



## Temporal changes in the genotypes of Pasmahepevirus balayani in southern Spain and their possible link with changes in pig trade imports

Pedro Lopez-Lopez<sup>a,b</sup>, Mario Frias<sup>a,b</sup>, Ana Belén Perez-Jimenez<sup>b,c,k</sup>, Carolina Freyre-Carrillo<sup>d</sup>, Juan A. Pineda<sup>b,e</sup>, Ana Fuentes<sup>b,f,l</sup>, Juan Carlos Alados<sup>g,m</sup>, Encarnación Ramirez-Arellano<sup>h</sup>, Isabel Viciano<sup>i</sup>, Diana Corona-Mata<sup>a</sup>, Javier Caballero-Gomez<sup>a,b,j</sup>, Ignacio Garcia-Bocanegra<sup>b,j</sup>, María A. Risalde<sup>b,j</sup>, Antonio Rivero-Juarez<sup>a,b,\*</sup>, Antonio Rivero<sup>a,b</sup>, for the HEPAVIR and GEHEP-014 Study Groups

<sup>a</sup> Service of Infectious Diseases, Hospital Universitario Reina Sofia, Instituto Maimonides de Investigación Biomédica de Córdoba (IMIBIC), Universidad de Córdoba (UCO), Córdoba, Spain

<sup>b</sup> CIBERINFEC, ISCIII – CIBER de Enfermedades Infecciosas, Instituto de Salud Carlos III, 28029 Madrid, Spain

<sup>c</sup> Clinical Microbiology Unit, Hospital Universitario Reina Sofia, Córdoba, Spain

<sup>d</sup> Clinical Microbiology Unit, University Hospital of Puerto Real, Cádiz, Spain

<sup>e</sup> Unit of Infectious Diseases and Microbiology, Hospital Universitario de Valme, Seville, Spain

<sup>f</sup> Clinical Microbiology Unit, Hospital Universitario Clínico San Cecilio, Granada, Spain

<sup>g</sup> Clinical Microbiology Unit, Hospital Universitario de Jerez, Cádiz, Spain

<sup>h</sup> Infectious Diseases, Microbiology and Preventive Medicine Unit, Virgen Macarena Univ. Hospital, and Department of Medicine, University of Sevilla/Biomedicine Institute of Sevilla, Sevilla, Spain

<sup>i</sup> Infectious Diseases, Microbiology and Preventive Medicine Unit, Hospital Universitario Virgen de la Victoria, Málaga, Spain

<sup>j</sup> Departamento Sanidad Animal, Grupo de Investigación en Sanidad Animal y Zoonosis (GISAZ), UIC Zoonosis y Enfermedades Emergentes ENZOEM, Universidad de Córdoba, Campus de Rabanales, Edificio Sanidad Animal, 14014 Córdoba, Spain

<sup>k</sup> Instituto Maimónides de Investigación Biomédica de Córdoba (IMIBIC), Córdoba, Spain

<sup>l</sup> Instituto de Investigación Biosanitaria Ibs. Granada, Granada, Spain

<sup>m</sup> Instituto de investigación e innovación biomédica de Cadiz (INIBICA), Spain

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### ABSTRACT

**Introduction:** Pasmahepevirus balayani (HEV) is an endemic zoonotic disease ranked as a major cause of acute hepatitis in Europe. Most infections occurring in Europe are due to the endemic several subtypes of genotype 3, through the consumption of raw or undercooked pork, observing a genotype geographical distribution pattern among countries. Because of global changes in the pig and pork trading markets, subtype distribution might vary. We aimed to evaluate the temporal distribution of HEV genotypes in patients from southern Spain with acute hepatitis to determine whether these changes were related to the pig import trade during the study period between 2018 and 2022.

**Methods:** Prospective longitudinal study including patients with acute hepatitis from southern Spain between 2018 and 2022. HEV RNA and antibodies were tested in all patients. In patients with detectable HEV RNA, genotype was obtained. To determine the number of imported pigs and their origins, we checked the official data from the Spanish statistics on international trade of Spanish Minister of Industry during by country of origin during the same study period.

**Results:** A total of 659 patients with acute hepatitis were included in the study. Among them, 162 (24.5%) had at least one marker (IgM or RNA) of acute HEV infection. Among the 71 patients with detectable viral RNA, genotypes could be obtained for 58 (81.6%). The most prevalent HEV genotype was 3f ( $n = 48$ ; 78.6%), showing a decreasing prevalence of over time, from 100% in 2018 to 70.6% in 2022. Since 2021, the emergence of other genotypes has been determined. A significant increase in the number of animals imported was observed since the beginning of the study. Denmark experienced a significant rise, from 0.03% in 2018 of total imports to 10.4% in 2022.

\* Corresponding author at: Virología Clínica y Zoonosis, Instituto Maimonides de Investigación Biomédica de Córdoba (IMIBIC), Avenida Menedez Pidal, s/n. 14004, Córdoba, Spain.

E-mail address: [arjvet@gmail.com](mailto:arjvet@gmail.com) (A. Rivero-Juarez).

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*Conclusions:* HEV molecular diversity is changing in Spain, could be linked to changes in fattening pig import origin.

## 1. Introduction

Paslahepevirus balayani (HEV), previously known as hepatitis E virus, is an endemic zoonotic disease found and a major cause of acute hepatitis in European countries [1]. In recent years, diagnosis of this virus has been widely implemented throughout the continent [2], which has improved the knowledge about its clinical impact and epidemiology in this setting. Regarding its clinical impacts, HEV is associated not only with acute hepatitis, which ranges from mild to severe, but also with chronic hepatitis in individuals with underlying immunosuppression [3]. In the same way, the clinical spectrum of these infections has recently expanded, and there are reports of extrahepatic manifestations related to the peripheral and central nervous systems, even in the absence of liver damage [4]. For this reason, HEV is considered a major health threat.

The vast majority of HEV infections occurring in Europe are due to the endemic genotype 3 through the consumption of raw or undercooked meat, with swine (both domestic and feral) being the main hosts [5,6]. The number of genotype 3 subtypes has significantly increased in the last standard classification [7], with 20 subtypes currently proposed. Although many of these have been found in domestic pigs, there seems to be a geographical distribution pattern among countries [8]. Spain is one of the global leaders in terms of pork exports and is currently the top European country engaging in pig farming, accounting for 22.1% of all European pork production [9]. Therefore, pork imports from other countries are rare, and the origin of pork consumed in Spain is mainly produced in this country. For this reason, the distribution of genotype 3 subtype seems to be constant over time [10]. Nevertheless, because of global changes in the pig and pork trading markets, the subtype distribution might vary over time. This point needs to be assessed to identify the emergence of new subtypes that could indicate changes at the pig farm and/or at food chain levels.

Here, we aimed to evaluate the temporal distribution of HEV genotypes in a cohort of patients from southern Spain with acute hepatitis to determine whether these changes were related to the pig import trade during the study period.

## 2. Methods

### 2.1. Study design and population

This was a prospective longitudinal study in which patients with acute hepatitis were recruited from seven reference hospitals in Andalusia (southern Spain) during the last five years (i.e., between 2018 and 2022, November 1st). These hospitals cover 5 out of the 8 provinces of Andalusia. For those individuals with negative serological and molecular markers of the common causes of acute viral hepatitis (e.g., Hepatitis C virus, Hepatitis B virus, Hepatitis A virus, Epstein-Barr virus, and cytomegalovirus), serum samples were collected and shipped to the Clinical virology and zoonoses lab of the Instituto Maimonides de Investigacion Biomedica de Cordoba (IMIBIC) for HEV infection evaluations.

### 2.2. HEV screening

To evaluate HEV IgM and IgG antibodies, commercial kits developed by Wantai Diagnostics were used (Beijing Wantai Biological Pharmacy Enterprise Ltd., Beijing, China). Positivity to IgM antibodies was confirmed by immunoblotting using a recomLine HEV IgG/IgM® kit (Mikrogen Diagnostik, Neuried, Germany).

HEV evaluations of serum samples were conducted using a qPCR method that was developed and validated by our group [11]. For PCR, RNA was extracted from 200- $\mu$ L serum samples using an automated procedure (QIAcube. QIAgen. Hilden, Germany) using the QIAamp MinElute virus spin kit (QIAgen. Hilden, Germany).

### 2.3. Sequencing and genotype assignment

For samples that were positive for HEV, nested PCR amplification of a 420-nt region located at ORF2 was carried out [11]. The amplicons were sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction Kit on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). SnapGene software (Version 6.1; GSL Biotech, [snapgene.com](https://www.snapgene.com)) was used for sequence analysis. The consensus sequence was obtained using SeqMan Software SeqMan NGen® Version 12.0 (DNASTAR, Madison, WI). Subtype assignments and phylogenetic analyses were performed using the HEVnet genotyping tool (<https://www.rivm.nl/mpf/typingtool/hev/>) (Mulder et al., 2019) and confirmed by BLAST.

All sequences are available in GenBank (accession numbers: MN628557 to MN628567, MT250081 to MT250083, MN537838, MN914126, MN914127, MT776550 to MT776554, MT854329, MW143072, OM236461 to OM236465, MZ964415, MZ964416, OL741633 to OL741655, ON720840 to ON720847, ON921072 to ON921078, and OP793784 to OP793790).

### 2.4. Changes in the pig trading market

To determine the number of imported pigs and their origins, we checked the official data from the Spanish statistics on international trade of Spanish Minister of Industry (available at: <https://datacomex.comercio.es>). We searched the numbers (in kg) of live pigs (under 50 kg) that were imported for farming from 2018 to 2022 by country of origin (including all countries). We calculated the percentages by country with respect to the total yearly amounts.

### 2.5. Statistical analysis

In the cohort, HEV prevalence was calculated globally and for each year of the study. The distributions of HEV genotypes were also calculated for the whole study period and by year of sampling.

### 2.6. Ethical statement

This study was designed and conducted in accordance with the Declaration of Helsinki. The Ethics and Clinical Trials Committee (CEIC) of Andalusia approved the study protocol, and informed consent was obtained from each patient (reference 4535). The SSPA Biobank coordinated the collection, processing, handling, and assignment of the biological samples used in this study in accordance with standard procedures established for this purpose (agreement S2100110).

## 3. Results

### 3.1. Population

Six hundred fifty-nine individuals suffering from acute hepatitis were included in the study. Among them, 162 (24.5%) had at least one marker (IgM or RNA) that was indicative of acute HEV infection.

Table 1 shows the number of individuals tested each year and

**Table 1**

Patients tested by year and exhibiting IgM antibodies and/or viral RNA during the study period.

Year	Tested, n	IgM positive, n (%)	HEV RNA detected, n (%)
2018	65	15 (23)	9 (13.8)
2019	53	15 (28.3)	16 (30.1)
2020*	38	24 (63.1)	10 (26.3)
2021	180	25 (13.8)	16 (8.8)
2022	323	37 (11.4)	20 (6.2)
Total	659	116 (17.6)	71 (10.7)

number of patients (n).

\* During 2020, because of the SARS-CoV-2 pandemic and limited reactivity, the number of tested individuals significantly decreased, which limited the analysis to only highly suspicious HEV infections.

number of samples that were positive for IgM antibodies and for viral HEV RNA. The global prevalence of HEV infection in our study was 10.7%. The positivity rates decreased over time, from 13.8% in 2018 to 6.2% in 2022.

### 3.2. Genotype temporal distribution

Among the 71 patients with detectable viral RNA, genotypes could be obtained for 58 (81.6%) of these patients. Table 2 shows the genotype distributions by year determined by sampling. The most prevalent HEV genotype was 3f ( $n = 48$ ; 78.6%). The prevalence of this genotype decreased over time, from 100% in 2018 to 70.6% in 2022. Since 2021, the emergence of other genotypes was detected (Table 2).

### 3.3. Spanish fattening pig imports during the study period

Additionally, we investigated the amounts and origins of pigs imported into Spain during the study period (Table 3). A significant increase in the number of animals imported was observed since 2018 (the first-year sample). The main exporting country during the entire study period was the Netherlands, but there was a significant decrease during the years of this study. In contrast, imports of fattening pigs from Denmark experienced a significant rise, from 0.03% in 2018 of total imports to 10.4% in 2022.

## 4. Discussion

Spain is one of the major pork-producing countries globally [9], which indicates that the origin of most pork consumed in the Spain is autochthonous [10]. For this reason, because genotype 3f is the principal and nearly unique HEV subtype circulating in the swine reservoir at the national level, this correlated with the prevalence of this genotype in human cases [10]. In fact, in a recent report that evaluated the prevalence of HEV subtypes in Spain between 2009 and 2019, genotype 3f represented approximately 90% of all cases, which was consistent during the whole study period [10]. Interestingly, our study found that the molecular variability of HEV genotype 3 has changed since 2021, suggesting a progressive subtype substitution. Consequently, the dominance of genotype 3f is challenging due to the emergence of new

**Table 2**

HEV genotype distribution by study year.

Year	HEV genotype						Total
	3c	3e	3f	3 l (p)	3 m	3na	
2018	0	0	7	0	0	0	7
2019	0	0	10	0	1	0	11
2020	0	0	7	0	0	0	7
2021	0	0	12	0	3	1	16
2022	1	1	12	1	1	1	17
Total	1	1	48	1	5	2	58

Porcine parvovirus balayani (HEV), proposed (p), and not assigned (na).

subtypes that might suggest modifications at the reservoir level during the last two years.

Since 2021, Spain has been ranked as the top European country in terms of pig farming and pork production and exports. This was linked to the African swine fever virus pandemic (ASFV), which has significantly decreased pork production in China (since 2019) and Germany (since 2021) [12,13]. Consequently, Spain, an area free of ASFV, experienced a greater demand for pork exports, which increased the number of animals produced. To meet this demand, it was necessary to increase the number of imported fattening pigs, which included trade with other European countries (Table 3), and the main exporting countries were the Netherlands and Portugal, with a substantial increase in imports from Denmark (Table 3). Changes in the differences in origins and percentages of the total amounts might explain the presence of new subtypes in our cohort in 2021 and 2022. The Netherlands is the main European pig-exporting country, with the HEV 3c genotype being the most prevalent [14,15]. This could suggest that this genotype might be increasing in other European countries, as we found in our study and in another study conducted in Spain [10]. In this way, this effect on the emergence of genotype 3c was observed in Scotland [16], where all sequences that have been identified in domestic pigs farmed in the United Kingdom were consistent with genotype 3e before the increase in pork imports from continental Europe. Similarly, subtype 3c has replaced the dominant genotype, 3f, in recent years [17], with an increase in prevalence from 8.2% in 2005–2011 to 25.5% in 2012–2017. Similarly, in Italy, the introduction of genotype 3c, related to swine importation, occurred in 2005–2006, which replaced the dominant genotype, 3f [18]. For this reason, introduction of pigs carrying other genotypes might progressively change the genotype distributions in human cases in other countries. This phenomenon could be occurring in Spain with the introduction of a new European HEV subtype. In this sense, the emergence of the new subtype, 3 l(p), in 2022 indicated in our study might be linked to the significant increase in pig imports from Denmark (Table 3). This subtype was first described in pigs from this country in 2012 (GenBank Accession number: KU747141- KU747144). Because the main countries to which Denmark exports pig and pork products inside the European Union are Italy and Spain [19], it is expected that this new genotype will be found in these countries. In fact, genotype 3 l (p) is described for the first time in Spain in the present study, and previously in swine from Italy [20]. Because the fattening pig trade to Spain from Denmark will increase in coming years, the number of human cases of this genotype could also raise.

The prevalence of HEV infections seemed to decrease over time in our study. This decrease is independent of the number of samples tested when considering 2021 and 2022 overall, when more patients were screened. In Germany and Denmark, a decline in HEV seroprevalence despite an increased number of infection notifications was observed [21,22]. Nevertheless, the increase on HEV screening is not correlated with the increase in acute HEV infection notifications across European countries that occurred in the same period [23]. This could also be related to replacement of HEV subtypes in these countries. Genotype 3efg seems to be more severe than the genotypes located in clade 3abchijklm [24,25]. In fact, in Germany, this lower severity associated with clade 3abchijklm is associated with an increased presence of strains belonging to this clade, with a corresponding decrease in strains belonging to genotypes 3f and 3e [26]. Because genotypes 3c and 3 l(p) belong to the less severe clade, it is possible that there are more asymptomatic individuals associated with infections by these genotypes, leading to misdiagnoses because of lower patient sampling levels for microbiological analysis. This point needs to be evaluated for other periods.

The main limitation of our study is that the analysis does not include parallel molecular analyses in swine. Nevertheless, from previous studies conducted by our group on animal populations from the same setting, we know that between 2015 and 2018, the genotypes circulating in both domestic pigs and wild boars were 3f and 3 m [27–30] and were

**Table 3**

Amounts (kilograms) and countries of origin of fattening pigs imported into Spain during the study period (2018–2021).

Country	2018 kg, (%)	2019 kg, (%)	2020 kg, (%)	2021 kg, (%)	2022* kg, (%)
Germany	153,172 (0.75)	1,558,849 (6.3)	1,479,885 (4.5)	1,415,713 (3.1)	900,431 (2.5)
Belgium	620,306 (3.05)	1,527,500 (6.2)	1,984,787 (5.4)	1,702,346 (3.7)	2,287,870 (6.3)
Denmark	73,250 (0.03)	166,634 (0.6)	1,056,372 (2.9)	4,820,260 (10.6)	3,760,270 (10.4)
France	127,989 (0.63)	13,061 (0.05)	214,751 (0.5)	43,695 (0.09)	158,002 (0.4)
Hungry	–	–	–	–	275,526 (0.7)
Italy	–	182,071 (0.73)	624,427 (1.7)	33,989 (0.07)	34 (<0.001)
Luxemburg	–	–	–	–	1,422,582 (3.9)
Netherlands	18,130,107 (89.3)	18,706,183 (75.9)	25,957,250 (71.2)	32,205,513 (70.9)	24,543,978 (67.8)
Portugal	1,256,543 (6.1)	2,456,252 (9.9)	5,154,068 (14.1)	5,154,068 (11.3)	2,816,228 (7.78)
United Kingdom	–	60,990 (0.02)	–	–	–
Total	20,295,442	24,616,649	36,471,540	45,375,584	36,164,921

Source: <https://datacomex.comercio.es/Data>. Official data from the Spanish statistics on international trade (Spanish Minister of Industry).

\* 2022 data are available up to the end of the third trimester of the year (30 September 2022).

not the other emerging genotypes identified in 2021 and 2022. New studies to update the molecular diversity in pigs in this setting are warranted to confirm this. Finally, despite the main source of infection in Europe is the consumption of pork products, it has been recognized other transmission routes. Nevertheless, because we do not have epidemiological data of the cases other source of infection, different to pork consumption, cannot be discarded.

## 5. Conclusions

The main results obtained in our study suggest that a change in the genotype diversity of HEV in patients suffering from acute hepatitis in southern Spain during the past five years. The emergence of new genotypes might be related to changes in fattening pig import quantities. Based on this, to include information about the global pig trade together with epidemiological and molecular data could be useful in anticipating the emergence of new HEV genotypes. Recommendation about monitoring HEV on exported and imported animals should be evaluate by Health Authorities.

## Author contributions

ARJ was involved in the study design and conception, data interpretation, drafting the manuscript, study supervision and obtaining funding. PLL, MF and JCG were involved in the serological and molecular determinations. All authors were involved in data acquisition and critical review of the manuscript. AR was involved in the study design and conception, data interpretation the drafting of the manuscript and obtaining obtention.

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## Conflicts of interest

The authors declare that they have no competing interests. Neither the authors nor their institutions have at any time received payment or services from a third party for any aspect of the submitted work (data monitoring board, study design, manuscript preparation, or statistical analysis).

## Data availability

Data will be made available on request.

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