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First detection of hepatitis E virus in Central Argentina: Environmental and serological survey

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ABSTRACT

Background: The hepatitis E virus (HEV) is an emergent causative agent of acute hepatitis worldwide, transmitted by fecal-oral route. In Argentina it is considered rare, so differential laboratory testing is not routinely performed. Besides, in Argentina's central area epidemiological and molecular characteristics of HEV are still unknown.

Objectives: Provide evidence of local circulation of HEV by molecular detection on environmental samples and by serological survey in healthy adult population of Córdoba city, Argentina.

Study design: Environmental surveillance was conducted in river and sewage samples collected between 2007 and 2009–2011. Viral detection was performed by RT-Nested PCR of ORF-1 and ORF-2 partial regions. Anti-HEV IgG was determined by EIA in 433 serum samples collected between 2009 and 2010.

Results: HEV was detected in 6.3% of raw sewage samples and in 3.2% of riverine samples. Nucleotide sequencing analyses revealed that all isolates belonged to genotype 3, subtypes a, b and c. The prevalence of IgG anti-HEV was 4.4%. Seroprevalence increased with the age of the individuals (OR: 3.50; 95% CI 1.39–8.87; $p = 0.0065$) and, although the prevalence was higher in low income population, no statistical relation was found between anti-HEV and socioeconomic level.

Conclusions: The environmental findings added to serological results, demonstrate that HEV circulates in central Argentina. Contamination of water with HEV could represent a route of transmission for local populations, which have a high number of susceptible individuals. This fact alerts local health care systems in order to include detection of HEV in the diagnostic algorithm of viral hepatitis.

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1. Background

Abbreviations: HEV, hepatitis E virus; RNA, ribonucleic acid; Nested RT-PCR, reverse transcription reaction followed by a nested polymerase chain; ORF, open reading frame; IgG, immunoglobulin G; anti-HEV IgG, anti-HEV IgG antibodies; EIA, enzyme immunoassay; hIgG, human immunoglobulin G; CO, cut-off; S, sample; HAV, hepatitis A virus.

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The hepatitis E virus (HEV) (*Hepevirus, Hepeviridae*) is the causative agent of human acute hepatitis E with a worldwide distribution [1,2], responsible for both sporadic cases and large hepatitis epidemics in developing countries [3,4]. It is a single strand, positive sense, RNA non-enveloped virus which is classified into 4 genotypes of mammalian HEV [5]. HEV is mostly transmitted by fecal-oral routes following ingestion of contaminated water or consumption of fruits and vegetables that have been washed with contaminated water [6]. Two epidemiological patterns are observed for HEV infection. In areas of high endemicity, hepatitis E is mainly caused by genotypes 1 (Gnt-1) and 2 (Gnt-2), and primarily transmitted via the fecal-oral route. The second epidemiological pattern occurs worldwide, and consists of sporadic cases of

hepatitis E of zoonotic and/or foodborne transmission, mainly from pigs, caused by genotypes 3 (Gnt-3) and 4 (Gnt-4) [1].

With the exception of Venezuela – where outbreaks of HEV Gnt-1 have been reported, no epidemics of hepatitis E have been documented in South America yet [7]. Sporadic cases E have been documented in Argentina (Metropolitan region), Brazil, Venezuela, Peru, Chile and Uruguay [7] by IgM detection and/or RNA amplification. Until recently, Gnt-3 was the only genotype of HEV detected in autochthonous cases [8–10]. But recent studies placed Gnt-1 as the responsible of an autochthonous case of hepatitis in Uruguay [11], showing more than one genotype circulating in Latin America. Gnt-3 has also been detected in swine and effluent samples from farms and slaughterhouses from Brazil [8,12].

Argentina is considered a low endemic country for hepatitis E [7]. However, seroprevalence data reported are scarce. Previous studies performed ten years ago in the metropolitan region of Buenos Aires showed seroprevalence rates of 0.15% in pediatric population [13], 1.8% in blood donors and 6.6% in HIV infected individuals [14]. Furthermore, human clinical cases of hepatitis E have been diagnosed in the same area [10,15–17]. HEV has also been detected in pigs of commercial farms in many provinces [18]. However, there are no studies on environmental monitoring or serological surveys involving healthy adult population in our country. The lack of epidemiological information is, in part, due to the absence of commercial kits for detection of IgG and IgM anti-HEV until the middle of 2013, when the National Administration of Medicine, Food and Technology (ANMAT) of Argentina approved their use in our country.

Environmental surveillance using molecular technology is an additional tool to determine the epidemiology of different viruses circulating in a given community [19,20]. Previous studies in our area have shown a correlation between virus detected in sewage and clinical cases [18], showing that this type of study is very useful for virus monitoring.

Herein, we present the data from the first study of environmental surveillance and serological survey of HEV in Córdoba, Argentina.

2. Objectives

The aim of this study was to provide evidence of HEV circulation in central region of Argentina. For that, molecular detection of HEV was performed on environmental samples, as well as detection of IgG-anti HEV in healthy adult population of Córdoba city.

3. Study design

3.1. Environmental samples collection

Wastewater samples ($n=48$) were monthly collected in the years 2007, 2009, 2010 and 2011 from the main pipe that enters the treatment plant which receives sewage discharges from about 61% of the population of Córdoba city (1,330,023 inhabitants, census 2010). Samples of the Suquía River ($n=31$) were collected seasonally during 2010 in eight sampling points that cover the whole of its course across Córdoba city. During spring, point 7 could not be sampled (Fig. 1).

For each sample, 1500 mL of water were collected in sterile plastic bottles, stored at 4 °C and transported to the laboratory for immediate analysis.

3.2. Sample concentration, viral extraction and reverse transcription

Samples were concentrated 100× by centrifugation and polyethyleneglycol precipitation (10). RNA was extracted from 140 μL of concentrated samples using a QIAamp® Viral RNA Kit (Qiagen GmbH, Germany). Then, reverse transcription was performed adding 10 μL of extracted RNA to 10 μL of mix containing: 1 μL Reverse Transcriptase (ImPromII – Reverse Transcriptase – Promega, Madison WI, USA), 0.5 μL RNase Out (RNase Out Recombinant Ribonuclease Inhibitor, 40 U/μL – Invitrogen, CA, USA), 4 μL buffer 5× (ImPromII – Reverse Transcriptase – Promega, Madison WI, USA), 2.4 μL MgCl₂ 25 mM, 1 μL random primers (10 pmol/μL) (Promega, Madison WI, USA), 1 μL dNTPs 10 mM and 0.1 μL free RNase water (final volume of 20 μL).

3.3. PCR, Nested-PCR and molecular analyses

During this study, two Nested-PCR assays were performed, targeting ORF 1 and ORF 2 regions, following protocols previously described [21,22]. Amplification of ORF 2 was utilized as screening, and positive specimens were processed for ORF 1 detection.

Specific PCR products of 418 bp and 348 bp respectively were sequenced directly in both directions by Macrogen automatic sequencing service, Korea. Phylogenetic analyses were performed using MEGA software v5.0 [23]. Phylogenetic trees were constructed with neighbor-joining method and Kimura two-parameter as model of nucleotide substitution. Bootstrap values were determined with 2000 resamplings of the datasets. A consensus tree was generated and bootstrap values greater than 50% provide significant evidence for phylogenetic grouping.

3.4. Nucleotide sequence accession numbers

Nucleotide sequences analyzed in this work were deposited at GenBank under accession numbers KF751218–KF751221 for ORF 2 genomic region and KF765479 for ORF 1 genomic region (see Table 1).

3.5. Serum samples

A retrospective study was carried out with 433 serum samples from individuals who attended health care centers of Córdoba city during September 2009 and September 2010. The enrolled individuals were classified into three groups according to age (range 18–78 years old: younger than 30 years old, 31–45 years old and older than 46 years old) and two groups according to socioeconomic level (low-income population and middle/high-income populations) following a classification provided by the Municipality of Córdoba, which is based on the economic, social and educational level of each person [24]. The location of collected samples is shown in Fig. 1.

3.6. Serological test

A third generation enzyme immunoassay (EIA) for the determination of IgG specific antibodies against HEV (Diapro, Milan, Italy) was used. EIA microplates were coated with HEV-specific synthetic antigens encoding for conservative and immunodominant determinants derived from ORF2 and ORF3 of all genotypes. This EIA was performed strictly following the manufacturer's instructions. Test results were interpreted as ratio of the sample (S) and the cut-off (CO) (S/CO). Samples with ratio below 0.9 were considered negative, between 0.9 and 1.1 as equivocal result and above 1.1 were considered positive results.

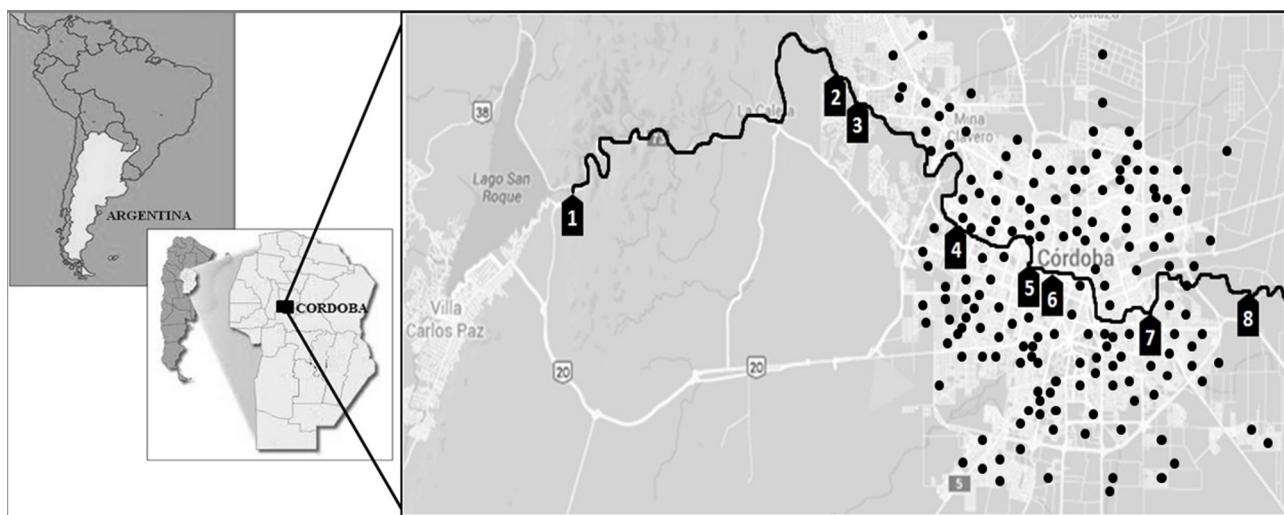


Fig. 1. Map showing the location of the city of Córdoba ($31^{\circ}23'51''S\ 64^{\circ}10'57''W$), in the province of Córdoba, Argentina, with the location of the sampling points throughout the Suquia River: (1) Funnel San Roque Dam, (2) Villa Warcalde Bridge, (3) San Antonio Ford, (4) Zípoli Bridge, (5) Ducks Island, (6) Centennial Bridge, (7) Sargento Cabral Ford and (8) San Jose Bridge. Sewage treatment plant is located between sampling points 7 and 8. This figure also shows the approximate location of the serum samples collected for this study as black dots.

3.7. Statistical analysis

Prevalence values were expressed as percentages and the univariate analysis (χ^2 test and the Fisher exact test) was performed to check out possible correlations between the serological marker and the risk factors assessed. Exact 95% confidence intervals (CIs) were calculated. Association between the variables was expressed as odds ratio (OR). Statistical significance was defined at $p < 0.05$.

The statistical package STATISTIC version 6.0 (2300 EAST 14th Street, Tulsa, OK, USA, 2005) for windows from Statsoft was used for the model fitting process.

4. Results

4.1. Environmental surveillance

HEV was detected in 6.3% of sewage samples (3/48: April-2007, September-2010 and March-2011) and in 3.2% of river samples (1/31: Spring-2010) by amplification of ORF 2 genomic region. From these, only one sample tested positive to ORF 1 amplification, probably due to a low sensitivity of the technique. The nucleotide sequencing analysis of both regions allowed assigning all HEV strains as Gnt-3. All samples formed distinct sequence clusters with high nucleotide similarity (Fig. 2). No segregation of the sequences was found and they grouped intermittently between samples from different geographic regions, years of isolation and origins (human or pig). Analyses of partial ORF2 sequences showed that samples obtained from the river in 2010 and the wastewater sample of 2007 (AmbRCbaArg02 and AmbSCbaArg04) clustered within subtype G3c and showed high homology between them. Sequence AmbSCbaArg03 was classified as G3b and AmbSCbaArg01 clustered within subtype G3a (Fig. 2).

4.2. Serological survey

The overall prevalence of IgG anti-HEV was 4.4% (19/433). The percentages for each group studied are shown in Table 2.

Seroprevalence increased with age (OR: 3.50; 95% CI 1.39–8.87; $p = 0.0065$): the higher prevalence was found in older than 46 years old group (8.1%), while in young adults (<30 years old) prevalence was low (0.7%) (Table 2).

Although higher anti-HEV prevalence was found in the low income population (5.9%), compared to middle/high income population (2.8%), this gap was not statistically significant. Nevertheless, in the group between 30 and 45 years, significant differences between both groups (middle/high income population vs. low income population) was found (0–8.6%) ($p = 0.0150$). It is necessary to increase the number of samples to corroborate these results.

5. Discussion

The epidemiology of viral hepatitis has changed in several parts of the world since the introduction of hepatitis A and hepatitis B immunization programs and the incidence of acute infections caused by these viruses has been declining [25]. Hepatitis E virus has been worldwide recognized as an increasingly important cause of acute hepatitis, but testing is not widely available.

This study reports, for the first time in Argentina, the presence of Gnt-3 HEV on environmental samples. Having found HEV in wastewater samples suggests its circulation in general population and its discovery in riverine samples would indicate the existence of an environmental reservoir in the city of Córdoba. This fact alerts local health care systems in order to consider HEV as a possible etiological agent of hepatitis, because it is not yet considered in the diagnosis algorithm of this group of pathologies. HEV has been associated with serious acute hepatitis disease in pregnant women and in patients with pre-existing chronic liver disease [26]. Chronic HEV infections with clinical relevance have been described in immunocompromised patients, such as organ transplant recipients [27], and HIV patients [28]. This previous reports, together with our findings, show the importance of the implementation of specific diagnostic of HEV in our area, not only in individuals with acute hepatitis, but also in other populations, like pregnant women and immunocompromised patients.

Waterborne transmission of HEV from watercourses in direct contact with humans poses a serious public health problem, especially in the summertime when these water sources can be used recreationally.

Phylogenetic analyses based on ORF-2 detection showed environmental circulation of HEV Gnt-3 (subtypes 3a, 3b, and 3c). Sequences of the ORF-1 region could not be obtained in all samples, possibly due to a sensitivity problem, as a result of the low number of viral particles present in water samples. This is also

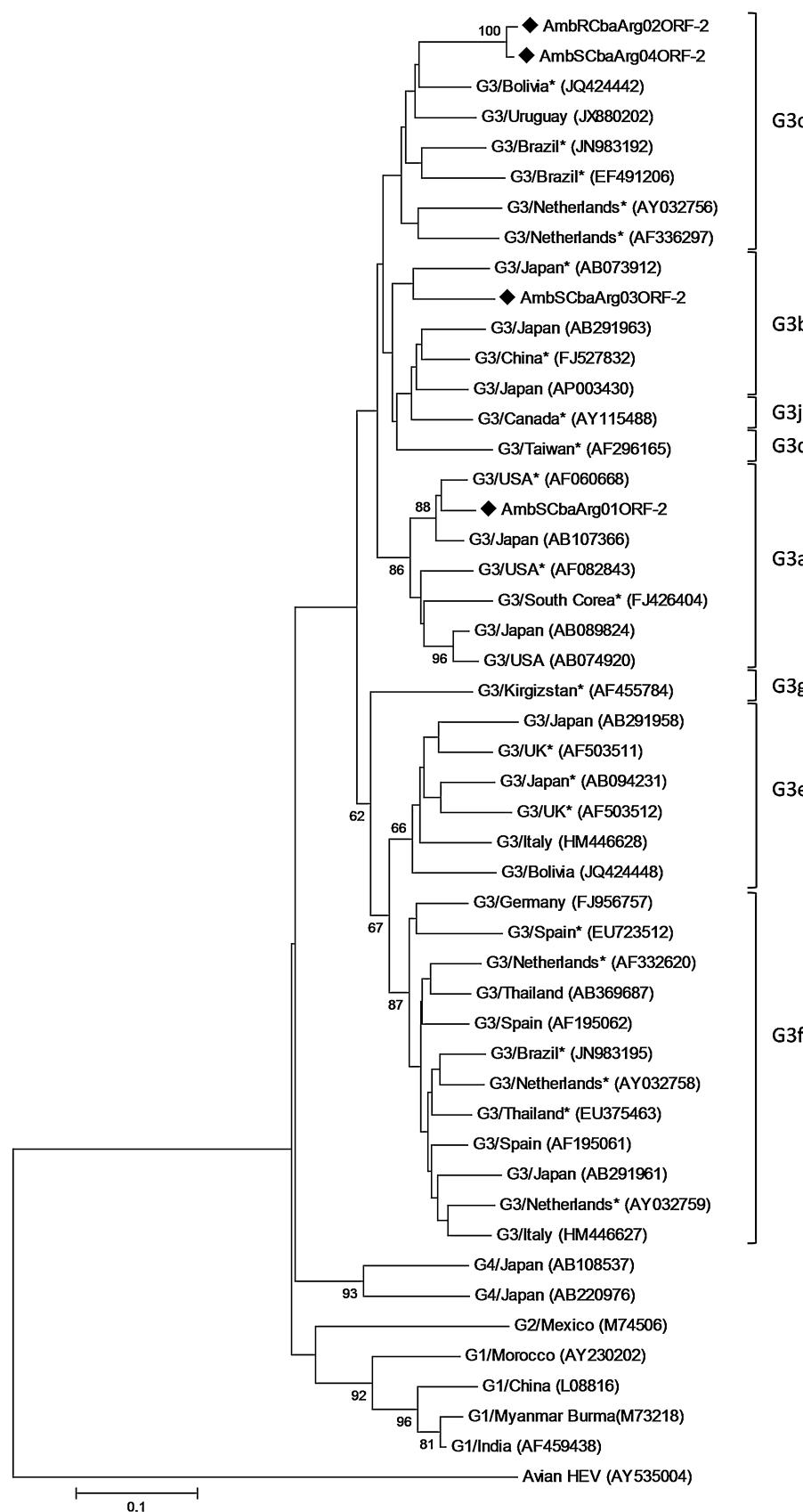


Fig. 2. Phylogenetic tree for subtyping studies reconstructed by the neighbor-joining method with common 280 nt ORF-2 sequences from 49 HEV isolates. Each viral strain is identified by the Genebank accession number, also there is indicated the name of the country of origin and its respective genotype. An avian HEV was included as outgroup. The Argentine environmental sequences are marked with a ♦. Asterisks indicate swine* HEV reference strains. Bootstrap values are indicated for the major nodes as a percentage of the data obtained from 2000 replicates (bar: 0.1 substitutions per site). AmbSCbaArg01 corresponded to sewage sample/September-2010, AmbRCbaArg02 to river sample/Spring-2010, AmbSCbaArg03 to sewage sample/March-2011 and AmbSCbaArg04 to sewage sample/April-2007, respectively.

Table 1

Source, date of sampling and accession numbers of the nucleotide sequences deposited in GenBank.

Sample ID	Source	Date	ORF 2 amplification – acc. number	ORF 1 amplification – acc. number
AmbSCbaArg01	Sewage	Sep-10	Yes – KF751218	Yes – KF765479
AmbRCbaArg02	River	Spring 2010	Yes – KF751219	No
AmbSCbaArg03	Sewage	Mar-11	Yes – KF7512120	No
AmbSCbaArg04	Sewage	Apr-07	Yes – KF7512121	No

Table 2

Percentage of individuals within each age group and socioeconomics characteristics with anti-HEV IgG serum antibodies.

Age (y)	N anti-HEV IgG+, %(n)	Middle/high income population anti-HEV IgG+, %(n)	Low income population anti-HEV IgG+, %(n)	p value
<30	0.7 (1/149)	1.4 (1/71)	0 (0/78)	NS*
31–45	4.4 (6/136)	0 (0/66)	8.6 (6/70)	0.0150
>46	8.1 (12/148)	6.8 (5/74)	9.5 (7/74)	NS*
Total	4.4 (19/433)	2.8 (6/211)	5.9 (13/222)	NS**

* A p value > 0.05 was considered non statistically significant.

evidenced in other reports [21,29]. Even though ORF-2 region has been extensively studied to infer phylogenetic relationships among HEV genotypes and subtypes [30–32], phylodynamic and coalescent studies among this region has been recently reported [12,29,33,34]. Detection of subtypes 3a and 3b agrees with subtypes previously detected in clinical samples from Argentinean patients [10]. Subtype 3c had not been previously described in our region.

Interestingly, the two sequences of 2010 (from river and sewage) did not cluster together, which could indicate more than one strain circulating simultaneously. Moreover, the clustering of the wastewater-2007 sequence with the riverine-2010 sequence, could show circulation of the same strain in the general population through several years.

Detection of IgG anti-HEV antibodies in general population contributes to improve and actualize the epidemiological knowledge of HEV circulation in specific geographic areas, such as the central area of Argentina, where there is no previous data. In this sense, conducting studies of IgG detection in individuals without previous history of acute hepatitis is a useful tool for estimating the presence of asymptomatic HEV infection [35]. Seroprevalence studies of HEV in the general populations of Latin America are scarce. The prevalence found in our population (4.4%) was slightly higher than that reported in Brazil (3.3%) [36], but lower than the found in Cuba (10%) [35]. Nevertheless, these studies are difficult to compare due to the different populations studied and different diagnostic tests used [37].

In accordance with previous studies, seroprevalence obtained of anti-HEV increased with age [35,38–40]. The low levels of anti-HEV in young adults (<30 years old) confirmed the susceptibility of this group to HEV infection, as reported in Cuba [41] and Brazil [39,42]. The high rates observed in elderly groups could be explained by a combination of cumulative exposure overtime and a cohort effect on prevalence, reflecting higher levels of HEV exposure in the past.

Some reports show the important role of low socio-economic factors in the transmission of water-borne infections [40,43]. In Córdoba, it has been reported that low income populations had higher prevalence of anti-HAV [44] antibodies. For HEV, more research is needed to establish whether the anti-HEV prevalence follows the same behavior. It is important to note that most of the people included in the study (90%) lived in houses with tap water, but some individuals lived in suburban areas where the potable water supply was not available. It cannot be excluded that HEV infection could be associated with the possibility of water contamination with sewage containing HEV. Another contamination source may be the use of recreational waters that are not appropriate for that purpose and may contain the virus. However, the international travel history, ingestion of raw or undercooked pork meat and risk occupations were not explored among the persons studied.

On the other hand, the findings of IgG anti-HEV show that there is a portion of the general population that has suffered subclinical infections. This absence of symptoms could be explained by the circulating genotype 3, found in environmental local samples, which produces mild or asymptomatic clinical presentation [1]. Sub-clinical HEV infection may well contribute to the perpetuation of the virus in endemic areas because it promotes its circulation in the community and its maintenance over the years.

The serological results, added to environmental findings, demonstrate that HEV circulates in central Argentina and that contamination of water could represent a significant route of transmission for local human populations. Sewage may act as a possible reservoir of HEV in this area. This fact alerts local health care systems in order to include detection of HEV in the diagnostic algorithm of viral hepatitis. Recent incorporation of serological kits and optimized molecular technology, will allow further investigations gain inside into the knowledge of this virus in our region.

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Competing interests

None declared.

Ethical approval

Ethics committee of the Health Ministry of the Province of Córdoba.

Authors' contributions

MMW designed the study, collection the data, performed the statistical analysis and drafted the manuscript; MBP, PAB and PDG contributed to the acquisition of samples and data. VER participated in revised the analysis plan, the data analysis and revised critically the manuscript. SVN, OCE, JMO and MAP revised the analysis plan

and made an important intellectual contribution in the content. All authors read and approved the final version of this manuscript.

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