20 years (1993–2012) in the federal state of Brandenburg, Germany.

MATERIALS AND METHODS

Sample Material

Fox cavity transudate samples were collected during an *Echinococcus multilocularis* surveillance program conducted in the German federal state of Brandenburg. Data on the hunting date, sex, age, and location were recorded for all samples (Conraths et al., 2003).

Hunting Statistics

The population density of red foxes in Brandenburg was deduced by the number of the yearly hunted foxes (**Supplementary Figure S1**) using the hunting index of population density (HIPD), which is calculated from number of foxes shot per km^2 and per year (Bögel et al., 1974).

Serology

Transudates were analyzed by the species-independent HEV-Ab ELISA (AXIOM, Buerstadt, Germany) according to the manufacturers protocol. This commercial kit is a double-antigen sandwich ELISA, which is based on recombinant HEV ORF2 protein as antigen. It is demonstrated as a species independent assay and can detect all immunoglobulin classes (IgG, IgM, and IgA). The specificity of the assay was checked by a modified strip immunoassay recomLine HEV IgG/IgM (Mikrogen GmbH, Neuried, Germany) as well as an in-house western blot. The strip immunoassay was carried out according to the manufacturers manual, but the secondary human antibody was replaced by horseradish peroxidase (HRP) conjugated goat anti-dog IgG (Dianova GmbH, Hamburg, Germany). Positive samples exhibit significant HEV specific signal especially for C-terminal part of ORF-2 capsid protein (**Supplementary Figure S2**). Western blotting was performed with recombinant ORF-2 protein encompassing 239 amino acids of the HEV genotype 3 capsid protein. In short, after SDS gel electrophoresis in a 16% acrylamide gel, protein was transferred to a PVDF membrane by semi-dry electroblotting. After blocking 30 min at room temperature with 5% skim milk (Difco) in PBS/0.1% Tween 20, the membranes were incubated for 1 h at RT with corresponding fox transudates in 1:100 dilution. The membranes were then washed three times for 10 min in PBS/0.1% Tween 20, and a secondary HRP-conjugated goat anti-dog antibody (Dianova GmbH, Hamburg, Germany) diluted 1:1,000 was incubated for 1 h. After a second washing step, the proteins were visualized by chemiluminescence detection with ECL substrate (ThermoFisher) and VersaDoc Imaging system (**Supplementary Figure S3**).

RNA Isolation and Molecular Analysis

RNA was extracted by QIAmp Viral Mini Kit (Qiagen GmbH, Hilden, Germany) as instructed by the manufacturer and HEV-specific genome copies amplified by a nested real-time RT-PCR protocol using primers targeting the RNA-dependent RNA polymerase (RdRp) region (Vina-Rodriguez et al., 2015; Hammerschmidt et al., 2017). In brief, reverse transcription was carried out with Superscript® III Reverse Transcriptase (Thermo Fisher Scientific Inc., USA) and primers HEV.RdRp_F1 (5′-TCGCGCATCACMTTYTTCCARAA-3′) and HEV.RdRp_R1 (5′-GCCATGTTCCAGACDGTRTT CCA-3′) according to the manufacturers' protocol, followed by 40 cycles of 20 s denaturation at 95°C, 30 s annealing at 50°C, and 1 min elongation at 72°C, finishing with 7 min at 72°C. Subsequently, a nested PCR followed using Maxima SYBR Green/Fluorescein qPCR Master Mix Kit (Thermo Fisher Scientific Inc., USA) and primers HEV.RdRp_F2b (5′-GTGCTCTGTTTGGCCCNTGG TTYMG-3′) and HEV.RdRp_R2 (5′-CCAGGCTCACCR GARTGYTTCTTCCA-3′) according to an established protocol (denaturation for 10 min at 95°C and 40 cycles of 15 s denaturation at 95°C, 30 s annealing at 50°C, 30 s elongation at 95°C, 50°C and 30 s elongation at 95°C). Finally, a melting curve analysis was performed starting with a temperature gradient from 68 to 94°C in increments of 0.2°C. Positive samples were determined by melting peaks and amplicons were excised and subsequently sequenced (Eurofins Genomics, Germany). Standard precautions were taken to prevent PCR contamination including a closed system for PCR amplification and detection. In addition,

preparation of PCR mastermix and primers, RNA-extraction, and final addition of RNA were carried out in separate laboratories.

Phylogenetic Analysis

Phylogenetic analysis was carried out with Geneious Tree Builder using Neighbor-Joining analysis. Genetic distances were calculated using the Tamura-Nei Method. Bootstrap values >70 are displayed at nodes. Phylogenetic analysis was carried out with a 280-nt fragment of the RNA-dependent RNA polymerase gene. Sequence of avian Hepatitis E virus was used as outgroup to root the tree.

Statistical Methods

A 95% confidence intervals (CI) were calculated using R (R Development Core Team, 2008) in *R studio*. Differences in prevalences were compared by the Fisher exact test.

RESULTS

Eight hundred and eighty fox transudates collected from 1993 to 2012 in the federal state of Brandenburg, Germany, were analyzed using in Axiom ELISA and displayed an overall high seroprevalence of about 81% on average, which varied between 48.9 and 100% over the years (**Figure 1**). The exact number is depicted in **Supplementary Table S1**. In order to confirm the immunoreactivity against HEV independently, selected samples were tested by a strip immunoassay (**Supplementary Figure S2**) and an in-house western blot (**Supplementary Figure S3**). The spatial distribution of positive and negative samples throughout the district are displayed in an overview map (**Figure 2A**) and in annual maps (**Figure 2B**). Samples were collected from all 12 districts within the federal state of Brandenburg, but the majority of the 516 samples originated from the two districts Ostprignitz-Ruppin and Prignitz, which are located in the North-West of Brandenburg (**Figure 2A**, selected section).

About 52.0% of the samples were from male individuals and 32.8% from female individuals. The majority of the samples (74%) derived from adult animals (born in a previous year) and 9.2% from juvenile foxes, i.e., animals born in the hunting year (1 April to 31 March) when they were sampled (**Table 1**). No statistically significant age- or sex-associated differences in the seroprevalence were found (**Table 1**).

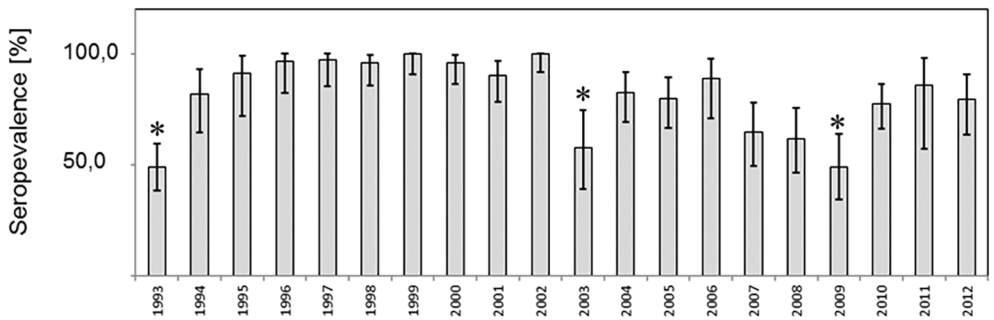

FIGURE 1 | Prevalence of antibodies to Hepatitis E virus among red foxes in the federal state of Brandenburg, Germany. **(A)** Prevalence estimates per year and the respective two-sided 95% confidence intervals are shown. Differences in prevalences were compared by the Fisher exact test und significant differences ($p < 0.05$) indicated by asterics.

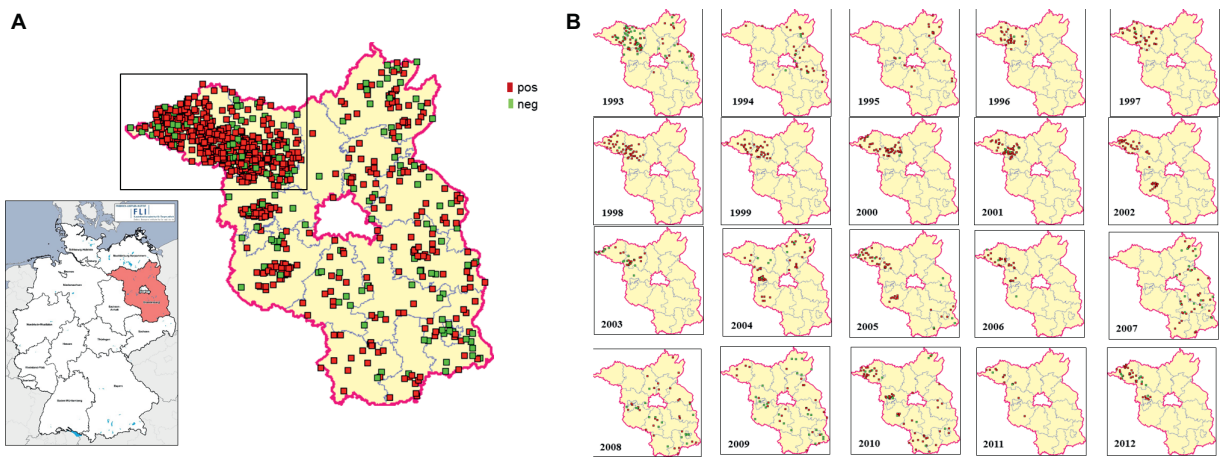

FIGURE 2 | Spatial distribution of HEV-tested fox samples in the federal state of Brandenburg, Germany. The geographic origin of each examined fox sample is plotted on the map. The districts of Ostprignitz-Ruppin and Prignitz, from where the majority of samples were obtained, are marked. Positive samples are represented by red dots, negative samples by green dots. Overview comprising 20 years **(A)** and annual distributions **(B)**.

From 1993 to 1994, a significant increase from 48.9 to 81.1% was observed and after 10 years, a significant decline from 100 to 57.6%, followed by a significant rise from 49 to 77% 6 years later 2009. A similar finding in the seroprevalence was observed for the samples collected in 1993 and 2003 when looking only to the districts Ostprignitz-Ruppin and Prignitz (**Supplementary Figure S4**).

All samples were subjected to a broad-range nested RT-PCR targeting the RNA-dependent RNA-polymerase (RdRp) gene. From one sample, a partial sequence of 280 nucleotides could be recovered (accession number: MN563782), which clustered to the *Orthohepevirus C* group. Phylogenetic analysis revealed high identity of 82–83% to a HEV isolate from a fox from the Netherlands and to a sequence of kestrel in Hungary (**Figure 3**). Pairwise comparison of the corresponding 90 amino acid fragment showed sequence identity of 90 and 89%, respectively, corresponding to 9 and 12 amino acid changes (**Supplementary Figure S5**).

TABLE 1 | Overview of analyzed fox samples. The age of each fox was determined as adult (born in a previous year) or juvenile (born in the year of sampling).

Sample characteristics		Positive [%]	Negative [%]	Total
Sex	Male	367 [80,1]	91 [19,9]	458
	Female	236 [81,7]	53 [18,3]	289
	Unknown	86 [64,7]	47 [35,3]	133
Age	Adult	522 [80,1]	130 [19,9]	652
	Juvenile	70 [86,4]	11 [13,6]	81
	Unknown	97 [66,0]	50 [34,0]	147

DISCUSSION

In this study, we investigated nearly 900 transudate samples from foxes collected in the federal state of Brandenburg collected during a period of 20 years and found a high seroprevalence for HEV in this population. This first report on HEV antibodies in foxes indicates therefore frequent HEV infections in this wild carnivore species. Neither age nor sex effects were observed which speaks for a general infection *via* the oral-fecal route probably by exposure to infectious feces.

Time kinetics showed an oscillation between 40 and 100% per year and a declining prevalence in 1993, 2003, and 2009. The underlying reasons are unknown, but it seems possible that the changes may have been triggered by variation of environmental factors or changes in the social organization pattern. In general, adult foxes are solitary hunters with low contact rates, but individuals have short- and long-term relationships and a seasonal community structure, e.g., during to cooperative raising of cubs (Dorning and Harris, 2019).

Seroprevalence studies from other members of the superfamily *Canidae* are limited. Raccoons and raccoon dogs had a prevalence of about 53.8 and 34.3%, respectively (Dähnert et al., 2018). A disparate picture is seen in dogs where seroprevalences ranged from 0.8% in the UK to 56.6% in Germany up to 88.5% in China (Liu et al., 2009; McElroy et al., 2015; Dähnert et al., 2018).

Only a single HEV genome sequence was extracted, which can be attributed to the age of the samples and a probably short and transient viraemic phase in foxes. In general, transudate fluids and blood/serum samples contain significantly smaller amounts

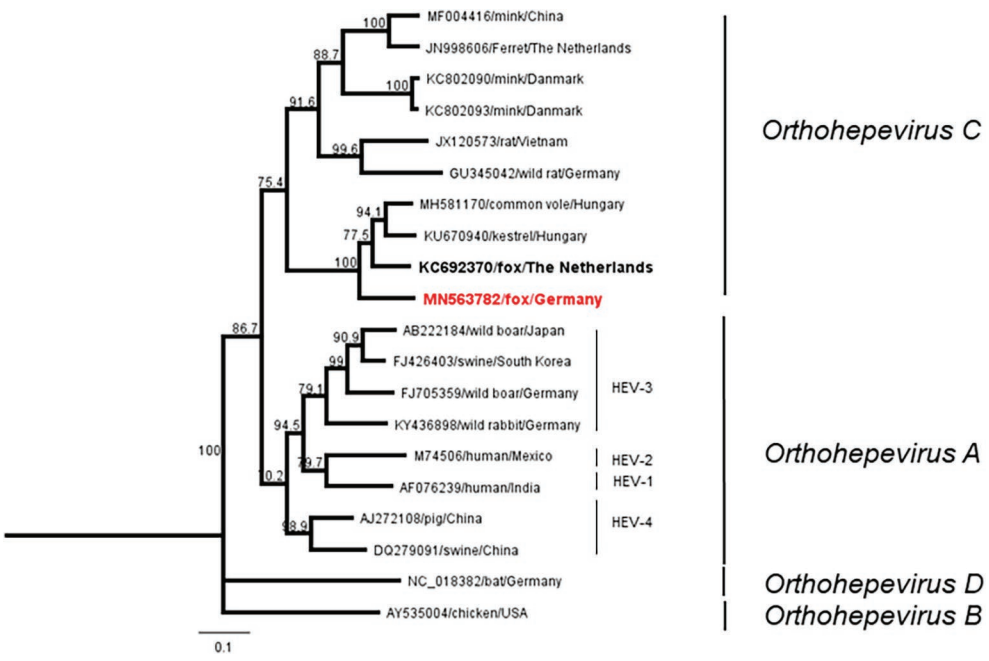

FIGURE 3 | Neighbor-joining phylogenetic tree based on partial RdRp sequences. Red boldface indicates fox sequence from Germany (this study). Boldface displays fox sequence from the Netherlands. Scale bar indicates mean number of substitutions per site.

of viral RNA compared to feces or tissue material. The obtained sequence showed a high identity to a fox feces derived HEV sequence isolated in The Netherlands (Bodewes et al., 2013), and both constitute a potential subclade together with other species including voles and a kestrel. Both sequences cluster with sequences from a kestrel and from vole-associated HEV strains (Reuter et al., 2016; Kurucz et al., 2019) and constitute one distinct clade within the *Orthohepevirus C* species. This includes also sequences isolated most recently from voles in Germany (Ryll et al., 2019).

The results demonstrate endemic HEV infections in a fox population over at least 20 years. As no HEV associated clinical signs in foxes are known to date, this species my perhaps even constitute a reservoir species (Haydon et al., 2002). However, direct information about virus shedding and subsequent infection is lacking. In addition, no information about the virulence of fox HEV and any possible impact on morbidity and mortality is available so far. At least no influence on yearly population density as displayed by means of the *hunting index of population density* (HIPD) could be observed (**Supplementary Figure S6**).

Questions regarding zoonotic character of fox-derived HEV remain open due to the lack of further sequence information and life virus. In general, foxes are the most widespread predators throughout the world and have been recently recognized as potential reservoirs of zoonotic pathogens including trematodes, cestodes, and nematodes (Mackenstedt et al., 2015) as well as *Babesia* spp. and *Theileria* spp. (Najm et al., 2014). In addition, the marked tendency of foxes to establish populations in suburban and urban areas should be kept in mind, as urbanization is a driving force for the emergence of zoonotic diseases across species and a major risk factor for the transmission of such agents to humans (Hassell et al., 2017). A significant example for dispersal of a fox derived zoonosis is alveolar echinococcosis caused by *Echinococcus multilocularis,* which displays transmission routes similar to HEV including fecal shedding and subsequent ingestion of the pathogen (Vuitton et al., 2015).

In principle, members of the species *Orthohepevirus* C may have zoonotic potential as illustrated by a rat HEV isolate that induced a persistent infection in a human patient (Sridhar et al., 2018). The reservoir for rat associated HEV are invasive *Rattus* species like *R. norvegicus* and *R. rattus* (Ryll et al., 2018) that – analogous to red foxes – globally expand to new (sub-) urban areas and thereby provide the appropriate environment for transmission of wild life associated HEV strains to the human population.

More studies are needed to isolate fox HEV from infected animals, to provide further sequence information about fox-associated HEV and to reveal exposure and infection routes between individuals. This will help to gain a deeper understanding of HEV infection patterns and emergence scenarios at the wildlife-livestock-humans interface.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the GenBank BankIt submission, accession number: MN563782.

ETHICS STATEMENT

Ethical review and approval were not required for the animal study because samples were collected from hunted animals during an Echinococcus multilocularis surveillance program conducted in the German federal state of Brandenburg.

AUTHOR CONTRIBUTIONS

ME and MG designed the research. ME, LD, and SS performed the experiments. RS, AV-R, and FC analyzed the data. FC provided the animal samples. ME and MG wrote the paper. FC revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.00115/full#supplementary-material

SUPPLEMENTARY TABLE S1 | Prevalence of antibodies to Hepatitis E virus among red foxes in the federal state of Brandenburg, Germany. Table compiling total number of tested fox samples. Prevalence estimates per year and the respective two-sided 95% confidence intervals are shown.

SUPPLEMENTARY FIGURE S1 | Number of hunted foxes in the federal state of Brandenburg. Source: hunting report Ministry of Rural Development, Environment and Agriculture (https://mlul.brandenburg.de/mlul/de/), German hunting organization (www.jagdverband.de).

SUPPLEMENTARY FIGURE S2 | Strip immunoassay recomLine HEV IgG/IgM. ELISA negative fox (1), positive fox (2), positive fox (3), samples. O2N N-terminal part of ORF-2 protein (HEV capsid protein, genotype 1 and 3) O2M central part of ORF-2 protein (HEV capsid protein, genotype 1). O2C C-terminal part of ORF-2 protein (HEV capsid protein, genotypes 1 and 3).

SUPPLEMENTARY FIGURE S3 | Western blot analysis of ELISA positive (lanes 1–4) and ELISA negative (lanes 5–9) fox samples.

SUPPLEMENTARY FIGURE S4 | Prevalence of antibodies to HEV in fox samples from the districts of Ostprignitz-Ruppin and Prignitz. Prevalence estimates and the respective two-sided 95% confidence intervals are shown. Differences in prevalences were compared by the Fisher exact test und significant differences ($p < 0.05$) indicated by asterics. No samples were received for this region 1995, 2007, and 2008.

SUPPLEMENTARY FIGURE S5 | Amino acid alignment of Fox HEV sequences. Fox HEV Germany (#MN563782), fox HEV, the Netherlands (#KC692370), common vole, Hungary (#MH581170), kestrel Hungary (#KU670940), and common vole Germany (#MK192413).

SUPPLEMENTARY FIGURE S6 | Development of the hunting index of population density (HIPD) of Brandenburg for the period 1993–2012.

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Insertions and Duplications in the Polyproline Region of the Hepatitis E Virus

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Recombinant strains of hepatitis E virus (HEV) with insertions of human genomic fragments or HEV sequence duplications in the sequence encoding the polyproline region (PPR) were previously described in chronically infected patients. Such genomic rearrangements confer a replicative advantage *in vitro* but little is known about their frequency, location, or origin. As the sequences of only a few virus genomes are available, we analyzed the complete genomes of 114 HEV genotype 3 strains from immunocompromised (n = 85) and immunocompetent (n = 29) patients using the single molecular real-time sequencing method to determine the frequency, location, and origin of inserted genomic fragments, plus the proportions of variants with genomic rearrangements in each virus quasispecies. We also examined the amino acid compositions and post-translational modifications conferred by these rearrangements by comparing them to sequences without human gene insertions or HEV gene duplications. We found genomic rearrangements in 7/114 (6.1%) complete genome sequences (4 HEV-3f, 1 HEV-3e, 1 HEV-3 h, and 1 HEV-3chi-new), all from immunocompromised patients, and 3/7 were found at the acute phase of infection. Six of the seven patients harbored virus-host recombinant variants, including one patient with two different recombinant variants. We also detected three recombinant variants with genome duplications of the PPR or PPR + X domains in a single patient. All the genomic rearrangements (seven human fragment insertions of varying origins and three HEV genome duplications) occurred in the PPR. The sequences with genomic rearrangements had specific characteristics: increased net load ($p < 0.001$) and more ubiquitination ($p < 0.001$), phosphorylation ($p < 0.001$), and acetylation ($p < 0.001$) sites. The human fragment insertions and HEV genome duplications had slightly different characteristics. We believe this is the first description of HEV strains with genomic rearrangements in patients at the acute phase of infection; perhaps these strains are directly transmitted. Clearly, genomic rearrangements produce a greater net load with duplications and insertions having different features. Further studies are needed to clarify the mechanisms by which such modifications influence HEV replication.

Keywords: hepatitis E virus, polyproline region, genomic rearrangement, virus-host recombinant variants, virus-virus recombinant variants

INTRODUCTION

The hepatitis E virus (HEV) is a significant human pathogen causing viral hepatitis worldwide. HEV is a member of the *Hepeviridae* family. The genus *Orthohepevirus* includes mammalian and avian strains while the genus *Piscihepevirus* infects Cutthroat Trout (Smith and Simmonds, 2018). The strains infecting humans belong to the *Orthohepevirus A* species. The most prevalent genotype in the industrialized countries, at least in Europe and America, is HEV genotype 3 (HEV-3); it has three major clades: HEV-3abjchi, HEV-3efg, and HEV-3ra (Oliveira-Filho et al., 2013; Smith et al., 2016). The first two clades are mainly found in humans, pigs, wild boar, and deer, and the third in humans and rabbits (Izopet et al., 2012; Abravanel et al., 2017). HEV-3 infections are frequently asymptomatic but they can result in severe acute hepatitis in patients with chronic liver disease (Kamar et al., 2017). HEV-3 can also lead to chronic infection, defined by replication that persists for over 3 months, in immunocompromised patients, including solid organ transplant recipients, patients with hematological disease, and those with an HIV infection. Patients with either acute or chronic hepatitis can suffer from extra-hepatic manifestations (Kamar et al., 2017).

The HEV genome is a single stranded positive-sense RNA about 7.2 kb long that has three open reading frames (ORFs). ORF2 encodes the capsid protein, ORF3 encodes a phosphoprotein involved in virus egress (Kenney and Meng, 2019), and ORF1 encodes a non-structural protein. This protein has several regions: a methyltransferase, a Y domain, a papain-like domain, a polyproline region (PPR), an X domain, a helicase, and an RNA-dependent RNA polymerase (RdRp). The length of the PPR can vary from 189 to 315 nt, depending on the HEV clade and/or subtype. The main PPR length of HEV-3f strains may be 228 nt (HEV-3f-short) or 315 nt (HEV-3f-long) (Lhomme et al., 2014; Nicot et al., 2018). The PPR may be involved in virus adaptation (Shukla et al., 2011; Purdy et al., 2012). The HEV strains infecting chronically HEV-infected patients can contain fragments of human genes, including the S17 ribosomal gene (RPS17), RPS19, human tyrosine aminotransferase gene (TAT), inter-α-trypsin inhibitor gene (ITI) (Shukla et al., 2011; Nguyen et al., 2012; Lhomme et al., 2014), and duplications of the PPR or PPR + RdRp (Johne et al., 2014; Lhomme et al., 2014). This suggests that a prolonged HEV infection favors genomic rearrangements in the PPR but the contribution of an impaired immune response to these recombinant events is not clear. Several *in vitro* studies have shown that a human fragment (RPS17, RPS19, ITI) inserted in the PPR confers a replicative advantage over variants with no human fragments (Shukla et al., 2011; Nguyen et al., 2012; Lhomme et al., 2014), while the duplication of part of the HEV genome (PPR + RdRp) permits HEV adaptation in A549 cell line (Johne et al., 2014). However, the mechanisms that promote virus growth and/or adaptation are largely unknown because of a lack of data.

This study used single molecular real-time (SMRT) sequencing to identify new recombinant HEV genomes, and determine their frequency, location, and origin. We estimated the proportions of variants with genomic rearrangements in each virus quasispecies and identified the features (amino acid composition and post-translational modifications) conferred by the genomic rearrangement and whether human insertions and duplications resulted in different features.

MATERIALS AND METHODS

Patients and Samples

We used stored plasma samples (stored at −80°C) from HEV-infected patients consecutively tested for HEV RNA between 2005 and 2016 in the laboratory of Virology at Toulouse University Hospital, National Reference Center for HEV, where French laboratories can send samples for diagnosis and genotyping. These patients were acutely (HEV replication persisting for less than 3 months) or chronically HEV-infected (HEV replication persisting for more than 3 months). We selected 114 samples containing high HEV virus loads (>100,000 copies/ml) for PacBio SMRT sequencing. The HEV RNA concentrations were determined using a validated real-time polymerase chain reaction (Abravanel et al., 2012). This non-interventional study was supported by Toulouse University Hospital Center. The samples used were part of a collection identified by the French authorities (AC-2015-2518).

The positive control for PacBio SMRT sequencing was the strain VHP6 (passage 6 of TLS-09/M48 virus from the feces of an HEV-infected patient) cultured on HepG2/C3A cells (Lhomme et al., 2014), with two different human genome insertions in the PPR: a fragment of the L-arginine/glycine amidinotransferase (GATM) gene and a fragment of phosphatidylethanolamine binding protein 1 (PEBP1), each variant representing 50% of the quasispecies. Both were characterized by shot-gun deep sequencing (454 GS Junior system). Briefly, six overlapping amplicons were generated. For the library preparation, amplicons were nebulized according to 454 shotgun protocol (Roche/454-Life sciences) and the purified fragmented DNA was further processed according to 454 GS Junior Library construction protocol (Roche/454-Life sciences). The sequencing run was carried out on a Genome Sequencer Junior according to manufacturer instructions (Roche-454 LifeSciences). Data analysis was done with GS *de Novo* Assembler and GS Reference Mapper software.

Single Molecular Real-Time Sequencing of the Complete Hepatitis E Virus Genome

Full length sequences of the HEV genomic RNA were generated as previously described (Nicot et al., 2018). Briefly, two long amplicons (4,500 and 4,200 bp) with an overlap of around 1,450 bp were amplified and then sequenced using P6-C4 chemistry on the PacBio RSII sequencer [at the Toulouse genomic platform[1]] to obtain the entire 7,250 bp HEV genome. The raw PacBio sequences were processed as previously described by a bioinformatics pipeline and manual processing to reconstruct the individual consensus sequences of each complete HEV genome. Several consensus sequences were sometimes obtained for a single

[1]https://get.genotoul.fr/

strain indicating the possible presence of different variants in the virus quasispecies. The HEV genotype was determined by analyzing the complete genome sequence by maximum likelihood phylogenetic analysis (Nicot et al., 2018); all the samples contained HEV genotype 3 (HEV-3). The proportion of each variant was estimated using the count related to each consensus read after the processing on Long Amplicon Analysis v2.0.

The detection of recombinant viruses by SMRT sequencing was validated using the positive control VHP6 characterized by shot-gun deep sequencing. Two variants with inserted fragments were detected using SMRT sequencing: one harboring a fragment of GATM and the other harboring a fragment of PEBP1 (**Supplementary Figure S1**). SMRT sequencing also enabled us to estimate the proportion of each: 50% for VHP6-GATM and 50% for VHP6-PEBP1. Thus, the use of SMRT is appropriate to detect inserted fragments and to determine their proportions.

Complete Genome Annotation

Each complete genome was annotated to determine the three open reading frames and the length of the domains in ORF1 (methyltransferase, Y domain, papain-like domain, PPR, X domain, helicase and RNA dependent RNA polymerase). All possible ORFs were determined with ORF Finder.[2] Each ORF was then submitted to BlastP versus the UniProtKB/SwissProt database[3] to find corresponding sequences and identify ORF1, ORF2, and ORF3. ORF1 was aligned with the best Uniprot BlastP result and the matching domains were collected to create a GFF file which annotated each complete genome.

Identification of Insertion or Duplication in the Hepatitis E Virus Genome

The annotated files were used to determine the length of each region in ORF1, ORF2, and ORF3 so as to identify strains with insertions. Analysis of the PPR took into account the fact that the length could vary from 183 to 315 nt, depending on the HEV clade. All sequences in each clade with longer than normal PPRs were considered to have insertions. The inserted segments were identified by aligning each complete genome sequence with the closest HEV sequence identified by BLAST on NCBI.[4] The origin of the inserted segment (human or HEV genome) was then identified by a BLAST on NCBI. The duplicated regions were determined by aligning them on the complete genome with MUSCLE. The sequences of the recombinant variants have been deposited in the Genbank database under accession numbers MF444083, MF444086, MF444119, MF444145, MF444152, and MN646689-96 (**Supplementary Table S1**).

Characterization of Insertions/ Duplications

The sequences of all the PPRs were identified with reference to the 11,938 sequences of *Orthohepevirus A* (including 338 complete HEV genomes) available in the Virus Pathogen Resource (VIPR) database.[5] Selected sequences were systematically searched to identify insertions so that they could be used, together with those identified by PacBio sequencing, for further analysis. The compositions of HEV PPR insertions/duplications were determined and their post-translational modifications predicted by analyzing a range of parameters. Potential ubiquitination sites were identified using the BDM-PUB server[6] with a threshold of >0.3 average potential score. Potential phosphorylation sites were identified using the NetPhos 3.1 server[7] with a threshold of >0.5 average potential score. Potential acetylation sites were identified using the Prediction of Acetylation on Internal Lysines (PAIL) server[8] with a threshold of >0.2 average potential score. Potential N-linked glycosylation sites were identified using the NetNGlyc 1.0 server[9] with a threshold of >0.5 average potential score. Potential methylation sites were identified using the BPB-PPMS server[10] with a threshold of >0.5 average potential score. Nuclear export signal (NES) sites were identified using the Wregex server[11] with parameters NES/CRM1 and Relaxed. Nuclear localization signal (NLS) sites were identified using SeqNLS[12] with a 0.86 cut-off. The amino acid composition (proportions of amino acids), physico-chemical composition, and net load were analyzed with R. Principal component analysis (PCA) is a mathematical algorithm that reduces the dimensionality of the data while retaining most of the variation in a data set. PCA allows to identify new variables, the principal components, which are linear combinations of the original variables (Ringner, 2008). PCA was done (excluding the amino acid composition due to redundancy with physico-chemical properties) to summarize and visualize the information on the variables in our data set (Abdi and Williams, 2010); each variable was then studied independently. An in-house R-pipeline based on the amino acid sequences and the results of each analysis was used to generate bar plots for amino acid composition. The amino acid compositions were assigned to one of two categories: sequences with insertions/duplications (including insertions of human genome and HEV genome duplications) and sequences without insertions/duplications. The other parameters were assigned to one of three categories: sequences with insertions, those with duplications, and sequences without insertion/duplication.

RESULTS

Characteristics of Hepatitis E Virus With Genomic Rearrangements

Complete genome sequences were obtained for HEV strains from 114 HEV-infected patients. Most patients were sampled

[2]https://www.ncbi.nlm.nih.gov/orffinder/
[3]http://www.uniprot.org/
[4]https://www.ncbi.nlm.nih.gov/
[5]https://www.viprbrc.org
[6]http://bdmpub.biocuckoo.org/prediction.php
[7]www.cbs.dtu.dk/services/NetPhos/
[8]http://bdmpail.biocuckoo.org/prediction.php
[9]www.cbs.dtu.dk/services/NetNGlyc
[10]https://omictools.com/bpb-ppms-tool
[11]http://ehubio.ehu.eus/wregex/home.xhtml
[12]http://mleg.cse.sc.edu/seqNLS/index.html

at the acute phase (92/114; 80%), of whom 29 were immunocompetent (29/92; 31.5%) and 63 were immunocompromised (63/92; 68.5%). The remaining 22 samples were taken from chronically infected immunocompromised patients during the chronic phase. Thus, 74.5% (85/114) of the samples were from immunocompromised patients: due to solid-organ transplantation (n = 61), hematological disease (n = 20), solid cancer (n = 2), or an immune disorder (n = 2). We found genomic rearrangements in the genomes of seven strains (7/114; 6.1%: 4 HEV-3f, 1 HEV-3e, 1 HEV-3 h, and 1 HEV-3chi-new). All the genomic rearrangements were found in immunocompromised patients (four solid organ transplant recipients and three patients with a hematological disease) (**Table 1**). Thus, the frequency of genomic rearrangements was 8.2% (7/85) in the immunocompromised patients. Three patients were acutely infected and four were chronically infected. All the genomic rearrangements were located in the PPR. The characteristics of each strain with genomic rearrangements are shown in **Table 2**. Virus-host recombinant variants were detected in six patients (**Figure 1A** and **Supplementary Figure S2**). Interestingly, one patient harbored two different recombinant variants (Hepac-93-2 and Hepac-93-3). Thus, seven recombinant host variants were identified in six patients. Another patient (Hepac-12) harbored three variants with duplications of HEV genes in the PPR (**Figure 1B**). The fragments of HEV genome were from the PPR + X domain (Hepac-12-1) or the PPR alone (Hepac-12-2 and Hepac-12-3) We found mixtures of variants with and without genomic rearrangements in the HEV from three patients infected for three months or less. We confirmed the sequences of all except one (Hepac-93-3, RNA18SP5) of these genomic rearrangement by Sanger sequencing (**Figure 1**). The discrepancy in Hepac-93-3 was due to a deletion of six nucleotides in the sequence obtained by SMRT.

Features of Polyproline Region With Insertion or Duplication

A search in the VIPR database identified eight additional recombinant strains with genomic rearrangement in the PPR: HQ709170 (HEV-3a) with an RPS17 fragment (Shukla et al., 2011), strain JN564006 (HEV-3a) with an RPS19 fragment (Nguyen et al., 2012), strains KC166952, KJ917704, KJ917720 and KJ917717 (all HEV-3f) with an ITIH2 fragment, a PPR + RdRp duplication, a TAT fragment and a PPR duplication

TABLE 1 | Characteristics of the patients infected by HEV-3 strain with genomic rearrangements.

HEV strain	Pathology of the patient	HEV diagnosis	Chronic/acute HEV infection at the time of diagnosis	Plasma HEV RNA concentration (log copies/ml)	Time between HEV infection and detection of genomic rearrangement
6	Chronic lymphoid leukemia	2014	Acute	7.5	<2 months
26	Renal transplant	2013	Acute	5.8	<2 months
94	Hepatic transplant	2013	Acute	6.6	<2 months
93	Hepatic transplant	2013	Chronic	7.5	3 months
154	Chronic lymphoid leukemia	2014	Chronic	5.3	5 months
64	Cardiac transplant	2009	Chronic	6.2	9 months
12	Light chain myeloma	2010	Chronic	7.7	10 months

TABLE 2 | Characteristics of the naturally occurring genomic rearrangements in the PPRs of seven HEV genotype 3 strains.

HEV strain	HEV subtype	Nature of insertion	Number of variants	Name of the recombinant variant	Position in PPR (nt)	Nature of the inserted fragment	Percent of quasi species
6	3h	Human insert	1	Hepac-6	84–239	Ring finger protein 19A (RNF 19A)	100%
26	3chi-new	Human insert	2*	Hepac-26-2	81–230	Human ribosomal protein L6 (RPL6)	21%
94	3f	Human insert	2*	Hepac-94-2	57–164	Ribosomal protein 17S (RPS17)	63%
93	3f	Human insert	3*	Hepac-93-2	166–222	Eukaryotic translation elongation factor 1 alpha 1 pseudogene 13 (EEF1A1P13)	12%
				Hepac-93-3	164–242	18S ribosomal pseudogene 5 (RNA18SP5)	31%
154	3f	Human insert	1	Hepac-154	170–247	Kinesin family member 1B (KIF1B)	100%
64	3f	Human insert	1	Hepac-64	181–318	Zinc finger protein 787 (ZNF787)	100%
12	3e	HEV duplication	3	Hepac-12-1	93–239	PPR + X-domain	67%
				Hepac-12-2	85–237	PPR	22%
				Hepac-12-3	84–239	PPR	11%

Strains with one wild type variant.

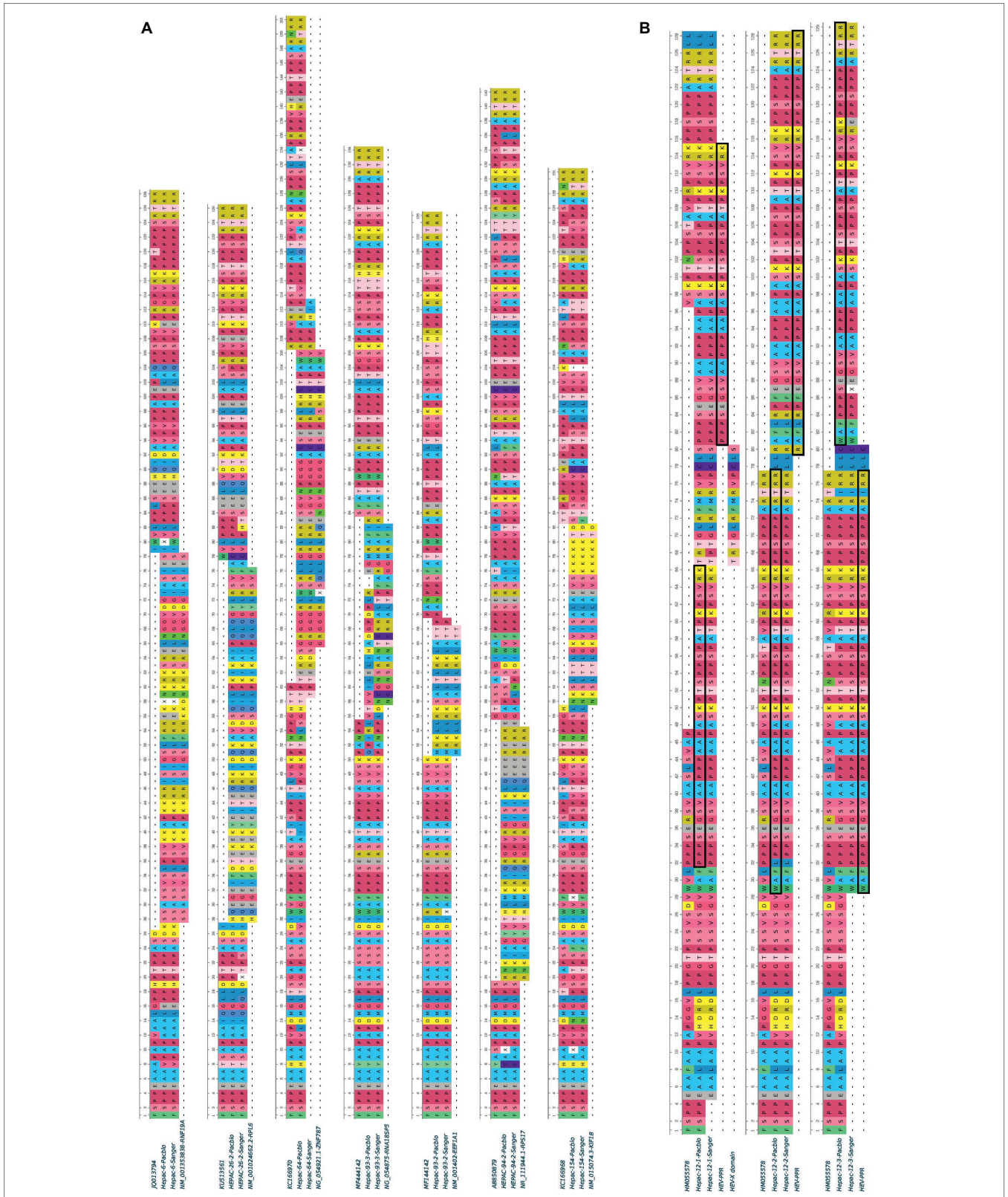

FIGURE 1 | Human fragment insertions and duplications in the PPR of seven HEV GC sequences obtained by SMRT and Sanger sequencing. **(A)** Human fragment inserts. Variants 93-2 and 93-3 were characterized in the same patient. **(B)** HEV genome duplication and reference sequences. PPR duplications are boxed. Hyphen: gap.

FIGURE 2 | Principal Component Analysis of HEV-3 PPR sequences with insertions or duplications. Individual observations, each dot represents a sample. Each clade (3 abjk, 3 chi, 3 efg short or long, and 3ra) is represented by a symbol. The axes show the first two principal components [dimension 1 (dim 1) and dim 2], with the fraction of explained variance in parenthesis. Variables of the two components are detailed in **Figure 3**.

(Lhomme et al., 2014), strain KC618402 (HEV-3c) with a PPR duplication (Johne et al., 2014), and strain KT591534 (HEV-3f) with a PPR duplication not reported as a recombinant virus. Thus, we analyzed 13 PPR sequences with human gene fragment insertions and seven PPR sequences with duplications of HEV genome fragments (**Supplementary Table S1**). As all the genomic rearrangements occurred in HEV-3, analysis of strain with genomic rearrangements only included HEV-3 sequences (n = 294). Principal component analysis (PCA) is a mathematical algorithm that reduces the dimensionality of the data and allows to identify new variables, the principal components, which are linear combinations of the original variables. PCA was used to determine whether some variables in the data set were specific to the genomic rearrangements. The PCA separated sequences with genomic rearrangements from sequences without genomic rearrangements (**Figure 2**). The two principal components represented 43.6% of the variance. A detailed analysis of the components indicated that variables like the net load, positive charge, ubiquitination, acetylation, and phosphorylation seemed to be associated with sequences with genomic rearrangements (**Figure 3**). The features of sequences without genomic rearrangements, including HEV-3f short and long, did not differ with the length of the PPR. The amino acid composition encoded by genomes with and without genomic rearrangements is shown in **Figure 4**. Sequences with genomic rearrangements had increased Arg, Cys, Gly, Lys, and Met contents and decreased Ala, Pro, Trp, and Val contents. Human gene insertions encoded peptides rich in

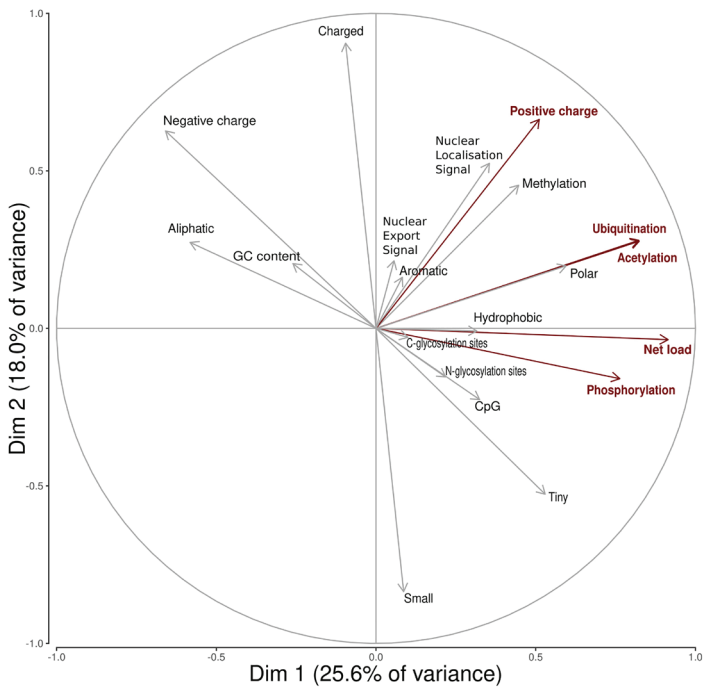

FIGURE 3 | Principal Component Analysis variable circles of correlation. Variables characterizing insertions/duplications are shown in red (positive charge, net load, ubiquitination acetylation, phospohorylation sites). Dim1 is mainly composed of net load (16.4%), ubiquitination (13.3%), acetylation (13.2%), and phosphorylation (11.4%). Dim2 is mainly composed of positive charge (12.2%).

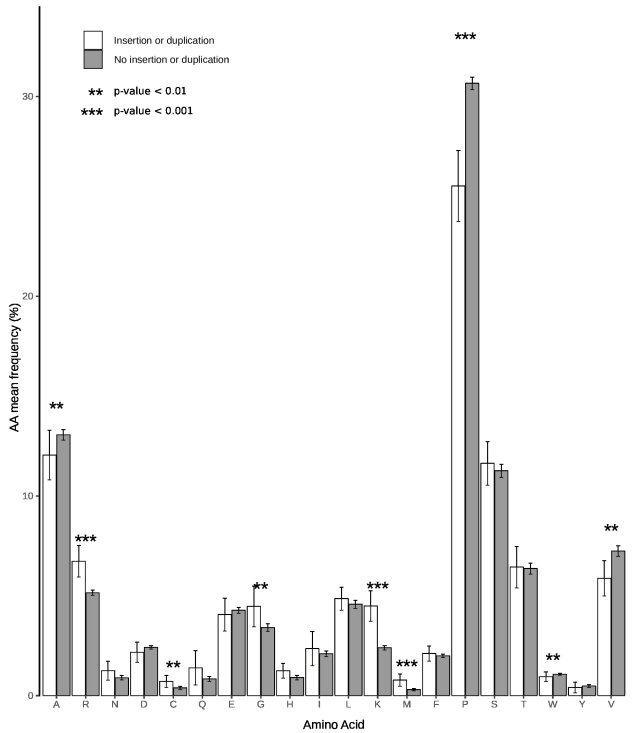

FIGURE 4 | Amino acid compositions of PPR sequences with and without insertions/duplications. White bars represent sequences with insertion/duplication (n = 20) and black bars sequences without insertion/duplication (n = 294). Statistical differences between groups are indicated by stars. A $p < 0.05$ was considered significant.

polar, positively charged amino acids (Arg, Asn, Gln, His, and Lys) and hydrophobic amino acid (Gly, Ile, and His) (**Table 3**). Insertions of HEV genome duplications encoded peptides rich in positively charged amino acids (Lys and Arg) and poor in negatively charged amino acid (Asp and Glu), while PPR sequences with genomic rearrangements had a significantly higher net load than sequences without genomic rearrangements ($p < 0.001$) (**Table 3**). The increased net load due to insertions resulted from increases in positively charged amino acids, whereas the increases caused by duplications were mainly due to fewer negatively charged amino acids. Sequences with genomic rearrangements had more ubiquitination ($p < 0.001$), acetylation ($p < 0.001$) and phosphorylation sites ($p < 0.001$) than did sequences without genomic rearrangements (**Table 3**), but there were no differences in methylation, N or O glycosylation sites.

DISCUSSION

We generated and analyzed the near complete genome sequences of 114 HEV strains and found genomic rearrangements in 7/114 (6.1%). All the recombination detected were in the PPR of the HEV genomes from immunocompromised patients. The 10 inserted fragments were of a human gene or a duplication of part of the HEV genome. We detected recombinant virus/host variants at the acute phase of infection and found pure or mixed populations of variants with or without genomic rearrangements. We have found that these genomic rearrangements increase the net load of the PPR, with different mechanisms

TABLE 3 | Impact of insertions on the amino acid composition, physico-chemical properties, and potential new regulation sites.

Variable	Sequences with human fragment insertions (n = 13)	Sequences with HEV genome duplication (n = 7)	Sequences without insertions/duplications (n = 294)	p (insertion/no insertion)	p (duplication/no duplication)
GC content (%)	48.5 (44.8; 51.9)	46 (45.3; 47.4)	46 (44; 49)	NS[±]	NS[±]
Small AA (%)	5.1 (2; 10.4)	5.5 (1.6; 12.8)	5.3 (1.9; 11.4)	NS[±]	NS[±]
Tiny AA (%)	8.3 (2; 10.7)	6.2 (1.7; 13.3)	6.7 (1.3; 12)	NS[±]	NS[±]
Positive charged AA (%)	4.2 (1.8; 7.1)	4.2 (0.82; 5.3)	2.7 (1.3; 4.5)	<0.01[±]	NS[±]
Negative charged AA (%)	3.2 (2; 4.5)	2.3 (1.6; 2.8)	2.9 (2.6; 4)	NS[±]	<0.01[±]
Charged AA (%)	3.8 (2; 5.7)	2.9 (1.5; 4.7)	2.7 (1.6; 4)	<0.01[±]	NS[±]
Aliphatic AA (%)	4.7 (3.6; 5.6)	4.2 (2.3; 5.5)	4 (2.7; 6.7)	NS[±]	NS[±]
Aromatic AA (%)	1 (0.75; 1.62)	0.8 (0.45; 1.6)	1.3 (0; 1.3)	NS[±]	NS[±]
Hydrophobic AA (%)	2.4 (1.8; 5.5)	2.4 (0.8; 4.7)	1.9 (1; 4.8)	0.04[±]	NS[±]
Polar AA (%)	2.4 (0.8; 6.5)	1.8 (0.8; 4.8)	2.5 (1; 4.8)	0.02[±]	NS[±]
Net load	5 (3; 6)	8 (5; 9)	0 (−1; 1)	<0.001[±]	<0.001[±]
Ubiquination sites	5 (3; 6)	6 (5.5; 6)	2 (1; 3)	<0.001[±]	<0.001[±]
Acetylation sites	5 (3; 6)	6 (5.5; 6)	2 (1; 3)	<0.001[±]	<0.001[±]
Phosphorylation sites	19 (16; 22)	19 (17; 19.5)	10 (8; 14)	<0.001[±]	<0.01[±]
Methylation sites	1 (0; 3)	0 (0; 0.5)	0 (0; 0)	<0.001[±]	NS[±]
Nuclear export signal sites (presence)	11 (84.6%)	7 (100%)	286 (97.3%)	<0.01[£]	NS[£]
Nuclear localization signal sites (presence)	3 (23.1%)	0 (0%)	1 (0.3%)	<0.001[£]	NS[¥]
N-Glycosylation (presence)	1 (7.7%)	0 (0%)	13 (4.4%)	NS[£]	NS[£]
C-Glycosylation (presence)	0 (0%)	0 (0%)	3 (1%)	NS[¥]	NS[¥]

Data are numbers unless otherwise indicated. Variables are expressed as medians and interquartile ranges for Wilcoxon test, number (%) for chi2 or Fisher's exact tests. AA: amino acids. NS: not significant. A p < 0.05 was considered significant.

[±]*Wilcoxon test.*

[¥]*Fisher's exact test.*

[£]*chi2 test.*

according to the nature of the inserted fragments: increase of positively charged amino acids in fragment from human genes and decrease of negatively charged amino acids in HEV gene duplication. Putative post-translation modifications were also found in recombinant variants.

We used SMRT PacBio sequencing to generate almost complete genome sequences. This third generation deep sequencing method can sequence single DNA molecules in real-time and generate long reads (Rhoads and Au, 2015). SMRT was used to identify genomic rearrangements because it enabled us to sequence longer fragments (up to 20 kb) than second-generation sequencing methods (< 500 bp).

All the genomic rearrangements obtained by SMRT sequencing except one were confirmed by Sanger sequencing, indicating that they are not artifacts. However, the fragment (RNA18SP5) inserted in one variant (Hepac-93-3) detected by SMRT was six nucleotides shorter than the sequence obtained by the Sanger method. This could be due to sequencing error not corrected by the bioinformatics pipeline, or it could reflect the presence of four variants: two detected by both methods and two others detected by either Sanger or SMRT sequencing.

All the new genomic rearrangements described herein were located in the HEV-3 PPR, as previously described by our group and others (Shukla et al., 2011; Nguyen et al., 2012; Johne et al., 2014; Lhomme et al., 2014). These recombinations were located at different positions in the PPR. Their presence in the PPR is not very surprising; the sequence encoding this region can vary in both composition and length depending of HEV clade and/or HEV subtype (Purdy et al., 2012; Lhomme et al., 2014). It is a region of great genetic flexibility: the PPR of HEV-3f viruses can be short (228 nt) or long (315 nt) due to a duplication of a PPR fragment (Purdy et al., 2012; Lhomme et al., 2014). A recent study also showed that HEV genomes harboring an epitope tag or NanoLuc in the PPR were found to be fully functional and allow for the production of infectious virus (Szkolnicka et al., 2019), confirming the remarkable flexibility of the PPR.

All the HEV genomic rearrangements described to date have been found in chronically infected patients (Shukla et al., 2011; Nguyen et al., 2012; Johne et al., 2014; Lhomme et al., 2014), but we have found genomic rearrangements at the acute phase in three HEV-infected patients. This raises the question of transmission of such recombinant variants at the acute phase. It is certainly more frequent in chronically HEV-infected patients; we reported a prevalence of 11% in chronically infected solid-organ transplant patients (Lhomme et al., 2014) and found that 8.2% of the immunocompromised patients in this study harbored recombinant variants. And four HEV strains had mixed populations of variants containing sequences with and without genomic rearrangements or different genomic rearrangements. Most of the mixed populations containing non-recombinant variants were found in patients infected for 3 months or less. Consequently, the time needed for recombinant variants to emerge still need to be clarified.

Several groups have shown that insertions of human fragments (RPS17, RPS19, ITI) (Shukla et al., 2011; Nguyen et al., 2012; Lhomme et al., 2014) give the virus a replicative advantage *in vitro* and that duplication helps it to adapt to cell culture systems (Johne et al., 2014). Although duplication of the virus genome has been found in several DNA viruses (Shackelton and Holmes, 2004), they appear to be infrequent in RNA viruses due to biological constraints, such as genome inflation (Simon-Loriere and Holmes, 2013). Duplications have been described in flaviviruses (Villordo et al., 2016), human respiratory syncytial virus (RSV) (Eshaghi et al., 2012; Schobel et al., 2016) and hepatitis C virus (HCV) (Le Guillou-Guillemette et al., 2015). Analysis of the RNA secondary structure of flavivirus 3'UTR revealed an association between RNA structure duplication and the ability of the virus to replicate in vertebrate and invertebrate hosts (Villordo et al., 2016). A 72-nucleotide duplication in the C-terminal region of the attachment glycoprotein gene of RSV genotype A was described (Eshaghi et al., 2012). As this glycoprotein is the target for neutralizing antibodies, such changes might alter the immunogenicity and pathogenicity of the virus. A duplication in the NS5A region of HCV has been described and may be associated with unfavorable evolution of the resulting liver disease, including possible involvement in liver carcinogenesis (Le Guillou-Guillemette et al., 2015). These strains with duplications were present in HCV genotype 1a and belonged to the same phylogenetic cluster. Several subtypes of HEV contain variants harboring duplications, although their impact on the pathophysiology of infection is still unknown. Duplications also occur in several RNA viruses but their locations differ: from the UTR, to structural and non-structural protein coding regions (Villordo et al., 2016), suggesting that they may influence virus function differently.

The present new, larger data set confirms earlier predictions that genomic rearrangements provide the PPR with putative new ubiquitination, acetylation, and phosphorylation sites (Lhomme et al., 2014). They also allow a higher net load. None of these features occurs in HEV-3f with a long PPR, suggesting that the differences are due to specific genomic rearrangements rather than PPR length. The fact that no new glycosylation or methylation sites were detected suggests that regulation sites are not acquired randomly. The peptides derived from the Kernow strain with reversed or reversed complementary insertions have fewer regulation sites, especially acetylation and ubiquitination sites, and they have no *in vitro* replicative advantage (Shukla et al., 2011). The conjugation of ubiquitin with a substrate usually leads to degradation of a peptide by the proteasome, and viruses, including HEV, can hijack the ubiquitin/proteasome system (UPS) (Karpe and Meng, 2012). The function of cellular enzyme can be modified by phosphorylation. Virus protein can also be phosphorylated (Jakubiec and Jupin, 2007): for example, phosphorylation of the hepatitis C virus NS5B has a regulatory role in HCV RNA replication (Kim et al., 2004, 2009; Han et al., 2014). Similarly, acetylation of histone and nonhistone proteins modulates protein function or the intracellular distribution

of the acetylated protein (Sterner and Berger, 2000; Glozak et al., 2005). Acetylation of virus proteins can also modulate their function. Acetylation enhances the enzymatic activity of the HIV integrase and increases its affinity for DNA (Cereseto et al., 2005). More recently, it was shown that acetylation of highly conserved lysine residues might regulate specific functions of nucleoprotein in the viral life cycle of influenza A viruses, including viral replication (Giese et al., 2017). Lastly, we have found that the mechanisms by which human fragments and duplications increase the net load differ. Human fragment insertions increase the frequency of positively charged amino acids, while duplications seem to produce a small increase in positively charged amino acids and decrease the fraction of negatively charged amino acids. An increase in the net load in the V3 domain of the HIV glycoprotein 120 affects HIV tropism as the virus enters the host cell *via* the CXCR4 coreceptor rather than CCR5 (De Jong et al., 1992; Fouchier et al., 1992). The increase in the net load in the PPR of HEV could modify the way the virus proteins interact with host proteins. Although the lifecycle of HEV is not yet clear, we believe the PPR could regulate transcription and translation through ubiquitination, acetylation, or phosphorylation. These putative sites and their role must be confirmed by *in vitro* approaches.

In conclusion, we have described HEV strains with genomic rearrangements in patients at the acute phase of infection raising the possibility that such strains are directly transmitted. We have also shown that genomic rearrangements provide a higher net load with different features depending on the nature of the genomic rearrangement (duplication or insertion). Further studies are needed to clarify the role of these insertions/duplications by *in vitro* and conformational studies.

DATA AVAILABILITY STATEMENT

The sequences of the recombinant variants have been deposited in the Genbank database under accession numbers MF444083, MF444086, MF444119, MF444145, and MN646689-9.

ETHICS STATEMENT

Biological materials and clinical data were obtained for a standard virus diagnosis, following physicians' orders. This non-interventional study involved no additional procedures. Data were analyzed using an anonymized database. Such a protocol does not require written informed consent according to French Public Health law (CSP Art L 1121-1.1).

AUTHOR CONTRIBUTIONS

FN, SL, and JI designed the project, analyzed the results, and wrote the manuscript. NJ and FN performed the bio-informatics analyses. CD performed the biostatistical analysis. NK, J-MP, and LA provided the plasma samples. FA, CL, AR, MM, MD, and RC carried out the experiments. All the authors have approved the manuscript.

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SUPPLEMENTARY MATERIAL

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ORIGINAL RESEARCH
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Molecular Characterization of HEV Genotype 3 in Italy at Human/Animal Interface

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Hepatitis E virus (HEV) is an emerging public health issue in industrialized countries. In the last decade the number of autochthonous human infections has increased in Europe. Genotype 3 (HEV-3) is typically zoonotic, being foodborne the main route of transmission to humans, and is the most frequently detected in Europe in both humans and animals (mainly pigs and wild boars). In Italy, the first autochthonous human case was reported in 1999; since then, HEV-3 has been widely detected in both humans and animals. Despite the zoonotic characteristic of HEV-3 is well established, the correlation between animal and human strains has been poorly investigated in Italy. In the present study, we compared the subtype distribution of HEV-3 in humans and animals (swine and wild boar) in the period 2000–2018 from Italy. The dataset for this analysis included a total of 96 Italian ORF2 sequences (300 nt long), including both NCBI database-derived (n = 64) and recent sequences (2016–2018, n = 32) obtained in this study. The results show that subtype 3f is the most frequent in humans and pigs, followed by the HEV-3e, HEV-3c and other unassignable HEV-3 strains. Diversely, in wild boar a wider group of HEV-3 subtypes have been detected, including HEV-3a, which has also been detected for the first time in a human patient in Central Italy in 2017, and a wide group of unassignable HEV-3 strains. The phylogenetic analysis including, besides Italian strains, also sequences from other countries retrieved from the NCBI database, indicated that human Italian sequences, in particular those of HEV-3f and HEV-3e, form significant clusters mainly with sequences of animal origin from the same country. Nevertheless, for HEV-3c, rarely detected in Italian pigs, human sequences from Italy are more correlated to human sequences from other European countries. Furthermore, clusters of near-identical human strains identified in a short time interval in Lazio Region (Central Italy) can be recognized in the phylogenetic tree, suggesting that multiple infections originating from a common source have occurred, and confirming the importance of sequencing support to HEV surveillance.

Keywords: hepatitis E virus, hepatitis E, subtype, prevalence, zoonosis, Italy

INTRODUCTION

Hepatitis E virus (HEV) is a quasi-enveloped RNA virus with a single stranded genome positive-sense of approximately 7.2 kb. It belongs to the *Hepeviridae* family, genus *Orthohepevirus* which includes 4 species *(Orthohepevirus A-D)*. Species *A* is divided into 8 genotypes, of which genotype 1 and 2 (HEV-1 and HEV-2) infect only humans, HEV-5, HEV-6, and HEV-8 are only detected in animals (Forni et al., 2018) and HEV-3, HEV-4 and HEV-7 (Lee et al., 2016) are zoonotic. HEV-1 and HEV-2 have an intra-human cycle and cause large epidemics due to poor sanitary conditions in developing countries, while in Europe and the US HEV-3 is the most common; HEV-4 is mostly diffused in Asia. Both HEV-3 and HEV-4 are zoonotic and the main animal reservoirs are swine, wild boar and deer (Primadharsini et al., 2019).

In Europe, HEV-3 is considered an emerging foodborne pathogen; the number of patients with hepatitis E has been increasing in the last 10 years probably because of higher clinicians' awareness coupled to increased circulation of the virus (Kamar et al., 2012, 2017; Domanovic et al., 2017). In Europe, although HEV-3 is the most common in both humans and animals, cases of HEV-4 have been reported (Colson et al., 2012; Garbuglia et al., 2013) but in pigs this genotype has been detected sporadically (Monne et al., 2015). The transmission is mainly caused by consumption of undercooked or raw contaminated food of animal origin (pig, deer, and wild boar meat). In humans, hepatitis E is an acute hepatitis, usually self-limiting. In immunocompromised patients extra-hepatic manifestations and chronic infection have been described, but only HEV-3 may cause persistent infections (Kamar et al., 2015).

In Europe, the reported seroprevalence in the general population or in blood donors is highly variable, ranging between 6.1% and 52.5% (Mansuy et al., 2011; Capai et al., 2019); some hyperendemic areas with high seroprevalence have been described (Müller and Koch, 2015; Zaaijer, 2015; Adlhoch et al., 2016; Mansuy et al., 2016; Bura et al., 2017). The different seroprevalence values reported in different countries or regions of the same country may partially depend on different assays used (Norder et al., 2016; Sommerkorn et al., 2017) or on dietary habits such as consumption of raw meat (Slot et al., 2017) or raw dried pig liver sausages (Lucarelli et al., 2016). Furthermore, the seroprevalence observed in several European countries is higher than expected on the basis of reported cases, suggesting HEV underdiagnosis and/or asymptomatic infections (Ricci et al., 2017). In Italy, a mean seroprevalence of 8.7% has been observed in blood donors. Some Italian regions are considered hyperendemic, i.e., 10.0 to 15.0% seroprevalence is reported in Lazio, Umbria, and Marche and >22.0% in Abruzzo and in Sardinia (Spada et al., 2018); in the Lazio region, a retrospective study performed on people who received counseling and underwent serological tests for anti-HIV antibody between 2002 and 2011 showed an overall anti-HEV IgG prevalence of 5.38%, with a variation over time fluctuating within a 3-year period, and an increase of 4.0% per year of participants' age (Lanini et al., 2015). HEV-3 is the most frequently detected in humans, pigs and wild boar In Italy. The reported risk factors among confirmed hepatitis E cases are the consumption of undercooked pork meat and wild boar sausages (La Rosa et al., 2011). As a matter of fact, sequence-based direct evidence of foodborne transmission has been recently provided in an Italian citizen who acquired a HEV-3i strain from figatelli (pork liver sausage) bought in France (Garbuglia et al., 2015).

To date, only one serotype has been described and the only way to determine the source of infection or to trace back the contaminated food is based on sequence analysis (Tei et al., 2003; Takahashi et al., 2004; Colson et al., 2010; Dalton et al., 2011). HEV strains belonging to the same genotype are further classified in subtypes or genetic variants based on sequence p-distance among strains. The HEV-3 strains are classified in 12 subtypes (HEV-3a to HEV-3l) differently distributed worldwide. The HEV-3c, HEV-3e and HEV-3f are the most common in Europe (Lu et al., 2006; Lapa et al., 2015). In addition, several unassigned subtypes and uncommon strains have been recently proposed (Smith et al., 2016). HEV-3c, HEV-3e, and HEV-3f have been reported not only in humans and animals (pigs and wild boar) but also in contaminated food items of pork (pig liver sausage) and wild boar (sausages) (Colson et al., 2010; Di Bartolo et al., 2015; Garbuglia et al., 2015; Montone et al., 2019) and in the environment (Di Profio et al., 2019).

Changes over time in the subtype circulation have been observed in Europe. Subtype HEV-3f represented 90.0% of human infections in South Western France in the period 2003–2005, and the incidence of this subtype dropped to 65.0% during 2012-2014 due to the increase of subtype HEV-3c circulation (Lhomme et al., 2015; Nicot et al., 2018). In England, HEV-3e, HEV-3f, and HEV-3g predominated before 2009, while HEV-3c, which first appeared in 2008, become the predominant variant in 2012 (Ijaz et al., 2014; Harvala et al., 2019).

In Italy, only few data are available on HEV subtype circulation in humans. Few studies described the detection of HEV-3 strains in human cases (La Rosa et al., 2011; Romano et al., 2011; Festa et al., 2014; Garbuglia et al., 2015; Lucarelli et al., 2016; Alfonsi et al., 2018; Marrone et al., 2019) and their classification into the HEV-3c and HEV-3e subtype (Festa et al., 2014; Garbuglia et al., 2015).

Despite the increasing number of papers reporting HEV-3 detection in humans in Italy, the relationship among humans, swine and wild boar strains has not been extensively addressed (Montesano et al., 2016). The aim of the present study was to merge the sequence information of strains circulating in Italy in animals (swine and wild boar) and humans, to evidence possible genetic correlation and eventually trace the HEV origin of human strains circulating in Italy.

MATERIALS AND METHODS

Constitution of Sequence Dataset

Dataset of Italian Sequences

All HEV-3 sequences available in NCBI database (as of June 2019) were screened, and, for the purpose of the first part of study, all those of Italian origin (humans, pigs, and wild boar), overlapping to a 300 bp of ORF2, corresponding to nucleotide positions 5988–6287 nt of HEV complete genome Acc. n° NC_001434.1,

were selected and included in the alignment. Sequences identical to each other and/or shorter than 300bp were discarded. The genomic region of the ORF2 (300 bp) was selected because it is the most represented in the NCBI (Ricci et al., 2017) and overlaps with the genome fragment of the ORF2 sequenced in this study. The final dataset of Italian sequences retrieved from NCBI included 22 swine, 32 wild boar and 10 human strains collected from years 2000 (first swine strain described) to 2018. Since no recent HEV-3 Italian ORF2 sequences were available in the NCBI database, the initial dataset was integrated with 32 novel HEV-3 sequences not described before: $n = 23$ of human origin, obtained in the years 2016–2018, and $n = 7$ obtained in previous years, 2011–2015; 2 recent sequences (collected in 2018) of swine origin were included as well.

Dataset of Italian and Worldwide Sequences

Subsequently, Italian HEV-3 sequences were compared to those from pigs, wild boars and humans available in the database worldwide, using the NCBI BLASTn[1]. MEGA7 software[2] was used for the alignment. Reference HEV-3 sequences of established and recently proposed subtypes ($n = 15$) (Smith et al., 2016; Miura et al., 2017; De Sabato et al., 2018) and 130 sequences showing closest relatedness (≥93.0% nt. identity) to the novel Italian sequences were included in phylogenetic analysis, for a total of 145 sequences included in the analysis (**Table 1**).

Human and Animal Samples From Which the Novel HEV-3 Sequences Used in This Study Were Obtained

Following the designation of the Laboratory of Virology of the National Institute for Infectious Diseases "L Spallanzani" (INMI) as Regional Reference Center for HAV and HEV in late 2015, all diagnostic samples with HEV IgM were analyzed for the presence of HEV genomes and those resulting HEV-RNA positive were sequenced. In the period January 2016 to December 2018, 161 serum samples from anti-HEV IgM-positive patients were analyzed, and sequences were obtained from 44 patients, yielding 23 HEV-3 infections. Only randomly selected samples had been sequenced in previous years (7 HEV-3 strains). The study was approved by the INMI Ethical Committee, and the analysis performed after patient anonymization.

In 2018, 15 pool fecal samples were collected from pen floor in 2 pig farms housing weaners and tested for the presence of HEV RNA; HEV-3 sequences were obtained from both farms.

[1]http://blast.ncbi.nlm.nih.gov/

[2]https://www.megasoftware.net/

TABLE 1 | Dataset description of sequences of human and animal origin detected worldwide and used to build the tree.

	EU			Non-EU				Total
Subtype	**Hu**	**Sw**	**Wb**	**Hu**	**Mon**	**Sw**	**Wb**	
3*	1	1	1			2		5
3a	2	2		3		1		8
3b				1		1	1	3
3c	45	8	1	1				55
3e	6	3	1	2	1	4		17
3f	32	12		8		2		54
3g						1		1
3h	1							1
3i			1					1
3j						1		1
3k				2		1		3
3l	1	1						2
Total	88	27	4	17	1	13	1	151**

*, Not assignable to any subtype; **, 15 reference strains; Hu, humans; Sw, pig; Wb, wild boar; Mon, monkey; EU, European strains; Non-EU, non-European strains.

Laboratory Methods

Hepatitis E virus infection was diagnosed by detecting IgG/IgM anti-HEV antibodies using a commercial enzyme-linked immunosorbent assay (DIA.PRO, Milan, Italy), according to the manufacturer's instructions. For molecular analysis, nucleic acids were extracted with QIASYMPHONY automated instrument (QIAGEN, Hilden, Germany). Internal RNA control template QuantiFast (QIAGEN, Hilden, Germany) was added prior to lysis step and viral RNA extraction to monitor the presence of inhibitors and to check nucleic acid extraction efficiency by performing a quantitative Real-time with QuantiFast Pathogen RT-PCR + IC Kit (QIAGEN, Hilden, Germany). RNA obtained was reverse transcribed and amplified by One-STEP RT-PCR (QIAGEN, Hilden, Germany) following the manufacturer's recommendations. The amplification target was a 412 bp fragment within the ORF2 (positions 5953–6363 respect to the E116-YKH98C strain, AB369687) (Mizuo et al., 2002). Briefly, first round was performed using a PCR protocol with reverse transcription at 50°C for 30 min followed by a denaturation step of 15 min at 95°C, and subsequent 35 cycle at 94°C for 1 min, at 55°C for 30 s and 72°C for 1 min, followed by a final extension at 72°C for 7 min. Nested PCR was performed using TaqGold DNA polymerase (Applied Biosystem Forster, CA, United States) at 94°C for 15 min followed by 35 cycles at 94°C for 30 s, 56°C for 30 s and 72°C for 45 s.

RT-PCR products with expected size were purified using a QIAQUICK PCR products kit (Qiagen, Hilden, Germany); the PCR amplicons were sequenced with the second PCR round primer set, using the method of BigDye terminators, on the 3500 XL sequencing instrument (Applied Biosystem Forster, CA, United States). Two sequences were obtained from pigs (Acc. n° MN546866; MK689362) following the same protocols described above for humans. **Table 2** reports the accession numbers of the novel human sequences from Lazio Region (Central Italy) used in this study.

Phylogenetic Analysis

The maximum likelihood (ML) phylogenetic tree was constructed with the Tamura–Nei parameter model as suggested by the MEGA 7 software model test based on 1,000 bootstrap replications. Reference HEV-3 sequences of established and recently proposed subtypes ($n = 15$) (Smith et al., 2016; Miura et al., 2017; De Sabato et al., 2018) were included in the tree

TABLE 2 | Sequence information of human HEV-3 sequences from Lazio region.

Sequence ID_Collection date	GenBank acc. number	Subtype
2120_2011	MN509469	3c
2122_2011	MN509470	3e
1203_2012	MN509471	3f
1205_2012	MN444846	3f
1313_2013	MN444845	3f
1402_2014	MN444847	3e
1516_2015	MN444844	3f
1602_2016	MN444839	3c
1603_2016	MN444842	3f
1604_2016	MN444838	3e
1609_2016	MN444840	3f
1610_2016	MN444843	3f
1611_2016	MN444841	3e
1706_2017	MN432489	3f
1707_2017	MN444828	3f
1708_2017	MN444829	3f
1712_2017	MN444830	3f
1714_2017	MN444831	3f
1715_2017	MN444832	3f
1718_2017	MN444833	3f
1719_2017	MN444834	3f
1725_2017	MN444835	3f
1728_2017	MN444836	3e
1736_2017	MN444837	3a
1809_2018	MN444853	3f
1813_2018	MN444852	3f
1814_2018	MN444848	3c
1820_2018	MN444852	3f
1823_2018	MN444849	3f
1825_2018	MN444850	3f

for the HEV-3 assignment. Those sequences not belonging to any subtypes defined so far were aligned with sequences of the HEVnet dataset (Mulder et al., 2019) using the public HEVnet typing tool[3].

RESULTS

Subtype Distribution of Human and Animal Italian HEV-3 Strains

The first Italian HEV-3 sequences suitable for the present analysis dated back to 2000 for pigs, 2003 for humans and 2012 for wild boars.

Even though the time interval covered by the study sequences was not overlapping for the 3 HEV hosts, the overall subtype distribution is reported in **Table 3**.

As can be seen, subtype assignment was achieved in 75 over 96 sequences, wild boar sequences being the most frequent in the unassigned group. Among the successfully subtyped strains, subtype HEV-3f was predominant in all species, followed by HEV-3e in all but wild boar species; HEV-3a and HEV-3l were scarcely represented in all species, while HEV-3c seemed to be slightly more frequent in wild boar (18.7%) than in humans (15.0%), and rare in pigs (<5.0%) (**Table 3**). The three swine (KJ508211, KF888265, and KF939862) and the wild boar (MH836530) sequences were not assigned to any subtypes either using the list of references strains (Smith et al., 2016) or by HEVnet typing tools. Differently, the three wild boar clusters including unassigned sequences, each one represented by MF959765, MK390970, and MF959764 were assigned to three provisional novel subtypes by the HEVnet typing tools (named in the typing tools: 3u(p), 3w(p), and 3t(p), respectively). Since for the three clusters only one full genome was available, the Italian strains for which a full genome sequences are available would represent the reference strains of the putative novel subtypes.

TABLE 3 | Subtypes HEV-3 distribution of animal and human strains detected in Italy (this study and downloaded at the NCBI database; collected from 2000 to 2018).

Subtype	Human**	Swine	Wild boar
3a	1	0	2
3c	6	1	6
3e	7	7	0
3f	24	11	8
3l	1	2	0
Unclassified*	1	3	16
Total	40	24	32

***First human strain in Italy detected in 2003. *Not assignable to any subtype.*

Phylogenetic Relationships Between Human and Animal HEV-3 Sequences

We next performed phylogenetic analysis of the Italian strains to identify genetic correlations between human and animal sequences. Results are shown in **Figure 1**. Some Italian human strains detected in this study were identical or strictly related among each other (displaying nucleotide identity >99.0%), in particular 5 strains HEV-3f detected in 2017 (1707-2017; 1708-2017; 1714-2017; 1719-2017; and 1706-2017) formed a statistically supported cluster in the phylogenetic tree (**Figure 1**), with null (4 strains) or very short p-distance (1 strain, 99.8% nt id) among each other. Similarly, three human strains (HEV-3f) detected in 2018 formed a significant cluster with another human Italian strain detected in 2016 (1609-2016), displaying 99.4% nt id. to each other. Finally, two HEV-3e strains (1604-2016 and 1611-2016) both detected in 2016, were identical.

Among the Italian animal strains, identical sequences were not detected; the closest nucleotide identity was observed between two swine Italian strains detected in 2012 and 2013 and belonging to HEV-3l (99.9% nt.id) (KF939866 and KY766999).

Except for the subtypes HEV-3f, HEV-3c, HEV-3a, wild boar sequences included mostly HEV-3 sequences with unassignable subtype (n = 16), showing identical p-distance with different HEV-3 subtypes (**Figure 1**; indicated with 3*).

A unique significant cluster between animal sequences was observed, including one swine and one wild boar HEV-3f strain (KJ508208 and MH836539).

[3]https://www.rivm.nl/mpf/typingtool/hev/

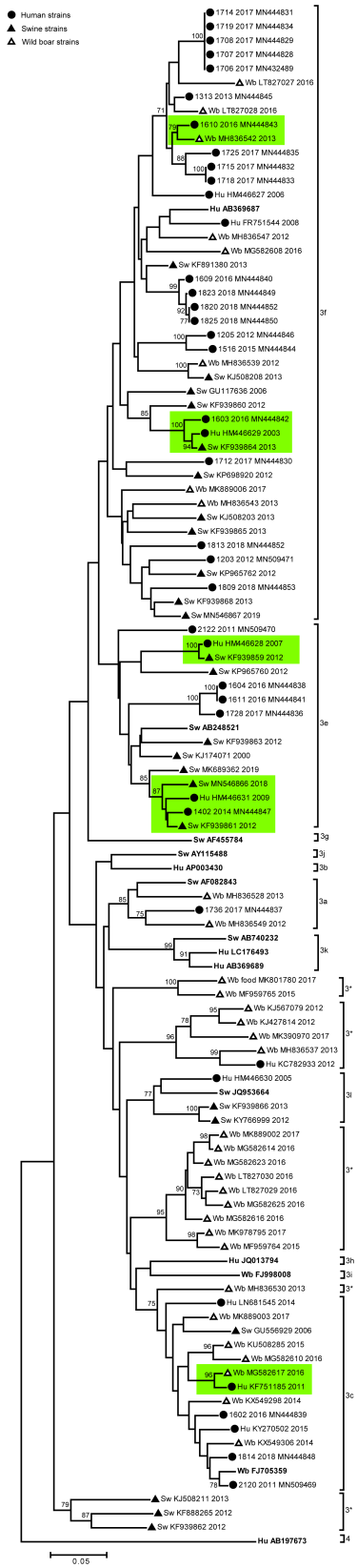

FIGURE 1 | Continued

FIGURE 1 | Phylogenetic analysis based on a 300 nt fragment of the partial ORF2 region of Italian HEV strains (n = 96) of human and animal (swine and wild boar) origin and 13 HEV subtype reference strains. The HEV-4 sequence (Hu AB197763 JP) was used as outgroup. The maximum likelihood tree was produced using the Tamura–Nei parameter model based on 1,000 bootstrap replications and bootstraps values >70 are indicated at their respective nodes. The Italian entries downloaded from NCBI database includes host (Hu, human; Sw, swine; Wb, wild boar) accession number and year of collection. The human Italian strains from this study were reported by sequence name and accession number. The HEV reference strains are reported in bold. Human and animal sequences forming strictly related clusters are highlighted in green. Symbol disclosure is included in the figure.

To address the possible autochthonous zoonotic origin of human infections, we considered clusters including both animal and human sequences (**Figure 1**). Although, the HEV-3f and HEV-3e subtypes are the most common among both Italian human and swine strains, no identical human and animal strains were detected. However, in the HEV-3f, HEV-3e and HEV-3c subtypes some clusters showed strict sequence correlations (highlighted in green in **Figure 1**); in particular the human 1603-2016 (HEV-3f) formed a sub-cluster (98.0% nt. id.) with both human (HM446629) and swine (KF939864) sequences described in 2003 and 2016, respectively. Similarly, in the HEV-3e cluster, the human strain 1402-2014 showed a 96.0–98.0% nucleotide identity (nt. id.) with one human (96.9% nt. id.; HM446631 in 2009) and three Italian swine strains: KF939861 (98.0% nt. id.; 2012), MN546866 (96.0% nt. id.; 2018), MK689362 (94.0% nt. id.; 2018). In these sub-clusters, human strains were detected over different years (2003 vs. 2016; 2009 vs. 2014) in different area of the country (northern and central Italy). The highest nucleotide identity (up to 99.0%) among animal and human Italian strains belonging to the HEV-3f, was displayed between human (HM446629, HM446628) and swine (KF939864, KF939859) strains detected in different years (2007 vs. 2012; 2003 vs. 2013), both originating from Northern Italy. Besides the high heterogeneity of Italian wild boar strains, mostly belong to unassignable HEV-3 subtypes, three strains, belonging to HEV-3f (MH836542) and HEV-3c (MG582617) displayed up to 99.0% identity with human strains detected in different years and areas of the country (**Figure 1**).

In the subsequent step, we included in the phylogenetic analysis all HEV-3 sequences sharing at least 93.0% nt id with the Italian sequences. The results are shown in **Figure 2**.

Some human strains from Italy, both HEV-3c and HEV-3f, were more strictly correlated to HEV-3 human strains described in Europe than with Italian strains. Among them, the HEV-3c Italian human strains (2120-2011, 1814-2018, 1602-2016, KY270502, KY270502, and KF751185) and only sporadically the HEV-3f (1712 2017, 1813-2018) displayed a nt. id. ranging between 93.0% and 99.0% with numerous sequences available online, mainly detected in humans in Europe, among which the highest genetic correlation (97.0–99.0% nt. Id) was observed with human strains reported in France, Netherlands and in the United Kingdom (MF4444071 FR; KR362815 GB; KY775016 NL; KR362795 NL; and MK355858 NL). Correlation with non-European sequences was only observed for two human Italian

FIGURE 2 | Phylogenetic analysis based on the 300 nt fragment of the partial ORF2 region of HEV-3 strains derived of human or animal origin. A total of 244 HEV-3 sequences have been included, and the HEV-4 sequence (Hu AB197763JP) was used as outgroup. The maximum likelihood tree was produced using the Tamura–Nei parameter model based on 1,000 bootstrap replications and bootstraps values >70 are indicated at their respective nodes. Each entry includes host (Hu, human; Sw, swine; Wb, wild boar; Mon, monkey), accession number and countries origin of strains. The human Italian strains from this study were reported by sequence name and accession number. Italian sequences are highlighted as follows: human sequences are in red, animal sequences (swine and wild boar) are in brown, of which swine sequences are in bold.

HEV-3f sequences (1715-2017 and 1718-2017), sharing 99.0% nt. id. to each other, and related to a non-European human sequence detected in Japan (LC192453).

The human strain 1736-2017 was classified as HEV-3a, 91.0% nt. id. with the prototype strain (AF082843, Meng), but displayed only a limited nucleotide identity (<93.0% nt. id.) with the other HEV-3a strains including the recently detected Italian wild boar strains (MH836549, MH836528), while the closest sequence (93.2% nt. id.) was that of a swine strain from Germany (KF303502). Among Italian animal strains not significant correlation with other non- Italian strains was observed. Nevertheless, within the HEV-3f, swine and wild boar strains (KJ508208 and MH836539) formed a supported and strict cluster with a human strain detected in the Netherland (KM820642) and another Italian swine strains was related to a strain detected in a patient in France (KR027099).

Similarly, the HEV-3c sequences detected in Italian wild boar are related to some human strains detected in non-Italian European countries (Netherland and United Kingdom).

DISCUSSION

Analyses conducted by sequence alignment and by establishing correlation among strains using phylogenetic analyses confirmed a genetic diversity of Italian HEV-3 strains. Despite the large variation in the number of sequences obtained for each year, the most common subtype was HEV-3f for both human and pig strains. The HEV-3f was predominant (n = 24) in humans both before and after 2016 when a systematic sequence collection of IgM HEV-positive sera in central Italy (Lazio Region) was established.

The HEV-3f was also the most commonly identified subtype in humans in Spain, France (Adlhoch et al., 2016) and in Belgium until 2015 (Suin et al., 2019). However, in some European countries a shift of subtypes has been observed among HEV-3 human strains, so that subtype HEV-3c is now predominant in England, Wales, Netherlands, and Germany (Suin et al., 2019). Also in Belgium the HEV-3c has become the most common subtype (since 2016), followed by the HEV-3f and HEV-3e, which are frequently reported in both humans and animal reservoirs (Adlhoch et al., 2016; Suin et al., 2019).

In Italy, this shift in HEV-3 subtype circulation was not observed either in humans or in animals. Among Italian pig sequences available online only one HEV-3c was identified and HEV-3c strains detected in wild boar are more strictly correlated to European human strains than to the Italian ones. This result could be attributed to wide circulation of pigs over European countries and the HEV-3c may have been imported to Italy. In Italy, the live import of piglets market (almost 1.6 million head) is mainly from Denmark and Netherlands (AHDB, Agriculture and Horticulture Development Board)[4] where the HEV-3c subtype has been described as the predominant subtype (Adlhoch et al., 2016).

Diversely, for the HEV-3f and, to a lesser extent, HEV-3e, human and pig Italian strains are more similar to each other than to European strains. The similarity of Italian human strains (e.g., HEV-3f), when observed, is not restricted to a short time interval in the time lapse considered in this study. No geographical correlation could be established, since most recent human sequences correspond to human cases which occurred in Lazio Region (Central Italy).

The phylogenetic analysis also indicated that human Italian sequences clustered not only with sequences of animal origin circulating in the same territory, but also with human and animal sequences from other countries, suggesting that meat/food from these countries may act as virus carrier. However, it is not easy to trace movement of HEV pig strains, because besides movement of live animals, the import/export of fresh and cured meat is frequent among European countries. However, the Italian pork export is lower than imports (AHDB)[5]. In Italy, which account for the 6.0% production of pig meat in Europe (1.470 thousand tonnes), pork meat is imported mainly from EU countries especially from Germany which is the main pig meat suppliers in Europe[6]. However, cured Italian pork products are also exported. In conclusion, understanding the trade of live pigs and pork would be important to control the HEV spreading. However, the complexity of trades makes impossible to predict strains movement but sequence analyses would help to understand their movement and possibly prevent spreading of emerging strains if arose.

The detection of identical human strains in the same year and frequently in the same month suggests the occurrence of small transmission clusters. This may be linked to a common source of infection but with the limited data available and the short sequence stretch analyzed, a definitive interpretation is difficult. It is noteworthy that only after 2016, when a stricter surveillance of cases supported by sequencing was established in Lazio Region (Central Italy) for HEV, could a suspected outbreak be hypothesized on the basis of sequence data. Most probably the number of sequences available before 2016 lead to a limited coverage to allow the identification of genetically correlated strains.

No identical sequences were detected among human and animal strains. This could be due to difficulties in tracing back the origin of infections because of the movement of animals or for the long incubation period of the infection, but may also be linked to the evolution of the strains in different hosts (Brayne et al., 2017).

In this study, the presence of the HEV-3a strain in a human case is firstly described in Italy. This subtype circulates predominantly in Japan and in the United States. More recently, the HEV-3a has also been described in Europe (Germany, Austria, Croatia, Hungary, and Belgium) (Reuter et al., 2009; Jemersic et al., 2019; Suin et al., 2019), although it can be considered still rare. In Italy, the HEV-3a has been recently described in wild boar (Di Pasquale et al., 2019) but it has never detected in other animal reservoirs. The Italian HEV-3a human and wild boar strains shared a limited nucleotide identity among them and with the other HEV-3a strains reported in Europe. The patients infected by HEV-3a identified in this study is a man who had recently traveled to Albania; he also reported the consumption of raw grocery in Italy (nearby Rome), but, among those who had been potentially exposed in that occasion, he was the only one who contracted HEV infection, therefore it is possible that the infection source could be located in Albania. However, molecular epidemiology of HEV in Albania is substantially lacking, therefore this hypothesis could not be demonstrated on a molecular basis. Our study has some limitations; in particular, most recent sequences included in the analysis are referred to cases occurred in a restricted Italian region (Lazio, Central Italy); in addition a limited number of sequences was available before 2016. However, independent from the yearly number of available sequences, the HEV-3 subtype

[4]https://pork.ahdb.org.uk/exports/export-markets

[5]https://pork.ahdb.org.uk/prices-stats/news/2018/april/italian-pork-imports-stable/

[6]https://ec.europa.eu/eurostat/data/browse-statistics-by-theme

frequency obtained from the whole set of human and animal Italian sequences did not seem to show gross fluctuations over time (not shown).

Overall, the results from this study provide for the first time a direct comparison of HEV-3 subtype distribution in humans and animals in a region that is experiencing a steady increase of incidence of this zoonotic infection. Despite the above mentioned study limitations, it may pioneer a more circumstantiated and robust exploration of the dynamics involved in HEV transmissibility at the human-animal interface, based on a larger availability of shared HEV sequences.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in NCBI, acccession numbers: MN546866, MN546867, MN509469, MN509470, MN509471, MN444846, MN444845, MN444847, MN444844, MN444839, MN444842, MN444838, MN444840, MN444843, MN444841, MN432489, MN444828, MN444829, MN444830, MN444831, MN444832, MN444833, MN444834, MN444835, MN444836, MN444837, MN444853, MN444852, MN444848, MN444852, MN444849, and MN444850.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Istituto Nazionale per le Malattie Infettive (INMI) Ethical Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AG and MC designed the research. MC supervised the study. AG and DL performed the sequences of human strains and analyzed the data. ID and LD performed the sequences of animal strains and phylogenetic analyses. ID wrote the manuscript together with LD and AG. All authors revised the manuscript and approved the final version for submission.

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Expression, Purification and Characterization of the Hepatitis E Virus Like-Particles in the *Pichia pastoris*

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Hepatitis E virus (HEV) is associated with acute hepatitis disease, which may lead to chronic disease in immunocompromised individuals. The disease is particularly severe among pregnant women (20–30% mortality). The only licensed vaccine against HEV, which is available in China, is the *Escherichia coli* purified recombinant virus-like particles (VLPs) encompassing the 368–660 amino acids (aa) of the viral ORF2 protein. The viral capsid is formed by the ORF2 protein, which harbors three glycosylation sites. Baculo virus expression system has been employed to generate a glycosylated VLP, which encompasses 112–608aa of the ORF2 protein. Here, we sought to produce a recombinant VLP containing 112–608aa of the ORF2 protein in *Pichia pastoris (P. pastoris)* expression system. The cDNA sequence encoding 112–608aa of the ORF2 protein was fused with the α-mating factor secretion signal coding sequence (for release of the fusion protein to the culture medium) and cloned into the yeast vector pPICZα. Optimum expression of recombinant protein was obtained at 72 h induction in 1.5% methanol using inoculum density (A_{600}) of 80 and at pH-3.0 of the culture medium. Identity of the purified protein was confirmed by mass spectrometry analysis. Further studies revealed the glycosylation pattern and VLP nature of the purified protein. Immunization of BALB/c mice with these VLPs induced potent immune response as evidenced by the high ORF2 specific IgG titer and augmented splenocyte proliferation in a dose dependent manner. 112–608aa ORF2 VLPs produced in *P. pastoris* appears to be a suitable candidate for development of diagnostic and prophylactic reagents against the hepatitis E.

Keywords: viral hepatitis, hepatitis E virus, open reading frame 2, virus-like particle, *Pichia pastoris*

INTRODUCTION

Hepatitis E virus (HEV) is a single-stranded, positive-sense RNA virus with a size of 27–34 nm, belonging to the family *Hepeviridae*. It is a major cause of acute viral hepatitis (Smith et al., 2014; Nan and Zhang, 2016). HEV is responsible for outbreaks and sporadic cases in both developing and developed countries. The disease is self-limiting and mostly resolve after the acute phase but can progresses to chronic hepatitis in some cases (Kamar et al., 2008; Purcell and Emerson, 2008). The mortality rate ranges from 0.5 to 3% in young adults and increases up to 30% in pregnant women (Chaudhry et al., 2015). The HEVs are classified into seven genotypes. Genotype 1and 2 viruses exclusively infect humans and no animal reservoir is yet known. Genotype 3 and genotype 4 are highly diverse and zoonotic with an expanded host range. Genotype 5 and genotype 6 viruses predominately infect wild boar whereas, genotype 7 viruses infect camel. All the genotypes are antigenically conserved and there is only one serotype, making the development of a univalent hepatitis E vaccine reasonable (Schlauder and Mushahwar, 2001; Emerson et al., 2006; Lu et al., 2006).

The HEV genome is approximately 7.2 kb and has three open reading frames (ORFs). ORF1 encodes a non-structural polyprotein with seven distinct domains: methyltransferase, Y-domain, papain-like cysteine protease, V domain, macrodomain, helicase, and RNA dependent RNA polymerase. ORF1 is followed by ORF2, which encodes the capsid protein, and ORF3, which overlaps with ORF2 and encodes a phosphoprotein that modulates host cellular activities and plays a role in release of the progeny virions (Mori and Matsuura, 2011; Nan and Zhang, 2016). HEV genotype 1 (G1-HEV) has a fourth ORF, which encodes the ORF4 protein that plays an essential role in viral replication (Nair et al., 2016). The ORF2 is a 660aa protein, which has three domains [shell (S), middle (M), and protruding (P)] (Cao and Meng, 2012) and three N-linked glycosylation sites (Zafrullah et al., 1999). Immune dominant epitopes of ORF2 protein are conserved among all HEV genotypes against which all neutralizing antibodies are targeted (Lu et al., 2006). Therefore, efforts to develop a safe and effective vaccine against HEV have focused on ORF2 protein. Homo-oligomerization ability of the ORF2 protein has been utilized to generate virus-like particles (VLPs), *in vitro* (Li et al., 2005a; Roldao et al., 2010).

Virus-like particles express viral antigen and epitopes on their surface, which may provide strong and long-lasting humoral and cellular immune responses. However, they lack viral genetic material. Therefore, VLPs may be a safe and effective strategy for vaccine development against viral diseases (Murata et al., 2003; Crisci et al., 2012; Syomin and Ilyin, 2019). Cervarix (Glaxosmithkline, United Kingdom), Gardasil and Gardasil9 (Merck, United States) are commercially available VLP-based vaccines against the HPV. Similarly, Engerix (Glaxosmithkline, United Kingdom), Recombivax HB (Merck, United States) and Sci-B-Vac (VBI Vaccines, United States) are commercially available VLP-based vaccines against the HBV. Further, VLP-based vaccines against the hepatitis C virus (HCV) and the human immunodeficiency virus (HIV) have generated promising results in preclinical studies (Murata et al., 2003; Olsson et al., 2007; Zhao et al., 2016).

In the case of HEV, different regions of the viral capsid protein have been expressed in bacteria, yeast and insect cell culture system (baculovirus/insect cells) to generate VLPs (Robinson et al., 1998; Li et al., 2005b,c; Simanavicius et al., 2018). The 368–606aa region of the ORF2 protein has been purified from the insoluble fraction of *Escherichia coli (E. coli)*, which assembles into VLPs, *in vitro* (Zhao et al., 2013; Wei et al., 2014). This VLP offers 100% efficacy in clinical trial against symptomatic hepatitis E and it is licensed for commercial use as a vaccine in China (Zhu et al., 2010; Li et al., 2015). Other smaller peptides such as E2 (394–606), E2s (459–606), which carry neutralizing epitopes, have been expressed in *E. coli.* These peptides also form VLPs, which show immunogenicity in primates (Li et al., 2005b, 2009; Zhang et al., 2005). By using baculovirus vectors, two variants of the ORF2 protein (56 kDa and 53 kDa) were purified from the insect cell line, of which the 53 kDa protein could self-assemble into VLPs that were slightly smaller than the native HEV particles and these proteins exhibited immunogenicity and protective efficacy in HEV challenged Rhesus monkeys (Tsarev et al., 1997; Guu et al., 2009; Xing et al., 2010). Further analysis of the ORF2 truncations revealed that removal of 111aa from the N-terminus and 52aa from the c-terminus (112–608) of G1-HEV ORF2 protein substantially enhanced VLP formation in insect cells and produced $T = 1$ VLP similar to the native virion (Li et al., 1997, 2004; Xing et al., 2010). The 112–608aa VLP exhibits all immunodominant neutralization epitopes and generates efficient humoral response in primate models (Khudyakov et al., 1999; Zhang M. et al., 2001; Li et al., 2004, 2011; Xing et al., 2010). The baculovirus-expressed N-terminally truncated rat HEV-3 capsid protein formed VLP of 35 nm in diameter, similar to native HEV particles having no RNA packaging inside and formed $T = 1$ virion (Yamashita et al., 2009). Compared to the baculovirus expression system, the yeast (*Pichia pastoris*) expression system has the advantage of ease of manipulation, high yield, and low production cost. *P. pastoris* has been successfully used for vaccine production against viruses such as hepatitis B virus (HBV), Coxsackie virus and human enterovirus 71 (Cregg et al., 1987; Wang et al., 2013; Zhang et al., 2016). In an earlier study, 382–674aa region of the capsid protein of HEV (named as p293 ORF2) was expressed in the *P. pastoris* as a His-tagged fusion protein. The secreted p293 ORF2 was purified from the culture supernatant and analyzed by electron microscopy, which revealed it to be assembled into VLPs of 30nm size (Yang et al., 2010).

In the present study, we expressed 112–608aa region of the ORF2 protein of g1-HEV in *P. pastoris as an* N-terminal His-tag fusion protein. ORF2 was secreted to the culture medium as an N-linked glycoprotein, which was purified by Ni-NTA affinity chromatography, followed by density gradient centrifugation. The purified protein was characterized and its immunogenicity was evaluated in mice. *P. pastoris* expression system appears to be a better alternative to the baculovirus expression system for production of 112–608aa VLP.

MATERIALS AND METHODS

Cloning and Generation of *Pichia* Transformant Containing pPICZαA-ORF2

The G1-HEV ORF2 region (112–608aa) was amplified from pSKHEV2 by PCR using the following forward and reverse primers: 5′AGCCGCGGCGGCCGCGCGGTCGCTCCGGC-3′ and 5′CATTGTTCTAGAAATGCTAGCACAGAGTGG3′. The PCR product was digested with *Not*I and *Xba*I restriction enzymes and ligated into the pPICZα vector predigested with the same enzymes. The resulting construct was named as pPICZα 112–608aa ORF2. The clone was confirmed by sequencing of the insert. pPICZα and pPICZα 112–608aa ORF2 vectors were linearized with *Bst*XI enzyme and electroporated into competent *P. pastoris* strain KM71H (Thermo Fisher Scientific, Massachusetts, United States). Transformants were grown on YPDS (1% yeast extract, 2% peptone, 2% dextrose, and 1M sorbitol) plates containing 100 μg/ml zeocin and incubated at 30°C in a humidified incubator. Single colonies from pPICZα and pPICzα 112–608aa ORF2 transformants, were inoculated in YPDS medium and incubated in a rotatory shaker (270 rpm) for a period of 16–18 h at 28.5°C till the absorbance (A_{600}) reached ∼ 2.0, followed by inoculation in BMGY (1% yeast extract, 2% peptone, 100 mM phosphate buffer, 1.34% Yeast nitrogen base, 0.02% Biotin, 1% glycerol) media. The culture was grown for a period of 16–18 h under similar conditions till the A_{600} reached ∼ 16.0. The culture was centrifuged and the pellet was re-suspended and grown in BMMY (1% yeast extract, 2% peptone, 100 mM phosphate buffer, 1.34% Yeast nitrogen base, 0.4 μg/mL Biotin) till A_{600} was ∼ 60–70. 1.5% methanol was added at 24 h interval till 72 h and culture was grown at 28.5°C, 270 rpm. The culture was centrifuged in a SW28 rotor in an ultracentrifuge (Beckman Coulter, Indianapolis, IN, United States) at 125,000 × *g* and medium was collected. Presence of ORF2 protein in the culture supernatant was detected by enzyme linked immunosorbent assay (ELISA), SDS-PAGE Coomassie blue staining and Western blot using anti-ORF2 antibody.

Enzyme Linked Immunosorbent Assay (ELISA)

Ninety-six well microtiter plates (Nunc, Thermo Fisher Scientific, Massachusetts, United States) were coated with 5 μl of culture supernatant mixed with 95 μl of 100 mM sodium bicarbonate buffer (pH 9.6) and kept at 4°C overnight. The plates were washed thrice in 200 μl/well of wash buffer (PBS + 0.1% Tween20, pH 7.4) and blocked with 200 μl/well of blocking buffer (PBS + 1% BSA) at 37°C for 2 h. Subsequently, plates were washed with wash buffer and incubated with anti-ORF2 rabbit polyclonal antibody (Nair et al., 2016) at a dilution of 1:1000 in assay buffer (PBS + 0.1% Tween20, 0.2% BSA) at 37°C for 2 h. Next, plates were incubated with HRP conjugated anti-rabbit IgG in assay buffer for 1 h at 37°C and washed three times in wash buffer. HRP activity was measured by colorimetry using TMB 3,3′,5,5′-tetramethylbenzidine, (Sigma, St. Louis, MO, United States) as the substrate. Values were measured at A_{450} using a multimode microplate reader (Synergy HT, BioTek, Vermont, United States).

SDS-PAGE and Western Blot Analysis

The protein samples were mixed with 2X Laemmeli buffer (50 mM Tris–HCl,100 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, and 20% glycerol), incubated for 5 min at 95°C and resolved on 10% SDS-PAGE gels. For the western blot analysis, the proteins were transferred to 0.2 μm polyvinylidene fluoride (PVDF) membrane (Pall Corporation, New York, NY, United States). The membrane was incubated in blocking buffer (5% BSA in PBS) for 1 h at room temperature and then incubated in anti-ORF2 polyclonal rabbit antibody diluted 1:1000 in buffer I [Phosphate buffer saline (PBS) + 0.1% Tween20 + 5% BSA] at 4°C overnight. The membrane was washed thrice with wash buffer (PBS + 0.1% Tween 20) and incubated with HRP conjugated goat anti rabbit antibody diluted 1:5000 in buffer I for 1 h at 25°C. After 3 times washing with wash buffer, blot was developed using a chemiluminiscence Substrate (Bio-Rad, California, United States).

Protein Purification by Immobilized Metal Affinity Chromatography

The culture medium was harvested by centrifugation at 7800 × *g* for 1 h, supernatant was mixed with equilibration buffer [5 mM Imidazole, 50 mM Tris–Cl (pH 7.5), 500 mM NaCl] containing 1 mM phenyl methyl sulfonyl fluoride (PMSF) and incubated with Ni-Agarose beads for 2 h. Washing was done with wash buffer (50 mM Imidazole in equilibration buffer) followed by elution of the bound proteins in 250 mM and 500 mM imidazole. For large scale purification, the culture supernatant was equilibrated with equilibration buffer (as mentioned above), loaded on to HisTrap FF Ni-Sepharose column (GE Healthcare, Illinois, United States) fitted to a FPLC (Fast protein liquid chromatography) system (AKTA purifier, GE healthcare, Illinois, United States). Washing was done in 4–50 mM imidazole gradient, followed by elution of the bound protein in a gradient of 50–500 mM imidazole. The eluted fractions showing protein peaks were analyzed by gel electrophoresis. The ORF2 containing protein fractions were pooled, concentrated and buffer exchanged to PBS using a 10 kDa centrifugal filter device (Pall Corporation, New York, NY, United States).

Mass Spectrometry

The ∼56 kDa band of protein was gel excised, placed in 1.5 ml microtube and centrifuged at 10,000 rpm for 5 min. 100 μl of destaining solution (1:1 ratio if 100 mM ammonium bicarbonate and 100% acetonitrile) was added and incubated for 30 min, centrifuged at 5000 rpm for 1 min at room temperature. The shrinked gel pieces were dried in speed-vac for 15 min at 30°C, mixed with trypsin buffer (13 ng/μl Trypsin, 10 mM ammonium bicarbonate + 10% acteonitrile) and kept on ice. After addition of trypsin buffer, pH was checked and 100 mM ammonium bicarbonate was added to obtain pH 7.0, followed by 90 min incubation on ice for 1 h, followed by incubation at 37°C overnight. Next day, the sample was centrifuged at 10,000 rpm for 1 min. The supernatant was collected and mass spectrometry was performed by MALDI MS-MS at the "Advanced instrumentation research facility" (Special

Centre for Molecular Medicine, Jawaharlal Nehru University, New Delhi, India).

Iodixanol Density Gradient Centrifugation

The protein sample was overlaid on top of 10–40% discontinuous iodixanol (Sigma-aldrich, St. Louis, MO, United States) gradient and centrifuged in SW 55Ti rotor in an ultracentrifuge (Beckman Coulter, Indianapolis, IN, United States) for 3 h at 100,000 × *g* without braking. Ten equal fractions were collected from top and processed further, as indicated.

Glycosidase Treatment

The purified protein was mixed with 10× glycoprotein denaturation buffer and incubated at 95°C for 5 min, chilled on ice and centrifuged for 10 s. Reaction mixture 2 (2 μl of 10× glycobuffer + 2 μl 10% NP40 + 6 μl H_2O) was prepared and incubated at 37°C for 1 h. The denatured protein sample and reaction mixture 2 were mixed and 1 μl endoglycosidase H or PNGase F enzymes added and incubated for 4 h at 37°C. Aliquots of the samples were separated on SDS-PAGE and analyzed by Coomassie Brilliant Blue staining and western blot using anti-ORF2 antibody.

Transmission Electron Microscopy

A Total of 5 μl of VLPs in suspension, at a concentration of 0.5 mg/ml, were adsorbed onto glow discharged Carbon-Formvar-coated copper grids for 2 min. The grids were then washed with PBS three times, followed by staining with 2% uranyl acetate. The grids were air-dried and examined in a Tecnai F20 electron microscope (FEI, Oregon, United States) operating at 200 kV.

Hepatitis E Virus Patient Serum Analysis

To detect the ORF2 specific antibody in the HEV infected patients, western blotting was performed using sera from HEV patients and healthy individuals. Informed consent was obtained from the donors as per the institutional ethics committee guidelines. 1:20,000 dilution of serum was used and 1:10,000 dilution of goat anti-human IgG-HRP conjugated secondary antibody was used.

Mice Experiments

The mice experiment protocol was duly approved by the Animal Ethics Committee of Translational Health Science and Technology Institute (THSTI), constituted under the provisions of CPCSEA (Committee for the Purpose of Control and Supervision on Experiments on Animals), Government of India. Animals were housed in the small animal facility of the THSTI and fed on standard pellet diet and water under pathogen-free conditions.

A total of eight groups of 6–8 week old male mice ($n = 5$) were immunized with 1 μg, 3 μg, and 5 μg ORF2 VLP in PBS or 1 μg, 3 μg, and 5 μg VLP emulsified with ALUM (1:1 volumetric ratio) by intraperitoneal route. Mice were boosted twice with the same dose of immunogens at 2 weeks interval. Two control groups were re-injected with PBS and PBS + ALUM, respectively. Blood samples were collected before each immunization and sera was prepared and stored at –80°C.

Evaluation of Antigenicity

The titer of ORF2 specific IgG level in the serum obtained from each mouse at indicated time points was measured by ELISA. 96 well microtiter plates were coated with 100 ng purified 112–608 ORF2 protein in bicarbonate buffer for 16 h, followed by incubation with blocking buffer at 37°C for 2 h, as described in the method for ELISA. Subsequently, twofold serially diluted serum samples (in assay buffer) started at 1:100 were used as a primary antibody to analyze the antibody titer. Next, plates were incubated with HRP conjugated anti-rabbit IgG in assay buffer for 1 h at 37°C and washed three times in wash buffer. HRP activity was measured by colorimetry. Absorbance was measured at 450 nm.

Cell Proliferation Assay

Cell proliferation assay was done with Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay Kit (Promega, Wisconsin, United States). Splenocytes were isolated from the spleen of the immunized and control mice on day 43 and cultured, as described previously (Kushwaha et al., 2012). Briefly, spleens were aseptically removed, gently macerated and passed through a sterile nylon cell strainer of 70 μm (BD Biosciences, California, United States). The cell suspension was centrifuged at 453 *g* in a swing-bucket rotor and the supernatant was discarded. Cells were resuspended in 0.84% chilled ammonium chloride solution (to lyse the erythrocytes), centrifuged at 453 *g* in a swing-bucket rotor and the supernatant was discarded. Next, cells were washed twice in RPMI medium, followed by resuspension in the RPMI medium containing 10% Foetal bovine serum (FBS). Cells were counted and seeded into 96-well plates at a density of 10×10^4 cells/well and incubated at 37^0C with 5% CO_2. 24 h post-incubation, 5 μg of purified 112–608 ORF2 protein was added to the cultured cells. After 24 h, 20 μl MTS dye was added to each well and incubated for 4 h, followed by measurement of the absorbance at 490 nm. The proliferation was assessed by the stimulation index (SI), calculated according to the formula: SI = (experimental OD – control OD)/control OD.

Statistics Analysis

Data are presented as mean ± standard errors of triplicate samples (SEM). Data are representative of two or more independent experiments. Data was analyzed using GraphPad Prism. Pairwise comparisons of values were performed using student's *t*-test and multiple comparisons were analyzed by one-way ANOVA.

RESULTS

Expression of 112–608aa ORF2 Protein in *Pichia pastoris*

The two clones (clone C1 and D1) of pPICZα 112–608aa ORF2 and pPICZα vector were linearized using *Bst*XI enzyme and

electroporated into *P. pastoris*, strain KM71H. Zeocin positive colonies were selected on YPDS medium supplemented with zeocin. To verify 112–608aa ORF2 expression, zeocin positive clones were grown in BMGY medium supplemented with 1% methanol. Level of 112–608aa ORF2 protein in the culture medium at different time point was measured by ELISA, which showed that clone D1 expressed more 112–608aa ORF2 compared to clone C1 (**Figure 1A**). Western blot of the culture medium using anti-ORF2 antibody confirmed the expression of 112–608aa ORF2 in both clones (**Figure 1B** upper panel). Coomassie brilliant blue staining of aliquots of the sample is shown in the lower panel (**Figure 1B**). Clone D1 was selected for optimization of culture parameters. ELISA data showed that the highest yield of 112–608aa ORF2 was obtained by 72 h incubation with 1.5% methanol (**Figure 1C**). pH analysis of the culture medium demonstrated that pH 3.0 is optimal for the maximum yield of the 112–608aa ORF2 (**Figure 1D**). Further, cell density of 80 (A_{600} = 80) favors maximum yield (**Figure 1E**).

Purification of 112–608aa ORF2 Protein

The culture media containing His-tagged 112–608aa ORF2 protein was incubated with Ni-agarose beads followed by removal of unbound protein by washing with 50 mm imidazole. Ni-agarose bound 112–608aa ORF2 was eluted in 250 mM and 500 mM imidazole (**Figure 2A**). Next, His-trap FF Ni-sepharose column was used to purify 112–608aa ORF2 from the culture medium by FPLC. Ni-sepharose bound 112–608aa ORF2 was eluted in a 50–500 mM imidazole gradient. The elution fractions showing strongest peak of protein were analyzed by SDS-PAGE and Coomassie brilliant blue staining, which showed that fraction 66–90 were enriched with the ORF2 protein (**Figure 2B**). These fractions were pooled and imidazole buffer was exchanged with PBS (pH 7.4) (**Figure 2C**). An aliquot of the protein was analyzed by MALDI-MS, which confirmed it to correspond to the HEV ORF2 protein (**Figure 2D**).

The purified protein was overlaid on top of 10–40% iodixanol gradient and subjected to ultracentrifugation. Total 10 fractions were collected from the top and analyzed by SDS PAGE followed by Coomassie Brilliant Blue staining. Fractions 1–3 were enriched with 112–608aa ORF2 protein (**Figure 2E**). These fractions were pooled and buffer exchanged with PBS (pH 7.4).

Characterization of the Purified 112–608aa ORF2 Protein

Open reading frames 2 protein contains three N-linked glycosylation sites (Zafrullah et al., 1999). Susceptibility to deglycosylation enzymes, endoglycosidase H (endo H) and PNGase F was used to determine the glycosylation status of the purified protein Endo H cleaves the N-linked glycans between the two *N*-acetylglucosamine (GlcNAc) residues in the core region of the glycan chain on high-mannose glycans, leaving one GlcNAc still bound to the protein while PNGase F is a glycoamidase that cleaves the bond between the innermost GlcNAc and asparagine residues, releasing the entire sugar chain. Both Endo H and PNGase F could deglycosylate 112–608aa ORF2, as evident from Coomassie brilliant blue staining and anti-ORF2 western blot of the samples (**Figure 3A**). Further, whether the purified 112–608aa ORF2 protein could form VLP, was assessed by transmission electron microscopy (TEM). Although the population obtained was heterogeneous, a large proportion of particles of 22 nm diameter were clearly visible, suggesting that the purified 112–608aa ORF2 protein assembled into VLPs (**Figure 3B**).

Next, we evaluated if immunogenic epitopes were conserved in the purified 112–608aa ORF2 VLP. An ELISA was performed to measure the reactivity of the purified 112–608aa ORF2 VLP with anti-ORF2 antibody present in clinically confirmed HEV patient sera. As expected, HEV patient sera strongly interacted with the 112–608aa ORF2 VLP (**Figure 3C**). ELISA result was further confirmed by western blot of the 112–608aa ORF2 protein using the same sera. Significant reactivity was seen only in the presence of HEV patient sera and not in the sera from healthy individuals (**Figure 3D**).

Induction of Humoral and Cellular Immune Response by 112–608aa ORF2 Protein

To evaluate the immunogenic potential of the 112–608aa ORF2 VLPs, immunization assay was performed in Balb/c mice. Six-week-old male mice were injected with the 112–608aa ORF2 VLPs, as illustrated (**Figure 4A**). Anti-ORF2 IgG titers were determined in serum by ELISA. Sera obtained from each mouse at indicated time points were twofold serially diluted starting from 1:100 and the reciprocal of the highest dilution that had two times absorbance of control mice was taken as positive ORF2-specific antibody titer. All the analysis was carried out on the log2 transformed antibody titers with standard error. The anti-ORF2 antibody titration shows that 112–608aa ORF2 VLPs induced ORF2 IgG production (**Figure 4B**). Alum emulsified 112–608aa ORF2 VLPs further increased the anti-ORF2 IgG level (**Figure 4B**). The IgG response was enhanced in a dose dependent manner, the titer being 1:12765 and 1:4837 for 5 μg VLP + ALUM and 3 μg VLP + ALUM samples, respectively.

The cellular immune response elicited by the 112–608aa ORF2 VLPs was evaluated by splenocytes proliferation assay. The splenocytes collected from 5 μgVLPs, 3 μgVLP + ALUM and 5 μgVLP + ALUM immunized mice could be significantly induced to proliferate, compared to controls (PBS/PBS + ALUM) (**Figure 4C**). The stimulation index of 3 μgVLP + ALUM (p = 0.0248) and 5 μgVLP + ALUM (p = 0.0138) groups were significantly higher, compared to only VLP, respectively (**Figure 4C**).

Taken together, our data shows that pichia expressed 112–608aa HEV ORF2 was glycosylated, formed VLPs and elicited significant immune response.

DISCUSSION

All four mammalian HEV genotypes show homology in the amino acid sequence of the capsid protein (ORF2), which has the capability to self assemble into VLPs. 60 copies of the 112–608aa ORF2 protein assemble to form the VLP (Zhang J.Z. et al., 2001; Xing et al., 2010). The three domains of ORF2, S (118-314),

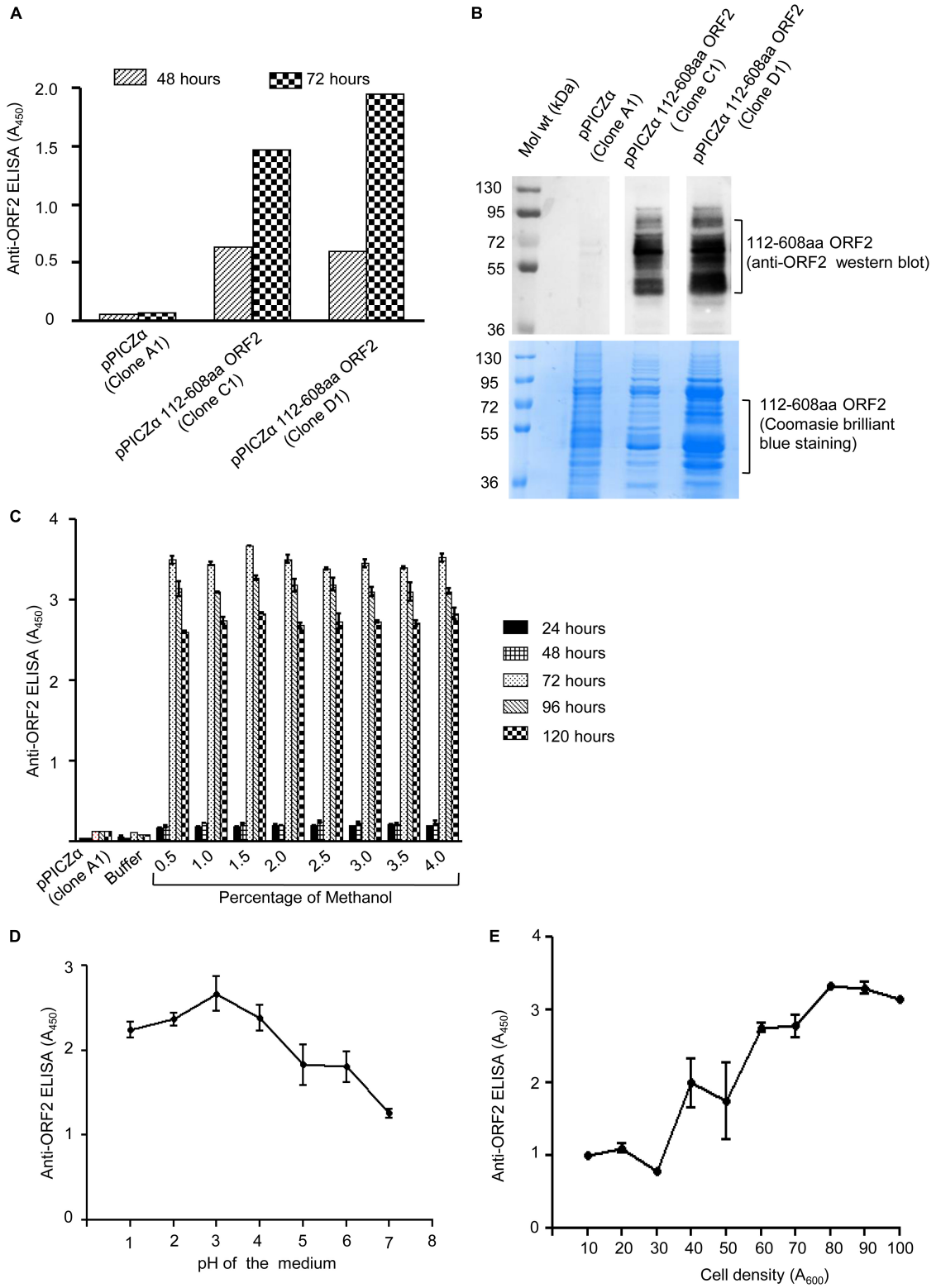

FIGURE 1 | Expression of 112–608aa ORF2 in the *Pichia pastoris*. **(A)** ELISA of the *Pichia pastoris* secreted 112–608aa ORF2 protein in the supernatant of induced culture using anti-ORF2 antibody at indicated time points. **(B)** Upper panel: Western blot of the 48 h induced protein samples shown in **(A)** probed with anti-ORF2 antibody. Lower panel: Coomassie Brilliant Blue stained image of protein sample shown in upper panel **(C–E)**. ELISA of the culture medium of pPICZα 112–608aa ORF2 (clone D1) and pPICZα vector using anti-ORF2 antibody in following conditions. **(C)** Increasing methanol quantity and induction period (pH-3.0, OD_{70}). **(D)** Increasing pH of the medium (72 h induction with 1% methanol at OD_{70}). **(E)** Increasing cell density (72 h induction with 1% methanol at pH-3.0).

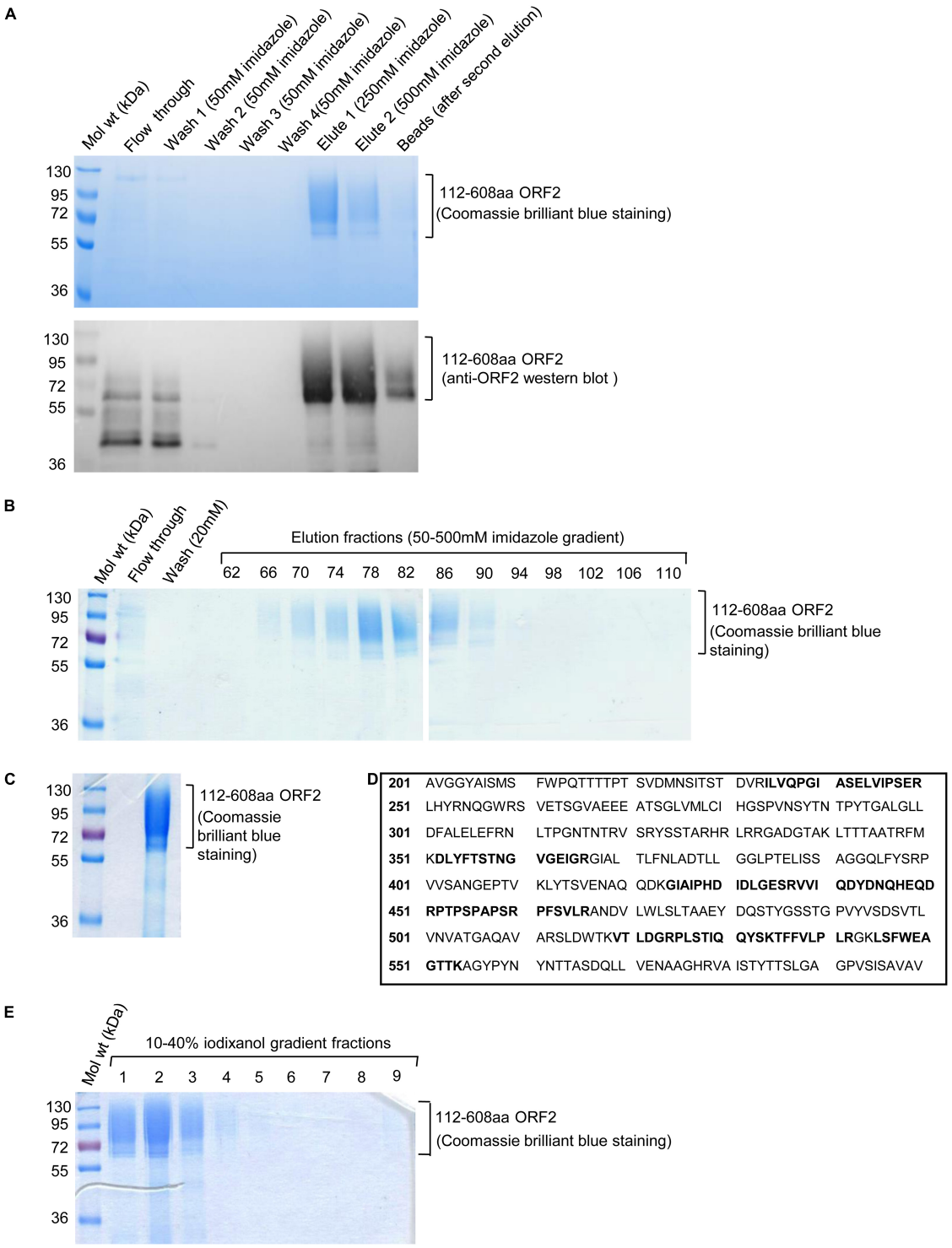

FIGURE 2 | Ni-agarose and Ni-sepharose affinity purification and identification of 112–608aa ORF2 protein. **(A)** Upper panel: Coomassie Brilliant Blue stained image of the indicated fractions collected during batch purification of the recombinant 112–608aa ORF2 protein from the culture medium using Ni-agarose beads. Washing and elutions were performed with the indicated concentration of imidazole in 50 mM Tris buffer. 32 μl beads were boiled in laemelli buffer and loaded (beads); Lower panel: anti-ORF2 western blot of the samples shown in upper panel. **(B)** Coomassie Brilliant Blue stained image of the indicated fractions collected during FPLC purification of the 112–608aa ORF2 protein from the culture medium using Ni-Sepharose column. Elution was performed using 50–500 mM imidazole gradient. **(C)** Coomassie Briliant Blue stained image of the 112–608aa ORF2 protein obtained after buffer exchange of fractions 66–90 (shown in B) in PBS. **(D)** Identification of the 112–608aa ORF2 protein by mass spectrometry analysis. Peptides identified by mass spectrometry denoted as bold letters. **(E)** Coomassie Briliant Blue stained image of iodixanol gradient fractions, as indicated.

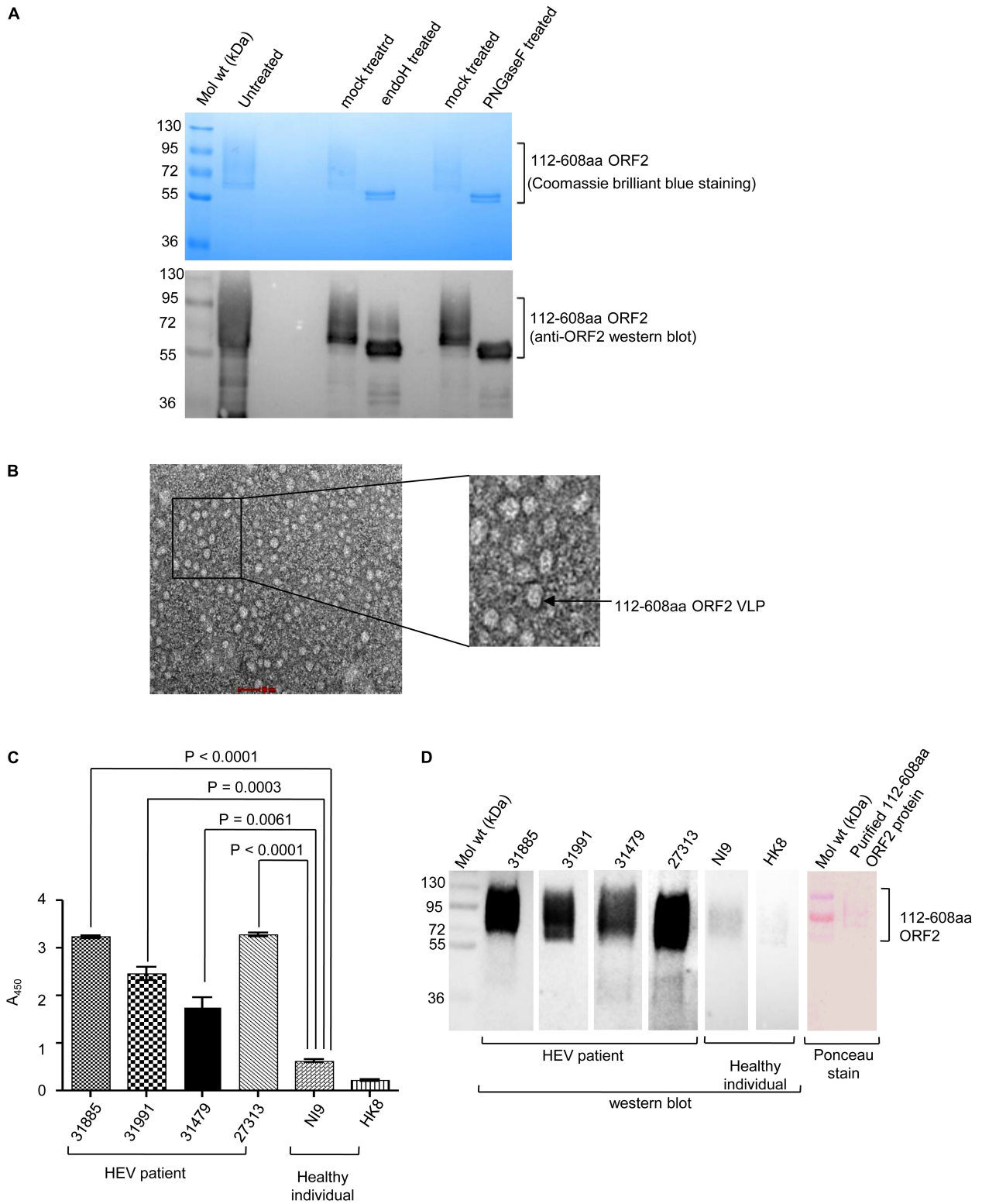

FIGURE 3 | Characterization of the purified 112–608aa ORF2 protein. **(A)** Upper panel: Coomassie Brilliant Blue stained image of the glycosidase treated purified 112–608aa ORF2 protein. Lower panel: Western Blot of aliquots of samples shown in upper panel with anti-ORF2 antibody. **(B)** Transmission electron micrograph of the purified 112–608aa ORF2 protein (scale: 50 nm, magnification: 55000X). **(C)** ELISA of healthy controls and HEV patients sera using purified 112–608aa ORF2 protein as antigen. Data are mean ± SEM of triplicate samples. **(D)** Left panel: Western blot of the purified 112–608aa ORF2 using sera from the indicated samples; Right panel: Ponceau staining of a representative western blot.

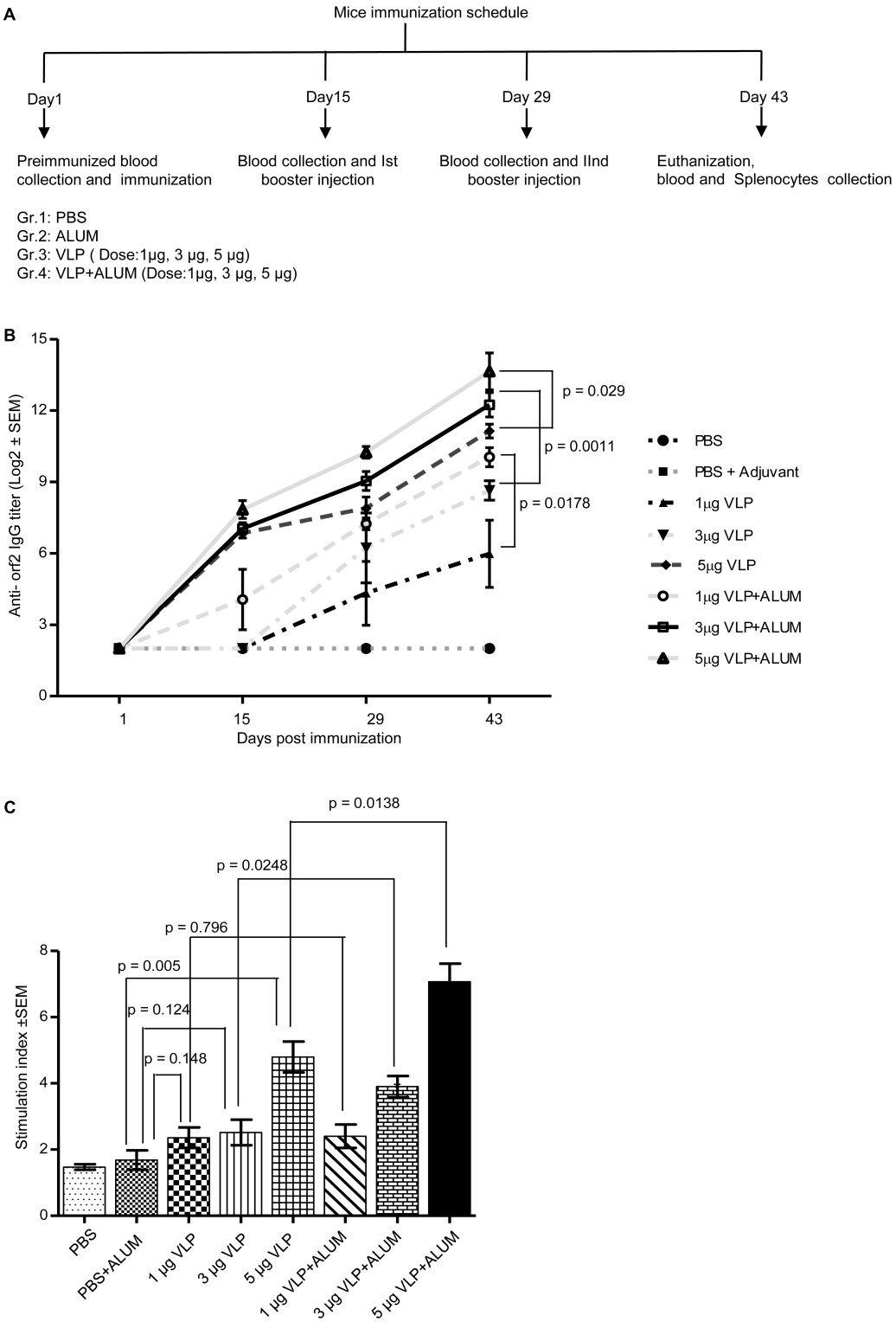

FIGURE 4 | Evaluation of immunogenicity of the purified 112– 608aa ORF2 protein. **(A)** Schematic of mice immunization schedule. **(B)** Antibody titer profile of ORF2 specific IgG in the sera of mice immunized with purified 112–608aa ORF2 protein with or without ALUM, as indicated. The sera of all animals (n = 5) were collected before each immunization and twofold serially diluted starting from 1:100 for ELISA analysis. The reciprocal of the highest dilution showing two times absorbance of control mice was taken as positive antibody titer. Data represented as log2 transformed antibody titers and negative titer value was set as log value 2.0 for statistical analysis. **(C)** Cell proliferation assay of the cultured splenocytes harvested from the immunized mice, stimulated for 24 h with 5 µg purified 112–608aa ORF2 protein. The Stimulation index denotes the ratio of values obtained for immunized mice to that of the control mice. Data represented as mean ± SEM of 5 samples.

M (315-453), and P (454-606) play measure role in VLP formation. The S and M domains are highly conserved between genotypes and are the fundamental structural units in mature viral particles (Simanavicius et al., 2018). Epitope mapping studies demonstrate that monoclonal antibody binding sites are present on the S and M domain rather than the P domain (Meng et al., 2001; Zhang J.Z. et al., 2001).

The current cell culture system of HEV is not efficient enough to produce plenty of viruses for vaccination purpose (Tanaka et al., 2007). Moreover, recombinant ORF2 VLP also holds importance for developing diagnostic assays for HEV infection. Therefore, there is a lot of focus to produce HEV VLPs through recombinant means. Here, we used *P. pastoris* expression system to produce glycosylated 112–608aa ORF2 protein, which is secreted to the culture medium in the form of VLP. This VLP includes the S domain (absent in the 368–606aa ORF2 VLP currently used as HEV vaccine in China), which has been shown to be crucial for stabilization of the capsid shell (Xing et al., 2010).

Analysis of a limited number of HEV patient sera indicates that the 112–608aa VLPs retain the antigenic epitopes of the ORF2 protein. Since it is easy and economical to purify these VLPs, their diagnostic potential may be explored. Evaluation of immunogenicity of 112–608aa ORF2 VLPs in mice revealed that alum emulsified VLPs elicit stronger immune response compared to non-alum VLPs. Other adjuvants may be explored to identify the ideal combination formula.

Recently, it was reported that glycosylated and cleaved ORF2 proteins are most abundant in the infected patient sera and the same protein forms are highly recognized by patient antibodies (Montpellier et al., 2018; Ankavay et al., 2019). In this context, the 112–608aa ORF2 VLPs will be useful to evaluate the role of glycosylation status of ORF2 in mediating the immune response and protection from infection. Efforts are underway to generate non-glycosylated 112–608 ORF2 VLP and compare its protective efficacy to that of the glycosylated VLP. VLPs are considered to be good vaccine candidates, as they closely resemble native virus particles, without being infectious. Indeed, VLPs are being used as a licensed vaccine product for human papillomavirus (Olsson et al., 2007) and HBV (Murata et al., 2003). Therefore, the next step should focus on evaluating the efficacy of the *Pichia* expressed ORF2 VLPs in an infectious animal model of HEV. These VLPs may also be engineered to display additional antigenic epitopes from HEV or other pathogens. Future studies should aim at exploring such possibilities.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Ethics Committee of Translational Health Science and Technology Institute (THSTI), constituted under the provisions of CPCSEA (Committee for the Purpose of Control and Supervision on Experiments on Animals), Government of India.

AUTHOR CONTRIBUTIONS

JG and MS contributed to the experimental design and data analysis. JG, SK, and MS wrote the manuscript. JG, SK, AS, SG, and CS performed the experiments. MB, Shalimar, BN, GB, NK, and CR-K provided material, analysis tools, and suggestions.

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Hepatitis E Virus ORF2 Inhibits RIG-I Mediated Interferon Response

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Understanding the dynamics of host innate immune responses against a pathogen marks the first step toward developing intervention strategies against the pathogen. The cytosolic pattern recognition receptor retinoic acid-inducible gene I (RIG-I) has been shown to be the major innate immune sensor for hepatitis E virus (HEV). Here, we show that HEV capsid protein (ORF2), a 660 amino acid long protein, interferes with the RIG-I signaling. Interestingly, only the full length ORF2 protein but not the 112-608 ORF2 protein inhibited RIG-I dependent interferon response. Both synthetic agonist and virus induced RIG-I activation was modulated by ORF2. Interference of interferon response was confirmed by reporter assays involving different interferon inducible promoters, qRT PCR, ELISA, and immunofluorescence microscopy. Neither glycosylation nor dimerization of the ORF2 protein had any effect on the observed inhibition. Further analyses revealed that the ORF2 protein antagonized Toll-like receptor (TLR) pathways as well. ORF2 inhibited signaling by RIG-I and TLR adapters, IPS-1, MyD88, and TRIF but was unable to inhibit activation by ectopically expressed IRF3 suggesting that it may be acting at a site upstream of IRF3 and downstream of adapter proteins. Our data uncover a new mechanism by which HEV may interfere with the host antiviral signaling.

Keywords: HEV, ORF2, RIG-I, interferon, innate immunity

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INTRODUCTION

Hepatitis E virus (HEV) has emerged as a leading cause of viral hepatitis since its discovery in the 1980s (Purcell and Emerson, 2008). The disease is self-limiting in healthy individuals but leads to chronicity and severe liver damage in patients with compromised immune system such as organ transplant patients (Melgaço et al., 2018). Due to its small size, HEV can easily cross the blood–brain barrier causing neuropathological manifestations (Shi et al., 2016). In rare cases, other extrahepatic manifestations such as musculoskeletal, hematological, renal, and immunological diseases have also been reported (Bazerbachi et al., 2015). A recent report estimated that 70,000 HEV-related deaths occur each year (Melgaço et al., 2018). The case fatality rate is particularly high in pregnant women (25–30%) (Melgaço et al., 2018).

Current anti-HEV treatments are limited to ribavirin therapy which cannot be administered to pregnant women due to its teratogenic effects (Todt et al., 2018). Additionally, in chronic

cases it leads to ribavirin induced anemia in 50% of the patients (Kamar et al., 2016; Van De Garde et al., 2017). PEGylated interferon is used in combination or as an alternative to ribavirin. However, it cannot be used for the high risk group of transplant patients due to fears of graft rejection (Kamar et al., 2016). Recent years have seen an increase in the number of reported HEV cases due to improved detection techniques, highlighting the need for developing better interventions against HEV.

Understanding viral interactions with the host and their roles in immune-modulation events during infection could lead to improved treatment regimes. The cytosolic pattern recognition receptor retinoic acid-inducible gene I (RIG-I) has been shown to be the major innate immune sensor for HEV (Xu et al., 2017). Classically, activation of RIG-I upon recognition of the viral RNA leads to its association to its adapter protein IPS-1 (also known as MAVS, VISA, and Cardif) and induction of a downstream cytokine, interferon beta (IFN-β) through a signaling cascade. Although the HEV RNA is recognized by RIG-I, downstream production of IFN-β upon recognition was low during HEV infection (Yin et al., 2017). These observations indicate that HEV might be modulating the RIG-I pathway to delay or subdue the host antiviral response. Furthermore, HEV ORF1 proteins, such as X, PCP (Nan et al., 2014b), and methyl transferase (MeT) (Bagdassarian et al., 2018; Kang et al., 2018) have been shown to lower IFN-β induction by inhibiting the RIG-I signaling. HEV ORF2 has been shown to inhibit the NF-κB signaling pathway by preventing proteasomal degradation of the IκBα protein (Surjit et al., 2012).

Here, we report that the full length HEV ORF2 protein (FL ORF2) inhibits RIG-I signaling in mammalian cells. Further characterization revealed that ORF2 interferes with the toll-like receptor mediated signaling as well. In the presence of FL ORF2, IFN-β induction and NF-κB activation are significantly lowered.

MATERIALS AND METHODS

Cloning and Site Directed Mutagenesis

All the HEV plasmids were constructed by PCR amplification of HEV protein domains from genotype 1 HEV (HEV-1, pSK-HEV2) and genotype 3 HEV (HEV-3, pSK-P6). HEV replicons were a kind gift from Dr. Suzanne Emerson, National Institute of Allergy and Infectious Diseases, NIH, Maryland, United States. Digested amplicons were cloned into linearized pUNO-mcs vector (Invivogen) at specified sites with Flag, Myc, or HA epitope tags (PCP, RdRp, and ORF2 with the FLAG tag, MeT and helicase with the myc tag, and Domains X, Y and ORF3 with the HA tag). pUNO-IRF3 HA was subcloned from a commercially available vector. Clones were confirmed by restriction digestion and DNA sequencing. pUNO RIG-I, pUNO IPS-1, and pUNO MyD88 were purchased from Invivogen (United States). pcDNA-TRIF myc was a kind gift from Dr. Stanley Lemon, University of North Carolina, United States. IFN-β Firefly Luc reporter plasmid was a kind gift from Dr. R. Lin, McGill University, Canada. NF-κB Firefly Luc, ISRE Firefly Luc, ISG56 Firefly Luc, pRLTKLuc *Renilla* and cmvRLLuc *Renilla* reporter plasmids were purchased from Promega. Plasmid information is given in **Supplementary Table S1**. A detailed list of primers is given in **Supplementary Table S2**.

Point mutations were introduced at different positions as indicated using PCR-based site directed mutagenesis to generate the glycosylation and dimerization mutants. Details of all the primers used to generate mutants are given in **Supplementary Table S3**. Clones were sequenced to confirm successful mutagenesis.

SDS PAGE and Western Blotting

For SDS PAGE, cell lysates in laemmli buffer (60 mM Tris-Cl Buffer pH 6.8, 2% SDS, 10% glycerol, 0.01% BPB, 0.1% β-mercaptoethanol) were incubated at 95°C for 3 min prior to loading and separated on 8–12% acrylamide gels with 0.1% SDS. Separated proteins were transferred to a PVDF membrane. Blocking was done using 5% non-fat milk for 1 h at room temperature. Primary antibody incubations were done for 16 h at 4°C in 5% blocking buffer containing the respective primary antibody at 1:1000 dilution. Proteins were detected using appropriate HRP tagged secondary antibodies (1:5000).

Maintenance of Cell Lines and Transfections

HEK293T and Huh7 (WT and stable) cells were maintained in Dulbeco's Modified Eagle Medium (DMEM) Glutamax supplemented with 10% FBS with penicillin and streptomycin at 37°C, 5% CO2. Stable cells were maintained in 4 μg/ml of blasticidin. DMEM was replaced with RPMI medium for THP-1 cells all other conditions were constant.

For transfections, cells were seeded at 70–80% confluency. Lipofectamine 2000 transfection reagent (Life Technologies) was used for DNA transfection at 1:1 ratio (1 μl Lipofectamine per μg of DNA). DNA concentrations are mentioned individually for each experiment in figure legends.

Virus Infections

Purified Sendai virus (SeV) was a kind gift from Prof. Debi P Sarkar, University of Delhi, India. Cells were infected at an experimentally optimized dose of 40 HAU/ml for HEK293T and 100 HAU/ml for Huh7. All infections were done for 12–16 h in serum free DMEM containing penicillin and streptomycin.

Purified Japanese Encephalitis virus (JEV) was a kind gift from Prof. Sudhanshu Vrati, Regional Centre for Biotechnology, Faridabad, India. Cells were infected with JEV at 0.5 multiplicity of infection (MOI). Infections were done in serum free DMEM containing penicillin and streptomycin for 3–4 h and replaced with DMEM with serum containing penicillin and streptomycin. Cells were incubated for 12 h to ensure optimum induction.

Luciferase Assays

Firefly luciferase cloned under the IFN-β promoter (IFN-β Luc) was used as the reporter for measuring IFN-β promoter induction. *Renilla* luciferase cloned under thymidine kinase promoter (pRLTKLuc) or CMV promoter (CMVRL Luc) was used as an internal control reporter for HEK293T or Huh7

cells, respectively. For RIG-I assay, RIG-I plasmid and reporter plasmids were transfected into HEK293T or Huh7 cells. 24 h post-transfection, a synthetic 5' triphosphorylated small double-stranded RNA (3pdsR27), SeV, or JEV (as described for individual experiment) were used to induce the pathway. RIG-I was replaced with the IPS-1 plasmid for IPS-1 assay, myc-TRIF for TRIF assay, MyD88 for MyD88 assay, and HA-IRF3 for IRF3 assay. IPS-1 or IRF3 over-expression results in constitutive activation of IFN-β promoter. Wherever applicable, individual HEV clones were transfected along with the corresponding plasmids described above. Luciferase activity was measured 24 h post-transfection using Promega Dual Glo luciferase assay kit following manufacturer's protocol. Firefly luciferase values were normalized with *Renilla* luciferase values and the data were plotted as % fold change where the test samples were compared to the induced positive control values taken as 100%.

RNA Isolation and Quantitative RT (qRT) PCR

Total RNA was isolated from HEK293T or Huh7 cells using TRIzol (ThermoFisher Scientific, United States) reagent, as per manufacturer's instructions. 1 μg of the RNA was used for cDNA synthesis with random hexamers using the GoScript first strand synthesis kit (Promega, United States). Gene specific primers were used to quantify IFN-β, ISG56, and NF-κB transcripts by SYBR green-based relative quantitation using the LC96 Real Time PCR machine (Roche, Life Science, United States). Test gene Ct values were normalized to the GAPDH Ct values and results were plotted using the ΔΔCt method. List of primers is present in **Supplementary Table S4**.

Generation of Stable Cell Lines

Stable cell lines were created in human hepatoma cell line, Huh7. FLAG-tagged HEV-1 FL ORF2, 112-608 ORF2 constructs or empty vector were digested with *Not*I restriction enzyme. 2 μg of the purified linearized plasmids were transfected into Huh7 cells using lipofectamine 2000 reagent at 1:1 ratio (Life Technologies, United States). 24 h post-transfection, media was replaced with DMEM containing 10% FBS and 4 μg/ml blasticidin, repeating after every 72 h till cells in the un-transfected control were completely dead. The blasticidin resistant cells were propagated under continued blasticidin selection. Presence of ORF2 proteins was confirmed by western blot every 2 weeks. Cells were said to be stably expressing the proteins when it was consistently observed for more than 2 months.

Immunofluorescence Assay

Huh7 cells transiently transfected with the FL ORF2 and 112-608 ORF2 plasmids were seeded on coverslips at 60% confluency. Cells were mounted in ProLong anti-fade gold reagent containing DAPI (ThermoFisher Scientific, United States). Cells were fixed with 2% paraformaldehyde in 1X PBS and ORF2 proteins were visualized with goat Anti-FLAG primary and anti-goat Alexa fluor 596 secondary antibodies.

For visualization of IRF3 translocation, pUNO Huh7, ORF2 Huh7, and 112-608 Huh7 stable cells were seeded on coverslips at 60% confluency and infected with JEV at 0.5 MOI. Cells were then fixed with 2% paraformaldehyde in 1X PBS. Rabbit anti-IRF3, mouse anti-JEV, and goat anti-FLAG antibodies were used as primary. Anti-rabbit Alexa-488 and an anti mouse Alexa-596 secondary antibodies were used. Cells were mounted in ProLong anti-fade gold reagent containing DAPI (ThermoFisher Scientific, United States). Slides were visualized using the Olympus FV3000 confocal microscope.

ELISA

Undifferentiated THP-1 cells were transfected with the FL ORF2 or 112-608 ORF2 plasmid along with a vector control. SeV infection was given at 40 HAU/ml 12 h post-transfection. Media was collected from the samples 24 h post-infection by centrifugation at 1000 *g*, 20 min, 4°C. 100 μl of the media was used to quantify secreted IFN-β protein levels using a sandwich ELISA kit (SEA222Hu, Aspira chemical, United States) as per manufacturer's instructions.

Purified IFN-β protein standard provided in the kit was processed in the same way as the test samples to obtain the standard curve (at concentrations mentioned in the plot given in **Supplementary Figure S6A**). Values of the test samples were plotted as absolute values of IFN-β protein (pg/ml) deduced from the standard curve.

Statistical Analysis

Each experiment was performed at least thrice in triplicates. *P*-values were calculated using unpaired Student's *t*-tests.

RESULTS

IFN-β Production Is Inhibited by the HEV ORF2 Protein

In order to identify the HEV protein(s) that might be interfering with the RIG-I signaling, a reporter assay was designed in which RIG-I mediated activation of the IFN-β promoter was quantified by measuring the activity of firefly luciferase, as described earlier (Madhvi et al., 2017). For this, all the known protein domains of HEV Genotype I (HEV-1) and Genotype III (HEV-3) were cloned into the mammalian expression vector pUNO-mcs. Expression of the HEV protein domains was verified by western blotting, which confirmed their authenticity (**Supplementary Figure S1**). Effect of HEV-1 and HEV-3 HEV domains on RIG-I signaling was determined by measuring IFN-β promoter activation using luciferase assays. Briefly, plasmids expressing RIG-I and HEV protein domains were transfected in HEK293T cells along with reporter plasmids; firefly luciferase under IFN-β promoter and *Renilla* luciferase under thymidine kinase promoter. A synthetic 27 bp triphosphorylated double-stranded RNA agonist of RIG-I (3pdsR27) was used to induce RIG-I signaling (Ranjith-Kumar et al., 2009). Both HEV-1 and HEV-3 ORF2 inhibited RIG-I signaling (**Figures 1A,B**). HEV-3 PCP also inhibited RIG-I signaling as reported earlier (Nan et al., 2014b) but HEV-1 PCP did not show any inhibition (**Figures 1A,B**). Contrary

to the earlier report, MeT domains of both genotypes showed no significant effect on RIG-I signaling (Bagdassarian et al., 2018; Kang et al., 2018). ORF3 proteins of both genotypes showed increase in IFN-β activation as reported previously (Nan et al., 2014a). We focused on characterization of the effect of HEV ORF2 on RIG-I signaling. The ORF2 protein from both genotypes showed a dose dependent inhibition of IFN-β promoter activity and HEV-1 ORF3, used as a control, showed increased activation of IFN-β promoter (**Supplementary Figure S2**).

To determine whether ORF2 acts directly on RIG-I, we tested its effect on IPS-1, which is the adapter protein of RIG-I. Over-expression of IPS-1 activates RIG-I signaling in the IFN-β promoter reporter assay (Kawai et al., 2005). Both HEV-1 and HEV-3 ORF2 protein lowered the IPS-1 induced IFN-β promoter activation in a dose-dependent manner whereas HEV-1 ORF3 increased the IPS-1 induced IFN-β promoter activation (**Figure 1C**). This result suggests that ORF2 may be inhibiting RIG-I signaling by acting at a step downstream of RIG-I. Additionally, we also looked at the effect of ORF2 on ORF3 induced IFN-β promoter activation (**Figure 1D**). When co-expressed in HEK293T cells, ORF2 significantly inhibited IPS-1 induced IFN-β promoter activation in the presence of ORF3, suggesting that the inhibitory effect of ORF2 is dominant.

Next, to test whether ORF2 protein could inhibit interferon response during viral infection, we infected HEK293T cells with SeV, a known inducer of RIG-I pathway (Kato et al., 2006). To determine the specificity of SeV dependent RIG-I activation, an RNA binding incompetent mutant of RIG-I, RIG-I K858E was also used (Cui et al., 2008). Expression of RIG-I and K858E mutant was confirmed by western (**Supplementary Figure S1**). As expected, significant activation of IFN-β promoter was observed only in case of WT RIG-I upon SeV infection (**Figure 1E** and **Supplementary Figure S3**). ORF2 inhibited SeV induced activation of the IFN-β promoter while ORF3 was unable to do so (**Figure 1E**). As RIG-I signaling also results in NF-κB activation, we also checked the effect of ORF2 on NF-κB promoter activity. Similar to IFN-β promoter activity, both HEV-1 and HEV-3 ORF2 protein lowered NF-κB promoter activity, further confirming its antagonistic effect on the RIG-I signaling (**Figure 1F** and **Supplementary Figure S4**). Co-expression of ORF3 in SeV infected cells resulted in 2.5-fold activation of NF-κB promoter (**Figure 1F** and **Supplementary Figure S4**). As the ORF2 proteins from both genotypes showed similar inhibition of RIG-I signaling, we performed further experiments with HEV-1 ORF2.

Next, we tested a truncated form of the ORF2 protein, spanning the 112-608 amino acid region of the full length ORF2 protein. We first compared the expression of the full length (FL ORF2) and the truncated (112-608 ORF2) ORF2 proteins in the HEK293T cells (**Figure 2A**). The effect of 112-608 ORF2 protein on RIG-I signaling was assessed by IFN-β promoter reporter assays using 3PdsR27 and SeV as RIG-I inducers. In both cases, FL ORF2 inhibited IFN-β promoter activity but 112-608 ORF2 did not (**Figures 2B,C**). To test the observed inhibition is not due to toxic effects of the FL ORF2, cytotoxicity of these two proteins was tested in HEK293T cells, using the formazan-based reagent WST-1. Both FL ORF2 and 112-608 ORF2 did not show any significant cytotoxicity (**Figure 2D**). Next, transcript levels of IFN-β and a downstream interferon stimulated gene (ISG), ISG56 and NF-κB were measured by quantitative real-time PCR in the presence of FL ORF2 and 112-608 ORF2 in IPS-1 over-expressing HEK293T cells. As expected, IFN-β, ISG56, and NF-κB mRNA levels were significantly lowered in the presence of FL ORF2 but not 112-608 ORF2 (**Figures 2E–G**). Similar decrease in IFN-β mRNA level was observed in Huh 7 cells as well (**Supplementary Figure S5**). To confirm that the observed effect is reflected at the protein level as well, a sandwich ELISA was performed to measure the IFN-β protein. For this, THP1 cells transfected with FL ORF2, 112-608 ORF2 or a vector control were infected with SeV and the level of secreted IFN-β protein was measured using sandwich ELISA. IFN-β levels were calculated from a standard curve (**Supplementary Figure S6A**). In agreement with the result from other assays, IFN-β protein was significantly lower in the presence of FL ORF2 but not 112-608 ORF2 (**Figure 2H**). To ensure the expression of ORF2 and 112-608 ORF2 in THP-1 cells, a direct qualitative ELISA was performed. Purified ORF2 protein was used as positive control. Ectopically expressed ORF2 and 112-608 ORF2 proteins were expressed at similar levels (**Supplementary Figure S6B**).

HEV-1 ORF2 Glycosylation or Dimerization Has No Effect on Its Ability to Antagonize the RIG-I Signaling

FL ORF2 is glycosylated and 112-608 ORF2 is unglycosylated (Zafrullah et al., 1999; Jimenez de Oya et al., 2012). To test the role of glycosylation in the observed inhibition of IFN-β production by the FL ORF2 protein, we created glycosylation deficient mutants of FL ORF2. All three reported glycosylation sites of FL ORF2, such as N137, N310, and N562 (Zafrullah et al., 1999) were mutated individually (single mutants, SM), in pairs (double mutants, DM), or simultaneously (triple mutant, TM) (**Figure 3A**). Western blot analysis revealed that expression of all the mutants was quite similar to that of the wild type ORF2 (**Figure 3B**). In the IFN-β promoter reporter assay, none of the glycosylation mutants showed any significant difference compared to the wild type ORF2 in inhibiting the IPS-1 induced RIG-I signaling (**Figure 3C**).

HEV ORF2 protein self-associates and forms higher order structures (Li et al., 2009). To understand whether dimerization played any role in the inhibition of IFN-β promoter activation by FL ORF2, dimerization deficient mutants V598E and A602E were generated in FL ORF2 and 112-608 ORF2, based on a previously reported study (Li et al., 2009). The mutations did not affect the expression levels of these proteins (**Figure 3D**). Next, the effect of these mutants on RIG-I signaling was analyzed. IFN-β promoter reporter assay revealed that the V598E and A602E mutations inhibited 3pdsR27 induced RIG-I signaling, similar to the wild type ORF2 (**Figure 3E**). These data suggest that neither glycosylation nor higher order structures of FL ORF2 are a prerequisite for its observed inhibitory activity.

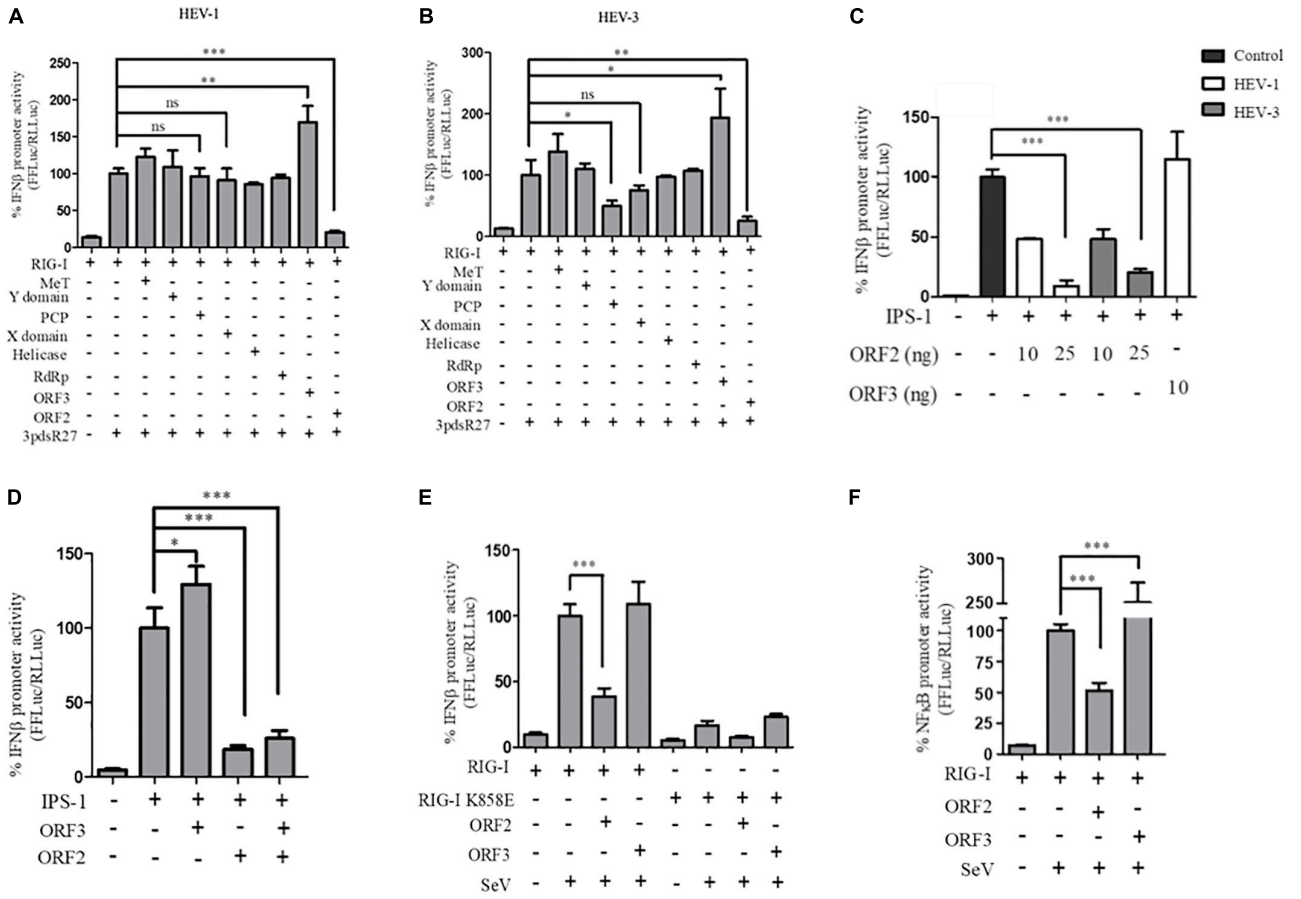

FIGURE 1 | HEV ORF2 protein inhibits IFN-β promoter activation by downregulating the RIG-I pathway. **(A)** Plasmids expressing HEV-1 proteins were transfected into the HEK293T cells (25 ng) along with RIG-I (0.5 ng) and IFN-β firefly and TK *Renilla* luciferase reporters. RIG-I was induced with 3pdsR27 24 h post-transfection. Luciferase assay was done 16 h post-induction. **(B)** Plasmids expressing HEV-3 proteins were transfected into the HEK293T cells (25 ng) along with RIG-I (0.5 ng) and IFN-β firefly and TK *Renilla* luciferase reporters. RIG-I was induced with 3pdsR27 24 h post-transfection. Luciferase assay was done 16 h post-induction. **(C)** For IPS-1 assay, 0.5 ng of IPS-1 was co-transfected with two different quantities of HEV-1 ORF2, HEV-3 ORF2 plasmids (10 and 25 ng) or 10 ng of HEV-1 ORF3 plasmid along with IFN-β firefly and TK *Renilla* luciferase reporter plasmids. Luciferase activity was measured 24 h post-induction. **(D)** HEK293T cells were co-transfected with 0.5 ng of IPS-1 plasmid, HEV-1 ORF2 or ORF3 plasmids (25 ng each) individually or in combination along with IFN-β firefly and TK Renilla luciferase reporters. Luciferase activity was measured 24 h post-transfection. **(E)** 0.5 ng of WT RIG-I or RIG-I K858E plasmid was co-transfected with 25 ng of HEV-1 ORF2 plasmid or ORF3 plasmid along with IFN-β firefly and TK *Renilla* luciferase reporter plasmids. Induction was given 24 h post-transfection with 40 HAU/ml of Sendai virus (SeV). Luciferase activity was measured 16 h post-induction. **(F)** Effect of the HEV-1 ORF2 and ORF3 on NF-κB activity. RIG-I assay was performed by co-transfecting 0.5 ng of the RIG-I plasmid, the HEV-1 ORF2 or the HEV-1 ORF3 plasmids (25 ng) along with the NF-κB firefly and TK *Renilla* luciferase reporter plasmids. SeV infection was given 24 h post-transfection. Luciferase activity was measured 16 h post-induction. All experiments were performed in the HEK293T cells. Values are mean ± SD, $n = 3$ (*** denotes p-values ≤ 0.001, ** denotes p-values ≤ 0.01, and * denotes p-values ≤ 0.05).

HEV-1 ORF2 Inhibits TRIF and MyD88 Induced IFN-β Production

To determine whether the observed inhibition of IFN-β promoter activity is specific to the RIG-I signaling pathway, IFN-β production through Toll-like receptor (TLR) adapters TRIF and MyD88 were analyzed in the presence and absence of HEV-1 ORF2 (**Figure 4**). Note that TRIF is an adapter for TLR3 and all other TLRs use MyD88. TLR4 can signal via both TRIF and MyD88 (Lester and Li, 2014). First, the expression of these adapter proteins was confirmed by western (**Supplementary Figure S1**). FL ORF2 inhibited TRIF and MyD88 induced IFN-β promoter activity (**Figures 4A,B**). TRIF induced IFN-β and ISG56 mRNA levels were also significantly reduced in the presence of FL ORF2 (**Figures 4C,D**) suggesting that ORF2 interferes with TLR signaling pathways.

IRF3 Nuclear Translocation Is Affected in the Presence of HEV FL ORF2

One of the proteins central to both the RIG-I and the TLR pathway is the transcription factor IRF3, which when activated undergoes phosphorylation, dimerization, and subsequent nuclear translocation (Seth et al., 2006). In order to monitor IRF3 nuclear translocation in the presence and absence of the FL and 112-608 ORF2 proteins, an immunofluorescence assay was performed. Due to the lack of SeV specific antibody for detection, JEV, which is also a known agonist of RIG-I pathway, was used

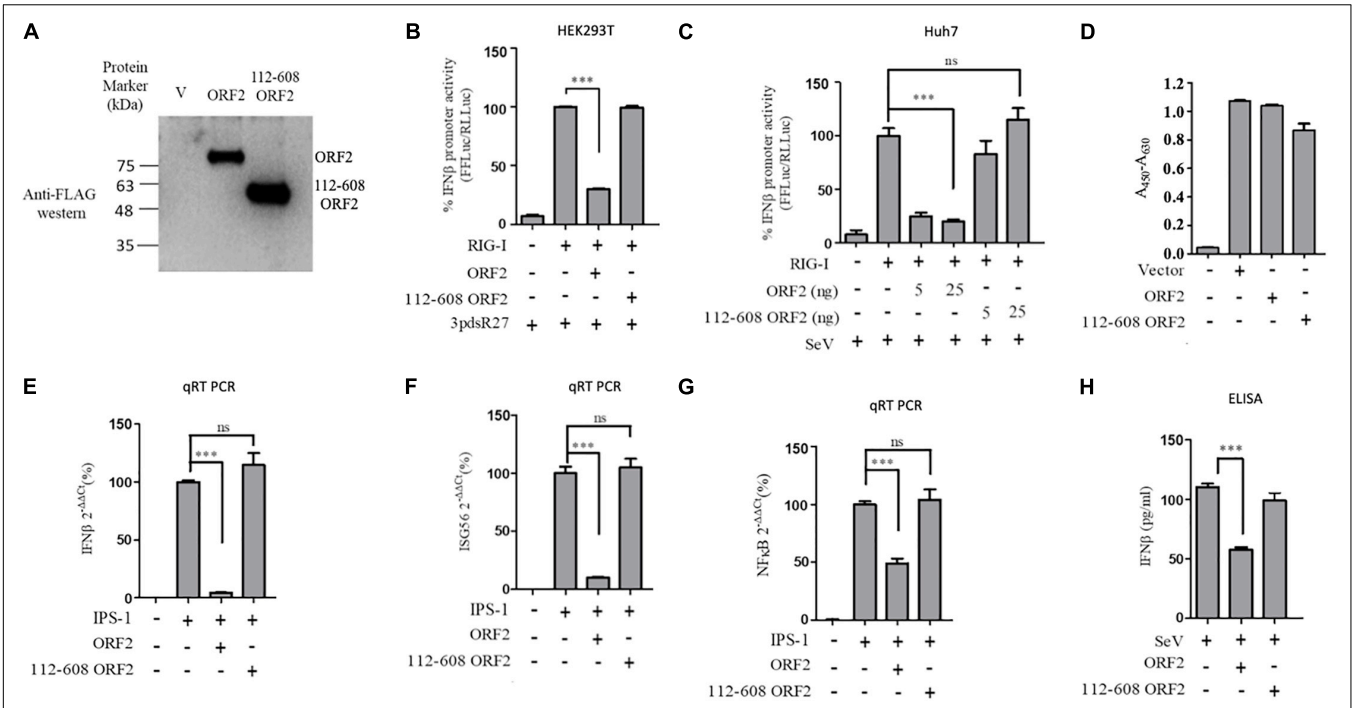

FIGURE 2 | HEV 112-608 ORF2 protein does not inhibit the IFN-β promoter activation. **(A)** Western blot analysis of the HEV-1 FL ORF2 and 112-608 ORF2 in HEK293T cells. **(B)** 0.5 ng of WT RIG-I plasmid was co-transfected with 25 ng of FL ORF2 or 112-608 ORF2 plasmids along with IFN-β firefly and TK *Renilla* luciferase reporter plasmids in HEK293T cells. Induction was given 24 h post-transfection with 3pdsR27. Luciferase activity was measured 16 h post-induction. **(C)** Plasmids expressing the FL ORF2 and 112-608 ORF2 were transfected into Huh7 cells (5, 25 ng) along with RIG-I (0.5 ng) and IFN-β firefly and CMV *Renilla* luciferase reporter plasmids. Induction was given 24 h post-transfection with 100 HAU/ml of SeV. Luciferase activity was measured 16 h post-induction. **(D)** For cell viability assay, 25 ng each of vector, FL ORF2 or 112-608 ORF2 plasmids were transfected into HEK293T cells. 48 h post-transfection, WST-1 reagent (diluted 1:10 with media) was added to the cells, incubated for 1 h and absorbance (A_{450}) was recorded. Values were normalized to that of the control absorbance taken at A_{630} and plotted as difference in the values (A_{450}–A_{630}). **(E)** HEK293T cells were co-transfected with IPS-1 (20 ng) and FL ORF2 or 112-608 ORF2 plasmids (1 μg each). RNA was isolated 24 h post transfection. Ct values corresponding to IFN-β or **(F)** ISG56 or **(G)** NF-κB mRNA levels were normalized to that of GAPDH Ct values and change in mRNA levels was calculated by the ΔΔCt method with respect to the un-induced vector control. **(H)** THP-1 cells were transfected with vector, FL ORF2, or 112-608 ORF2 plasmids. SeV infection was given 12 h post-transfection. Secreted IFN-β protein levels were estimated by a sandwich ELISA from media collected 24 h post-infection. Values are percent mean ± SD for qRT PCR and mean ± SD for ELISA. $n = 3$ for all experiments (*** denotes p-values ≤ 0.001, ns denotes p-values ≥ 0.05).

as an inducer (Chang et al., 2006). We first verified RIG-I pathway activation by JEV by measuring the RIG-I induced IFN-β promoter reporter activity. Infection with JEV led to a robust induction of IFN-β promoter activity, only when RIG-I was co-expressed, indicating that the RIG-I pathway is activated upon JEV infection. As with SeV, JEV induced IFN-β promoter activation was inhibited by the FL ORF2 but not the 112-608 ORF2 (**Figure 5A**).

Transient transfection often results in differential levels of expression in different cells depending on the transfection efficiency. Therefore, we established a hepatoma stable cell line that would constitutively express the FL ORF2 (ORF2 Huh7) or 112-608 ORF2 (112-608 Huh7) proteins. A vector control (pUNO Huh7) cell line was also generated. These cell lines were validated for the expression of the ORF2 proteins by western blotting (**Figure 5B**). The cell lines showed robust expression of FL ORF2 and 112-608 ORF2 proteins, which was consistently seen for more than 2 months (**Figure 5B**).

Next, the ability of stably expressed FL ORF2 to inhibit interferon response was determined by measuring the level of IPS-1 induced IFN-β promoter activation. For this, ORF2 Huh7, 112-608 Huh7, and pUNO Huh7 cell lines were transfected with IPS-1 and IFN-β promoter reporter plasmids. IFN-β promoter driven luciferase activity was significantly reduced in ORF2 Huh7 cells, but not in 112-608 Huh7 cells, keeping with the results obtained with transient transfection assays (**Figure 5C**). An immunofluorescence experiment was performed to check whether FL ORF2 and 112-608 ORF2 were expressed in all the cells at a comparable level. The ORF2 proteins were visualized with an anti-FLAG antibody and an Alexa-568 conjugated secondary antibody. Most of the cells showed similar levels of the two ORF2 proteins (**Figure 5D** middle column and right column). Next, these cells were infected with JEV to induce IRF3 phosphorylation and nuclear localization. A mock infection control was also used where pUNO Huh7 cells were incubated only with the infection media without JEV (**Figure 5E**, Mock).

High levels of IRF3 nuclear localization could be seen in control cells infected with JEV (**Figure 5E**, JEV) compared to mock. JEV infection was detected using antibody specific

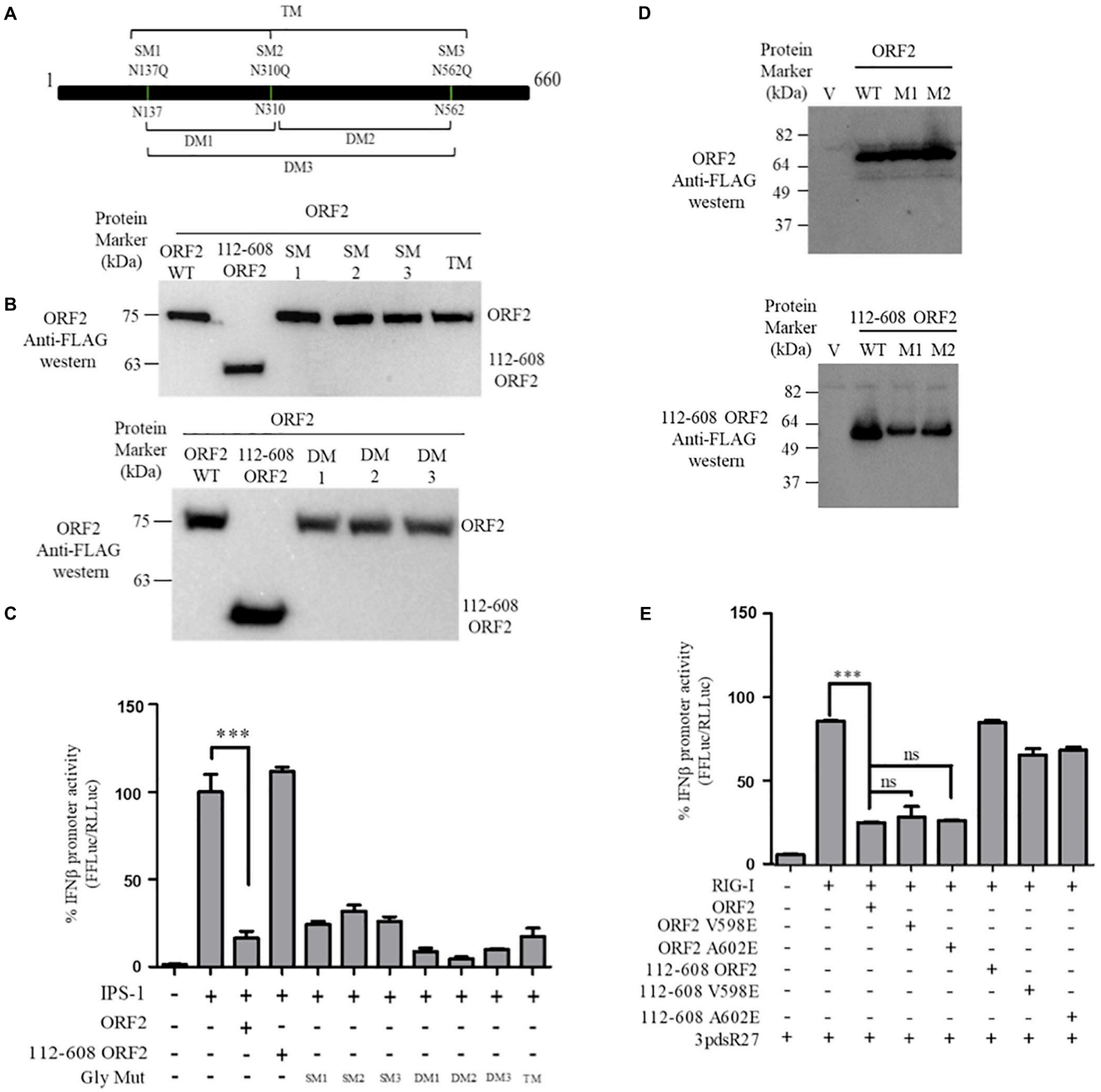

FIGURE 3 | ORF2 glycosylation and dimerization is not required for IFN-β inhibition: **(A)** A schematic showing the location of the three glycosylation sites and the mutations introduced in the FL ORF2. **(B)** Western blot analysis of WT and mutant FL ORF2 or 112-608 ORF2 in HEK293T cells. **(C)** For IPS-1 assay, 0.5 ng of IPS-1 was co-transfected with 25 ng of WT or mutant FL ORF2 or 112-608 ORF2 plasmids along with IFN-β firefly and TK *Renilla* luciferase reporter plasmids. Luciferase activity was measured 24 h post-induction. **(D)** Western blot analysis of the whole cell lysates of HEK293T cells expressing the WT FL ORF2 (top) or 112-608 ORF2 (bottom) and their two mutants, V598E (M1) and A602E (M2) ORF2. **(E)** RIG-I assay was performed with RIG-I (0.5 ng) and 25 ng of the WT and mutant ORF2 plasmids. Induction was given by transfecting 3pdsR27 24 h post-transfection. Luciferase activity was measured 16 h post-induction. Values are percent mean ± SD, $n = 3$ (*** denotes p-values ≤ 0.001, ns denotes p-values ≥ 0.05).

to its Glycoprotein E. However, in ORF2 Huh7 cells, IRF3 nuclear localization was significantly reduced (**Figure 5E**, ORF2 + JEV). Significant IRF3 translocation was observed in 112-608 Huh7 cells (**Figure 5E**, 112-608 + JEV). These data indicate that FL ORF2 inhibits translocation of IRF3 into nucleus.

To check whether ORF2 is directly acting on IRF3, we tested the effect of FL ORF2 on HEK293T cells overexpressing IRF3.

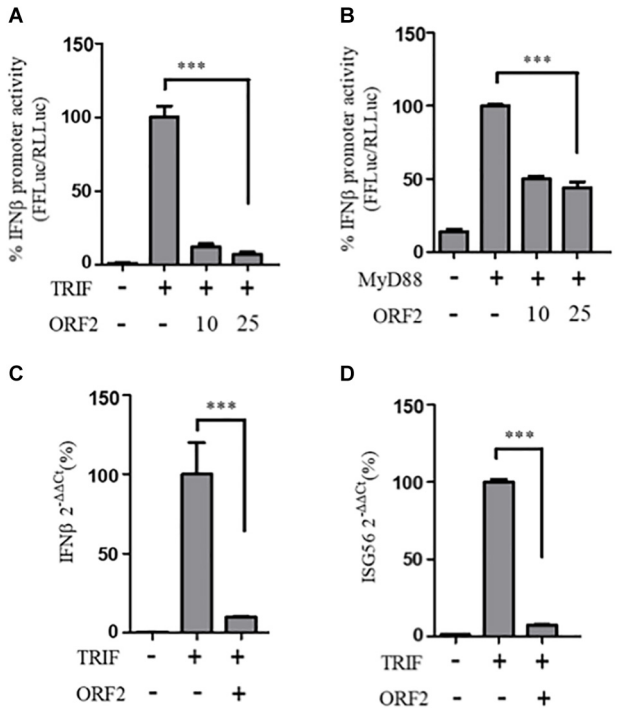

FIGURE 4 | HEV ORF2 protein inhibits TRIF and MyD88 induced IFN-β promoter activation. **(A)** TRIF assay was performed by co-transfecting 0.5 ng myc-TRIF plasmid with two different quantities of FL ORF2 (10 and 25 ng) along with IFN-β firefly and TK *Renilla* luciferase reporter plasmids. Luciferase activity was measured 24 h post-induction. **(B)** MyD88 assay was performed by co-transfecting 2.5 ng of MyD88 plasmid with two different quantities of FL ORF2 (10 and 25 ng) along with IFN-β firefly and TK *Renilla* luciferase reporter plasmids. Luciferase activity was measured 24 h post-induction. **(C)** Cells were co-transfected with TRIF (20 ng) and FL ORF2 plasmids (1 μg each). RNA was isolated 24 h post transfection. Ct values corresponding to IFN-β and **(D)** The ISG56 mRNA levels were normalized to that of the GAPDH Ct values and change in the mRNA level was calculated by the ΔΔCt method with respect to the un-induced vector control. All experiments were performed in HEK293T cells. Values are percent mean ± SD, $n = 3$ for all experiments (*** denotes p-values ≤ 0.001).

FL ORF2 was unable to inhibit IRF3 overexpression-induced activation of the IFN-β promoter (**Figure 5F**). Similar result was obtained in IRF3 overexpression-induced activation of the ISG56 and ISRE promoters (**Figures 5G,H**, respectively). Taken together, these data suggest that the FL ORF2 may be acting at a site down stream of adaptor proteins of RLR and TLR pathways but upstream of IRF3.

DISCUSSION

Rapid recognition of pathogens by pathogen recognition receptors is essential for mounting an effective immune response against it. Viruses have evolved multiple strategies to counter the innate immune recognition to replicate and spread efficiently. RIG-I is one of the key PRRs that recognize HEV (Xu et al., 2017). Earlier reports have shown that X, PCP, and MeT domains of HEV interfere with RIG-I signaling (Nan et al., 2014b; Bagdassarian et al., 2018; Kang et al., 2018). To identify whether other HEV encoded proteins inhibit interferon response, we ectopically expressed each protein domains of ORF1 along with ORF2 and ORF3 and assessed their ability to modulate RIG-I signaling. In our initial screen of HEV proteins, we identified ORF2 as a RIG-I inhibitor protein along with HEV-3 PCP. Compared to PCP, inhibition by ORF2 was higher and was seen in both genotypes, HEV-1 and HEV-3. These results point to an auxiliary role of the ORF2 protein in addition to encapsidation as a host immune regulator protein.

The full length ORF2 protein was previously shown to undergo processing to form the 112-608 aa product when expressed in insect cell lines (Zhang et al., 1997). It was hypothesized that the processed protein forms the viral capsid and is structurally similar to the full length ORF2 protein (Li et al., 1997). However, the 112-608 ORF2 did not inhibit IFN-β or NF-κB promoter activity whereas inhibition was consistently observed with the FL ORF2 protein. Interference of the RIG-I signaling was confirmed in different cell lines, using synthetic and viral RIG-I agonists, by reporter assays involving different interferon inducible promoters, qRT PCR, and ELISA and immunofluorescence microscopy. This difference between the effect of FL ORF2 and 112-608 ORF2 proteins on the RIG-I signaling could be due to lack of the N-terminal 111 or the C-terminal 52 amino acids in the 112-608 ORF2 protein. Studies have shown that the 112-608 aa region of the ORF2 forms T = 1 particles that are structurally distinct from the T = 3 native particles and do not encapsidate the viral RNA (Yamashita et al., 2009). Thus, the apparent lack of inhibition by 112-608 ORF2 could also be due to change in the structure.

Absence of the first 111 amino acids in 112-608 ORF2 also suggests that the protein lacks the signal sequence that translocates the protein to endoplasmic reticulum and hence is not glycosylated. Three potential glycosylation sites were identified for the HEV ORF2 protein (Zafrullah et al., 1999). Mutating these sites hindered capsid assembly and virus infectivity (Graff et al., 2008). Mutating the glycosylation sites did not alter the FL ORF2 protein induced IFN-β inhibition. This could mean that the structural features required for the FL ORF2 induced IFN-β inhibition are not altered in the absence of glycosylation.

In order to protect their genome, viral capsid proteins must assemble in a tightly packed capsid. Hence, the capsid proteins display strong self-interactions and often form dimers and hexamers, before the final assembly into mature virions, with or without the help of host proteins. It was shown that dimerization of the ORF2 protein plays a crucial role in recognition of the protein by host antibodies (Li et al., 2009). Thus, two of the residues (V598 and A602) that were shown to be critical for dimerization were mutated in FL ORF2 and 112-608 ORF2. V598 and A602 in context of 112-608 ORF2 were able to inhibit IFN-β promoter activity better than 112-608 ORF2. This could be due to altered structure of these mutants compared to that of 112-608 ORF2. However, this difference was not significantly high and was entirely absent in ORF2 indicating that formation of

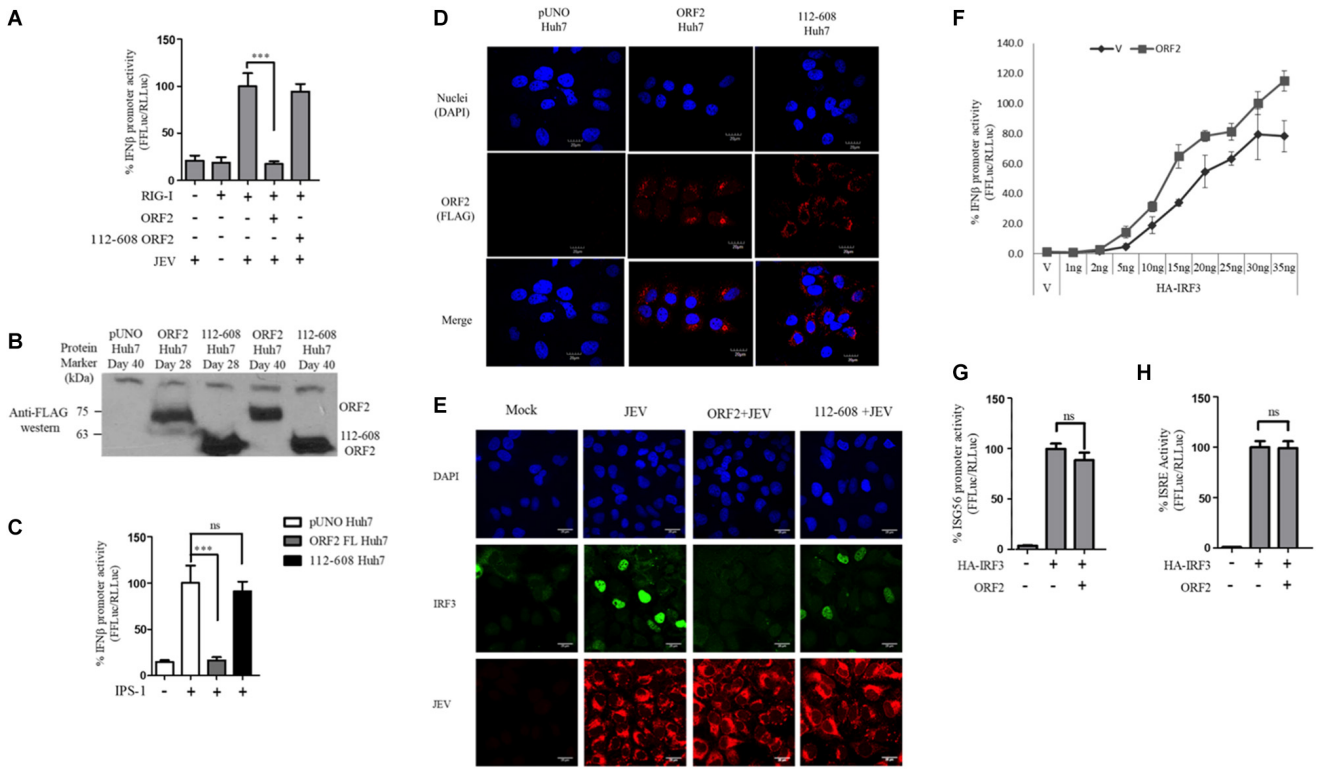

FIGURE 5 | IRF3 nuclear translocation is inhibited by the HEV ORF2 protein: **(A)** RIG-I assay was performed by co-transfecting RIG-I (0.5 ng) and FL ORF2 or 112-608 ORF2 plasmids with IFN-β firefly and CMV *Renilla* luciferase reporter plasmids in Huh7 cells. JEV infection was given at the 0.5 MOI for 4 h. Luciferase activity was measured 16 h post-infection. **(B)** Stable expression of FL ORF2 and 112-608 ORF2 proteins was monitored by western blot analysis. Cell lysates at day 28 and day 40 for each of ORF2 Huh7 and 112-608 Huh7 were probed with anti-FLAG antibody. **(C)** For IPS-1 assay, IPS-1 (5 ng) and IFN-β firefly and CMV *Renilla* luciferase reporter plasmids were transfected in pUNO Huh7, ORF2 Huh7, and 112-608 Huh7 stable cell lines. IFN-β promoter activity was measured 24 h post-transfection. **(D)** pUNO Huh7, ORF2 Huh7 and 112-608 Huh7 cells were treated with the goat anti-FLAG primary antibody and anti-Goat Alexa-568 (red) secondary antibody to visualize the ORF2 proteins. Nuclei were stained with DAPI. The scale represents 20 μm. **(E)** pUNO Huh7, ORF2 Huh7, and 112-608 Huh7 cells were infected with the JEV at 0.5 MOI for 24 h. Nuclei were stained with DAPI, IRF3 was stained with rabbit anti-IRF3 primary and anti-rabbit Alexa-488 (green) antibody and JEV was stained with mouse anti-JEV glycoprotein E antibody and anti-mouse Alexa-596 (red) antibody. **(F)** IRF3 assay was performed by co-transfecting increasing quantities of HA-IRF3 and 25 ng of FL ORF2 plasmids with IFN-β firefly and TK *Renilla* luciferase reporter plasmids. Luciferase activity was measured 24 h post-transfection. **(G)** IRF3 assay was performed by co-transfecting HA-IRF3 and 25 ng of FL ORF2 plasmids with ISG56 firefly and TK *Renilla* luciferase reporter plasmids. Luciferase activity was measured 24 h post-transfection. **(H)** IRF3 assay was performed by co-transfecting HA-IRF3 and 25 ng of FL ORF2 plasmids with ISRE firefly and TK *Renilla* luciferase reporter plasmids. Luciferase activity was measured 24 h post-transfection. Values are mean ± SD, $n = 3$ for all experiments. (*** denotes p-values ≤ 0.001, ns denotes p-values ≥ 0.05).

higher order structures by ORF2 may not be required for RIG-I antagonism.

While our manuscript was being prepared, an article was published, which showed a direct interaction of HEV ORF2 with TBK-1 (Lin et al., 2019). The authors suggest that ORF2 interacts with TBK-1 via its N-terminal arginine rich region and interferes with the RIG-I/MDA5 pathway. Our data further show that in addition to RLR HEV ORF2 also affects pathway TLR pathway. Analyses with adapter proteins of the RIG-I-like receptors (RLRs) and TLR pathways, IPS-1, TRIF, and MyD88 showed that the ORF2 protein inhibited signaling via these PRRs. Multiple studies have shown that MyD88 signaling pathway does not involve TBKI (Clark et al., 2011; Kawasaki and Kawai, 2014). Inhibition of MyD88 signaling by ORF2 suggests that it may be acting multiple sites in addition to TBK1. This suggests that ORF2 may be acting at a site common to multiple innate immune receptors. There is an inherent redundancy in different immune pathways as they converge downstream of the adaptors and utilize the same effector molecules for signal transduction (Takeuchi and Akira, 2010).

Both RLR and TLR families induce type I IFN production through a family of interferon regulatory factors (IRFs) (Fischer et al., 2010). In particular, IRF3 gets phosphorylated and forms a dimer, which then translocates to the nucleus and drives type I IFN transcription by binding to the interferon stimulated regulatory elements (ISREs) situated in the promoters of all the ISGs (Fischer et al., 2010). FL ORF2 was able to lower nuclear localization of IRF3 but it could not inhibit IRF3 over-expression induced IFN-β promoter activity. Taken together, these data indicate that the ORF2 protein targets innate immune signaling downstream of adapter proteins and upstream of the

IRF3. As both IFN-β and NF-κB axes of the RIG-I signaling and the TLR signaling are affected, the HEV ORF2 protein most likely brings about a universal suppression of the innate immune response. Further studies are required to understand the underlying mechanisms and identify the host proteins involved in the inhibition.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

SH and CR-K did experimental design and data analysis. SH, NJ, and CR-K performed the experiments. MS provided material, analysis tools, and suggestions. SH, MS, and CR-K wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.00656/full#supplementary-material

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