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Research Letter

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Unusual high prevalence of antibodies to hepatitis E virus in South Brazil

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One sentence summary: Unusually high seroprevalence of hepatitis E virus in South Brazil.

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ABSTRACT

Hepatitis E virus (HEV) is worldwide distributed and might cause acute or chronic hepatitis mainly in immunocompromised individuals. In previous studies we found a high prevalence of antibodies to HEV within blood donors in south Brazil and also within backyard-raised pigs. Here, we aimed to investigate the prevalence of anti-HEV antibody and HEV RNA within the general population from three major municipalities (Caxias do Sul, Passo Fundo and Santa Maria) in south Brazil. A total of 3000 blood samples were randomly obtained from clinical laboratories at each of the three municipality ($n = 1000$ each) to determine the presence of anti-HEV antibodies and HEV RNA. Overall, anti-HEV antibodies were detected in 574/1000 (57,4%) samples in Caxias do Sul, 655/1000 (65.5%) samples in Passo Fundo and 554/1000 (55.4%) samples in Santa Maria. The prevalence of HEV-positive samples increased steadily and significantly ($P < 0,001$) with age and was unusually higher within individual over 40 years. Despite of this, none of the pooled serum samples had detectable levels of HEV RNA. The high anti-HEV antibody prevalence suggests that the virus might be present on the environment and/or foodstuff and poses a permanent threat to immune-compromised individuals.

Keywords: epidemiology; hepatitis E Virus; seroprevalence; zoonosis

INTRODUCTION

Hepatitis E virus (HEV) genotypes and host range are expanding continuously. HEV belongs to the family *Hepeviridae*, genus *Hepevirus* (Purdy *et al.* 2017) and contains a single-stranded positive-sense RNA molecule with approximately 7.2 kb inserted within a non-enveloped icosahedral structured capsid of 27–34 nm in diameter. HEV can infect humans and several animal species (Yugo, Cossaboom and Meng 2014; Meng 2016) and to date at least eight different genotypes have already been detected (Nimgaonkar *et al.* 2017). Genotypes 1 and 2 infect humans and are transmitted through the fecal-oral route by contaminated water and is endemic in Asia, Africa and Central America; genotypes 3, 4 and 7, besides infecting different animal species like pigs, rats and camelids, might also infect humans and are considered zoonotic agents (Nan *et al.* 2017) mainly transmitted by

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direct contact with animals, animals' manure or consumption of undercooked animal meat (Pavio *et al*. 2016; Korsman *et al*. 2019). HEV RNA from genotype 3 has also been detected in human blood and transmission might occur by transfusion of blood or blood-related products or solid organs transplantation (Pas *et al*. 2012; Fischer *et al*. 2015; Rendon *et al*. 2016). Genotype 3 has been most frequently reported in developed countries in which might occur endemically in some regions. Genotypes 5, 6 and 8 have been detected recently and so far only in non-primate animals (Nimgaonkar *et al*. 2017).

Hepatitis E virus infection is considered a matter of public health and accounts for approximately 20 million cases reported worldwide each year (Nimgaonkar *et al*. 2017). In healthy individuals, HEV infection usually occurs unnoticed (Purdy and Khudyakov 2010), but in immunosuppressed patients, such as those infected by the human immunodeficiency virus (HIV), organ-transplanted individual, pregnant women or with preexisting liver disease, the infection might become chronic and/or persistent with increased mortality rates (Teshale, Hu and Holmberg 2010; Bura and Sikora 2017; Nimgaonkar *et al*. 2017). Recently, it was suggested that a previous infection by HEV might aggravate subsequent infection by hepatitis B and C viruses and could be related to the surge of hepatocellular carcinoma (Amougou *et al*. 2017). In most countries, infection by HEV is not notifiable and there are no guidelines related to HEV monitoring or surveillance even on blood or blood-derivatives destined to transfusion recipients (Adlhoch *et al*. 2019). In this sense, we and several groups already found a high proportion of blood donor with antibodies to HEV (Ali *et al*. 2008; Fischer *et al*. 2015; Yamada *et al*. 2015; Mansuy *et al*. 2016; Bura and Sikora 2017; Pandolfi *et al*. 2017; Moss *et al*. 2019) and, in some cases, even HEV RNA was detected within the blood donors' groups. These findings strengthen the need to turn HEV RNA screening obligatory at least in blood destined to immunocompromised recipients.

Recently, we developed an indirect enzyme-linked immunosorbent assay to detect anti-HEV antibodies in human blood and we found that 40.25% of the samples within blood donors were positive in our region (Ramos *et al*. 2016; Pandolfi *et al*. 2017). Our assay was further used to detect anti-HEV antibodies in pigs and we found a strikingly high prevalence within blood samples collected in 2012 (77.6%) and 2014 (65.5%) from noncommercial swine farms, i.e. swine destined to local consumption (da Silva *et al*. 2018), and this could be related to the high prevalence found in the blood donors' group within the same region. These findings led us to question whether HEV infection would be ubiquitously distributed in the general population of other regions of the state. Here we sought to detect anti-HEV antibody and HEV RNA in the general population of three major municipalities of RS and contribute to a better understanding on the epidemiology of HEV in South Brazil.

MATERIAL AND METHODS

Blood samples

The presence of anti-HEV antibodies was investigated in 3000 blood samples equally obtained from clinical laboratories at three major municipalities (Caxias do Sul, Passo Fundo and Santa Maria) in the state of Rio Grande do Sul (RS), south Brazil (Fig. 1). The samples were obtained from individuals that visited a physician and were requested to perform hematological or biochemistry evaluation for routine or any other healthy reason during April and May, 2019. After sampling and performing the requested blood analysis, the samples were then stored on the respective laboratory for up to 7 days at −20°C and then randomly selected by the laboratory supervisor to be forwarded to our laboratory at the University. The samples were then aliquoted and assigned a serial number keeping the record of origin, gender and age of each individual that was provided by each laboratory. Afterward, the samples were stored at −80°C up to use.

Anti-HEV antibody detection by ELISA

An indirect *in-house* ELISA, previously described and validated by our group (Pandolfi *et al*. 2017) was used to analyze all 3000 samples for the presence of anti-HEV IgG. To determine whether the sample was positive or negative for anti-HEV IgG, we analyzed the relation between the optical density (OD) of the samples and the OD of the negative control in each plate. Samples with a relation ≥2.5 were considered positive.

RNA extraction and one step RT-qPCR

RNA extraction was carried out using pooled serum samples (50 samples/pool). Each pool was made using 20 μL of serum and thoroughly vortexed. Then, 400 μL of each pool was used for RNA extraction using the Invitrogen® Purelink RNA/DNA Mini Kit as indicated by the manufacturer. The RNA was then stored at −80°C up to use. Sterile water and an additional sera pool were spiked with 50 mg of HEV-containing swine feces and used as control to assure efficient RNA extraction for further RT-qPCR amplification.

A one-step reverse transcription quantitative real time polymerase chain reaction (RT-qPCR) was carried out using primers and probe described previously (Germer *et al*. 2017) with minor modifications. Briefly, the 20 μL mixture contained 3 μL of template RNA, 10 μL of TaqMix, 0.4 μL of 1-Step RT Mix (PROMEGA® GoTaq Probe 1-Step RT-qPCR System Kit), 1.8 μL of *Primer Forward*, 1.8 μL of *Primer Reverse*, 0.4 μL of the probe labeled with FAN reporter dye and BHQ as a quencher, 0.35 μL of Carboxy-X-Rhodamine Reference Dye (CXR) and 2.25 μL of PCR grade water. Amplification and detection were performed using the StepOnePlus Real-Time PCR System (Applied Biosystems®). The RT-qPCR temperature were: reverse transcription of RNA at 45°C for 5 min and RT inactivation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 3 s and annealing at 60°C for 60 s. The positive controls consisted of RNA extracted from a serum pool, or water, spiked with 50 mg of HEV contaminated feces; negative control used water only (non-template control). Samples in which target amplification was detected within the 40 cycles were considered positive.

The study was approved from a scientific, methodological and ethical point of view by the University of Passo Fundo Ethical Committee on Research (protocol # 3.226.232).

RESULTS

Anti-HEV antibody prevalence

Out of 3000 samples, 1814 (60.5%) were from females and 1186 (39.5%) from males. Anti-HEV antibodies were detected in 655 (65.5%) samples from Passo Fundo, 574 (57.4%) samples from Caxias do Sul and 554 (55.4%) samples from Santa Maria. The prevalence of anti-HEV antibodies in females and males was statistically similar (Table 1). However, in all municipalities, there was a strong correlation between age range and the presence of anti-HEV antibodies that increased evenly and steadily with

Table 1. Gender and age range distribution of anti-HEV antibodies in the general population of three major municipalities in Rio Grande do Sul, Brazil.

Locality	Variable	Category	*n*	%	Positive (*n*)	Negative (*n*)	% positive[a]	% positive[b]	P
Caxias do Sul									
	Gender								
		Female	638	63.8	371	267	58.2	64.6	0524
		Male	362	36.2	203	159	56.1	35.4	
		TOTAL	1000		574	426	57.4		
	Age range								
		0–20	97	9.7	29	68	29.9	5.1	< 0001
		21–40	321	32.1	155	166	48.3	27.0	
		41–60	342	34.2	210	132	61.4	36.6	
		> 60	240	24	180	60	75.0	31.4	
		TOTAL	1000		574	426			
Passo Fundo									
	Gender								
		Female	579	57.9	372	207	64.2	56.8	0329
		Male	421	42.1	283	138	67.2	43.2	
		TOTAL	1000		655	345	65.5		
	Age range								
		0–20	92	9.2	36	56	39.13	5.50	< 0.001
		21–40	268	26.8	147	121	54.85	22.44	
		41–60	313	31.3	221	92	70.61	33.74	
		> 60	327	32.7	251	76	76.76	38.32	
		TOTAL	1000		655	345			
Santa Maria									
	Gender								
		Female	597	59.7	319	278	53.4	57.6	0128
		Male	403	40.3	235	168	58.3	42.4	
		TOTAL	1000		554	446	55.4		
	Age range								
		0–20	234	23.4	80	154	34.19	14.4	< 0001
		21–40	208	20.8	99	109	47.60	17.9	
		41–60	260	26.0	158	102	60.77	28.5	
		> 60	298	29.8	217	81	72.82	39.2	
		TOTAL	1000		554	446			

[a]Percentile of positive samples within the category.
[b]Percentile within positive samples.

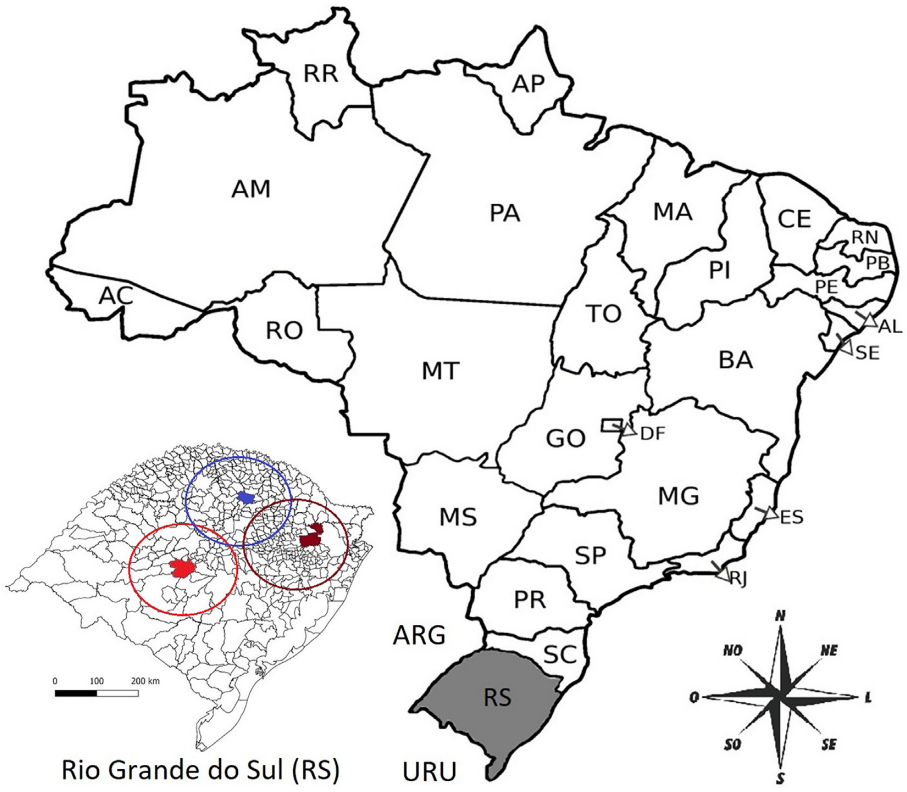

Figure 1. Geographical indication of samples origin. Samples were obtained from three municipalities in Rio Grande do Sul (south Brazil) that are considered major health center for surrounding region. The geographical localization of Santa Maria is indicated in red; Passo Fundo in blue and Caxias do Sul in dark red. The circles represent the estimated area covered by the laboratory in which samples were collected. One thousand serum samples were randomly collected in each municipality ($n = 3000$ total). Geographic location of Uruguay (URU) and Argentina (ARG) are indicated.

age ($P < 0.001$ for all municipalities; Table 1). When age range and gender were considered, there were no differences on anti-HEV antibody prevalence between females and males at any age range (not shown). Overall, out of 3000 samples, 1783 (59.4%) had anti-HEV antibodies.

HEV RNA on blood samples

All pooled serum samples were analyzed by one-step RT-qPCR and were considered negative for the presence of viral RNA in that no amplification of the target sequence could be detected within the 40-cycle reaction. All positive and negative controls gave the expected results to validate the assay (not shown).

DISCUSSION

In our previous studies, we found a high prevalence (40.25%) of anti-HEV antibodies in blood donors (Pandolfi *et al.* 2017), which are regarded as healthy individuals, and also within blood samples collected from backyard-raised pigs (da Silva *et al.* 2018), which are commonly found in small countryside villages and slaughtered for local consumption. Here, we reasoned whether the presence of anti-HEV antibodies was similar in the general population of three major Municipalities of RS, south Brazil. Overall, the highest rate of anti-HEV antibodies was found in blood samples collected in Passo Fundo (65.5%), followed by Caxias do Sul (57.4%) and Santa Maria (55.4%). The prevalence of HEV-positive samples was similar in men and women in all municipalities. As demonstrated in our previous study (Pandolfi *et al.* 2017) and confirmed by the data reported herein, the region of study might be endemic to HEV. In contrast, a much lower prevalence of HEV-positive samples was reported in other regions of Brazil including RS: e.g. 7.1% in Rio Grande, a port city in the southernmost part of RS (Moss da Silva *et al.* 2019), 10% in a municipality in the southern state of Santa Catarina (Passos-Castilho *et al.* 2016) and 20.7% in a small community in the southeastern region (de Almeida e Araújo *et al.* 2020). A similar contrasting difference in the prevalence of anti-HEV antibodies was observed in samples from different regions of France in which HEV-positive samples prevalence ranged from 8% to 86.4% (Mansuy *et al.* 2016). We could not find a reasonable event that could explain why anti-HEV antibody prevalence in RS in higher or whether individuals are more prone to infection. However, perhaps the high consumption of meat and local habit of constantly dealing with raw meat to prepare the traditional barbecue could be an important predisposing factor. In Brazil HEV genotype 3 is zoonotic and mostly found in humans and pigs (Pandolfi *et al.* 2017; da Silva *et al.* 2018); pig farming and consumption of pork and pork-derived foodstuff are higher in south Brazil compared to other regions of the country and this could account for the higher rate of infection detected in humans. Furthermore, pig manure spreading on land as a fertilizer might represent an additional risk of getting vegetables and watercourses contaminated with the virus (Mansuy *et al.* 2011).This hypothesis is supported by data indicating that anti-HEV IgG is higher in pork slaughterhouse workers when compared to the general population (Hoan *et al.* 2019) and by the presence of HEV in pig liver (Pavio *et al.* 2016). Other sources of infection should also be considered like ham, sausage and salami made up with

raw pork meat (Pavio *et al.* 2016) and widely consumed by general population. Although the presence of HEV in environmental samples like riverine water and sewage has not yet been investigated in Brazil, they should be also considered as potential source of infection to humans (Pisano *et al.* 2018).

In all municipalities, the prevalence of anti-HEV antibodies (namely, the rate of infection) augmented evenly and steadily with age suggesting that human population is continuously exposed to virus sources. In fact, previous studies elsewhere already highlighted similar data (Bura and Sikora 2017). This is an unwelcomed situation in that women at reproductive age have a high chance of contracting HEV infection which could be life-threatening during pregnancy. HEV infection could also be a burden to HIV immune-suppressed individual (Bura and Sikora 2017; Moss *et al.* 2019) and for individual with a preexistent liver pathological condition or solid organ transplant recipients (Pas *et al.* 2012). Also, a previous infection by HEV aggravates subsequent infection by hepatitis B and hepatitis C viruses (HBV, HCV) and accelerates the development of hepatocellular carcinoma (Amougou *et al.* 2017) which is the third most frequent cause of cancer death worldwide. Thus, underlying HEV infection could be an important trigger to other pathological conditions that otherwise would represent a lower burden to individuals and to the public health system.

Anti-HEV antibodies have been detected in the general population (Yamada *et al.* 2015), blood donors (Ali *et al.* 2008; Fischer *et al.* 2015; Mansuy *et al.* 2016; Pandolfi *et al.* 2017; de Almeida e Araújo *et al.* 2020) and individual belonging to specific groups considered at risk of infection (Bura and Sikora 2017; Moss *et al.* 2019), or with an underlying immune condition that could trigger the development of acute hepatitis (Amougou *et al.* 2017). Anti-HEV antibodies are indicative of infection and their presence correlates with HEV clearance; however, in antibody naïve donors, the time lapse between first HEV RNA detection in blood and detection of anti-HEV IgG might range from 8 to 230 days (Krain, Nelson and Labrique 2014; Kraef *et al.* 2018;) and HEV-RNA clearance might take as long as 230 days. Thus, blood samples might be free of anti-HEV antibody but viremic and, in this case, unsafe for donation. This already worrisome situation could be worsened on HEV endemic regions in which a high percentile of individuals, at any time, could be asymptomatically infected but still negative to anti-HEV antibodies, attending all criteria required to provide blood for donation. Thus, because serological and molecular HEV testing is not mandatory in most blood banks worldwide, HEV could be transmitted to recipients of blood and derivatives. Although the course of infection is mainly asymptomatic in healthy individual, it is considerably dangerous to immunocompromised individuals and pregnant women (Nimgaonkar *et al.* 2017). Considering that individual in need of blood or its derivatives might have an underlying pathological condition that could affect immune response to pathogens, an additional infection by HEV present on blood destined for donation, as already reported (Fischer *et al.* 2015; Moss *et al.* 2019), could represent a life-threatening event. In England, for instance, a retrospective study of blood donations found traces of genotype 3 HEV RNA in several samples that were used to prepare blood components for transfusion. Of these, 42% of the recipients showed evidence of infection and 10 patients developed persistent infection (Hewitt *et al.* 2014). HEV transmission, in this case, could be prevented by previously testing all blood samples for the presence of HEV, as already carried out in some European Countries (Adlhoch *et al.* 2019).

We used a highly sensitive one-step RT-qPCR to test pools of 50 serum samples. Although we found a high percentile of individuals with anti-HEV antibodies, no HEV RNA could be found within the blood samples evaluated. RNA extracted from a serum pool spiked with HEV-containing feces yielded a positive result on RT-qPCR indicating that RNA extraction and amplification were efficient. In similar studies performed elsewhere, the percentile of HEV RNA positive samples was usually low (Moss *et al.* 2019). In Austria, for instance, in a large-scale study, only 1 out of 8.416 (0.01%) blood donation were found positive to HEV RNA (Fischer *et al.* 2015). However, in that study anti-HEV antibody prevalence was much lower (13.55%) compared to our study. Here we also expected to detect HEV RNA in blood but the amount of HEV present on serum pools could be below the cut-off point of our HEV RT-qPCR. Nonetheless, considering the usually low rate of HEV RNA positivity, we are confident that all our samples were truly free of HEV.

In conclusion, we showed that in general 59.4% of the blood samples had anti-HEV IgG antibodies strengthening the previous suggestion that HEV is endemic and ubiquitously distributed in RS. We found no evidences of chronic carriers of the virus. Although the source of infection has not been investigated, the high prevalence found in humans correlates with the high prevalence previously reported in pigs at the same region raising the possibility that human contact with raw pork meat and consumption of pork-derived product could be a reliable infection route. Ongoing studies are directed towards finding the sources of HEV to humans in our region.

Authors' contribution

RZ contributed to the concept, clinical assessment, writing and reviewing. RLK, LMSE, BDK and IG carried out clinical assessment. RF contributed with the methodology and writing. LCK contributed with the conceptualization, formal analysis, writing, reviewing, study supervision and funding acquisition. All authors read and approved the final manuscript.

Ethical approval

This study was approved by the Committee on Research Ethics (protocol # 3.226.232) of the Universidade de Passo Fundo, and the procedures used conformed to the tenets of the National Commission for Ethic on Research (CONEP) of the Brazilian Ministry of Health.

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Conflict of interest. None to declare.

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