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Prevalence of *Paslahepevirus balayani* in commercial swine food products from Spain

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ABSTRACT

Paslahepevirus balayani (formerly known as hepatitis E virus) is an emerging cause of foodborne disease in Europe, transmitted mainly by the consumption of raw or undercooked pork. Since little is known about the presence of the virus in several pork products that are eaten uncooked, our aim was to evaluate the prevalence of *Paslahepevirus balayani* in groups of commercial pork products intended for human consumption subjected to different processing techniques. A total of 1265 samples of pork products from Spain were divided into four groups and tested for the presence of *Paslahepevirus balayani* RNA: unprocessed pig and wild boar meat frozen at −20 °C ($n = 389$), dry-cured pork products ($n = 391$), dry-cured and salted pork products ($n = 219$), and boiled products ($n = 266$) (none of these products contained pork liver). Five samples were positive for *Paslahepevirus balayani* RNA (overall prevalence: 0.4%; 95% CI: 0.17% - 0.92%). All positive samples were from unprocessed meat stored at −20 °C, with a prevalence in this group of 1.3% (95% CI: 0.42–3.44); two samples came from pigs (1.1%; 95% CI: 0.13–3.81) and three from wild boar (1.5%; 95% CI: 0.31–4.28). None of the pork samples in the other groups was positive. In conclusion, *Paslahepevirus balayani* was found in unprocessed swine products form Spain, but not in processed products intended to be consumed undercooked, demonstrating that transmission of this zoonotic virus by eating these pork products should be more seriously considered.

1. Introduction

Paslahepevirus balayani (formerly known as hepatitis E virus) is an emerging foodborne virus in Europe, and a major cause of acute hepatitis in this setting [1]. Of the 8 major genotypes, genotype 3 is endemic and widely distributed in Europe in both human and animal populations [2,3]. It is mainly transmitted to humans through the consumption of raw or undercooked meat derived from infected animals [4]. Pigs and wild boar are considered the main reservoirs of *Paslahepevirus balayani* in Europe and therefore pose a major risk of transmission to humans [3,5].

The seroprevalence of *Paslahepevirus balayani* detected in pigs raised on European farms suggests a high circulation of this virus among pig populations in Europe. Studies carried out in different European countries have detected seroprevalences between 8% to 92.8%; for example, 8% in Ireland [6], 22.0% in Portugal [7], 31.0% in Italy [8], 37.5% in Norway [5], 43.8% in Spain [9], 83.3% in Finland [10], 87.9% in France [11], and 92.8% in the United Kingdom [12]. A high prevalence of active viral infection (defined as detectable viral RNA in feces, serum, or liver) of between 0% and 75% has also been confirmed in European pigs, varying accordingto the type of sample tested and the country. In this regard, 0% was detected in feces in France [13], 9.1% in feces and

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16.8% in serum in Spain [14,15], 22.4% in positive feces in Finland [10], 32.9% in sera in Lithuania [16], 15.5% in liver and 53.3% in feces in Serbia [17], 17.5% in liver and even 75% in feces in Italy [18,19], which could represent a significant risk of transmission to humans and a major public health concern. Indeed, numerous foodborne outbreaks related to *Paslahepevirus balayani* have been identified in France, Italy, and Spain, linked to the consumption of roasted suckling pig [20], roasted or grilled wild boar meat [21], and cured pork liver sausage [22–24]. Because of this, the European Food Safety Authority (EFSA) is encouraging the development of integrated studies to determine the potential risk of *Paslahepevirus balayani* transmission in the European food chain [4].

Pork is the second most consumed meat in the world with upper-middle income countries being the major producers and consumers of pork products (OECD, 2020). This situation looks set to continue in the coming years, according to a forecast published by the Organization for Economic Co-operation and Development-Food and Agricultural Organization (OECD-FAO) [25]. The Spanish pork sector is of key importance to its national economy, as it is the leading producer of pork meat in the European Union, ranking first in exports worldwide [26]. The south-western area of Spain has a large trade in Iberian pork, which comes from a breed of domestic pig that is usually reared extensively, and wild boar meat. The typical pork products of this region, such as *salchichón* [27], chorizo [28] and cured ham [29], among others [30] are consumed raw or undercooked. Based on studies previously conducted by our group in Mediterranean ecosystems in southern Spain, the viral RNA prevalence in *Paslahepevirus balayani* infections of pigs and wild boar is over 20% [31,32]. However, no studies have been conducted in this area to assess the prevalence of the virus in pork-derived food products and consequently, the risk of foodborne transmission of *Paslahepevirus balayani* in products consumed in and exported from this setting. Therefore, the aim of our study was to evaluate the prevalence of *Paslahepevirus balayani* RNA in commercial pork and wild boar products from virus-endemic areas subjected to different types of processing for human consumption.

2. Materials and methods

2.1. Design and type of products analyzed

Commercially available pork products were purchased between October 2020 and July 2022 from various supermarkets and butchers in south-central Spain, including: (I) unprocessed blade bone meat, frozen at −20 °C (pork from domestic Iberian pigs and hunted wild boar); (II) dry-cured foods (*salchichón* and chorizo–types of large sausage made of minced pork– from white and Iberian pigs), (III) dry-cured and salted foods (cured ham from white and Iberian pigs), and (IV) boiled foods (*morcilla*– blood sausage– from white and Iberian pigs) (Fig. 1). For processing procedures associated with products in each group, see Tejada et al. [28] and Martín et al. [27] for group II foods, Bayarri et al. [29] and Navarro et al. [33] for ham products (group III), and Santos et al. [34] for blood sausages (group IV). None of these products contained pork liver.

2.2. Sampling procedures

For group I, a total of 150 g of blade bone meat was collected from the carcass; then, a 10-g portion was removed and homogenized using a scalpel and a disposable stick into a tube and promptly frozen at −20 °C for further analysis. For groups II and IV, the whole piece was collected, and a 10-g section was specifically cut from the center, where the product is less dry, and was then homogenized, mixing the different parts of the food present in each product, and placed in a tube for subsequent analysis. For group III, a piece of 100 g from a cured ham was collected, and 10 g of this was homogenized for analysis. Samples from groups II, III and IV were stored at a temperature between 5 °C and 8 °C until laboratory analysis (Fig. 1). All samples were analyzed within 7 days of collection.

2.3. Determination of the detection limit for each matrix group

PCR validation was conducted for each matrix group employed in the research. Initially, the WHO standard control *Paslahepevirus balayani* strain, consistent with genotype 3a and provided by the Paul-Ehrlich Institute (code 6329/10), was resuspended following the manufacturer's instructions. This process yielded 500 µL with a concentration of 125,000 IU/mL. Subsequently, 0.05 g of each food sample was combined and homogenized with 40 µL of the standard extract. Following this, each sample was subjected to duplicate extraction using manual lysis and a commercial RNeasy® Mini Kit (QIAgen, Hilden, Germany) with an automated procedure (QIAcube, QIAgen, Hilden, Germany), producing two elutes of 50 µL each and a final elution volume of 100 µL with a concentration of 100,000 IU/mL. Various dilutions (1/1; 1/10; 1/100; 1/1000; 1/10000) were then prepared from this elution. Following the dilution process, RT-qPCR for the detection of *Paslahepevirus balayani* was performed for each food sample and dilution, with six replicates conducted for each condition (Table 1), as explained in the next section.

2.4. RT-qPCR for the detection of Paslahepevirus balayani

For this, 1 g of different homogenized samples from all food groups was used. Viral RNA was extracted from 0.05 g of sample using manual lysis and a commercial RNeasy® Mini Kit (QIAgen, Hilden, Germany) with an automated procedure (QIAcube, QIAgen, Hilden, Germany). Due to the high fat content of group II products, which significantly

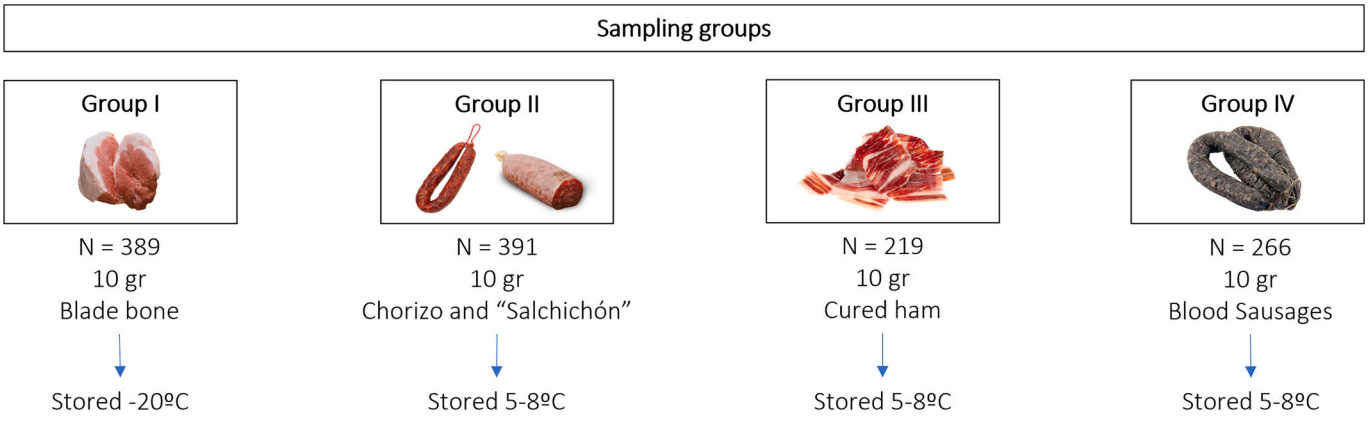

Fig. 1. Origin and types of foods sampled and storage conditions.

Table 1
Determination of the detection limit for each matrix group of swine-derived food products.

Group	I	II		III	IV
Concentration (IU/mL)	Blade bone	*Salchichón*	Chorizo	Blood sausage	Cured ham
10^5	6/6	6/6	6/6	6/6	6/6
10^4	6/6	6/6	3/6	6/6	4/6
10^3	1/6	5/6	2/6	4/6	0/6
10^2	0/6	0/6	0/6	0/6	0/6
10	0/6	0/6	0/6	0/6	0/6
Detection limit (IU/mL)*	5774	1168	21,435	1417	12,966

Legend: International units (IU); mililitre (mL).*Limit of detection calculated by probit analysis (at the 95% confidence level).

influences both the final volume to be collected for the extraction process as well as the detection capability of molecular methods, the protocol was modified by adding 700 µl of RLT buffer (instead of 600 µl) and using a disruption time of 40 s in the lysis process. Finally, the purified RNA was eluted in 50 µl volume. For the diagnosis of *Paslahepevirus balayani* infection, a one-step RT-PCR was used for the detection of viral RNA and an internal control, using sequence-specific probes (QuantiFast® Pathogen RT-PCR + IC, QIAgen, Hilden, Germany) developed and validated by our group [35]. Briefly, a broad-spectrum real-time RT-PCR (CFX Connect Real-Time PCR System) was used, with the primers FWD (20 µM) 5'-RGTRGTTTCTGGGGTGAC-3′ and RVS (20 µM) 5'-AKGGRTTGGTTGGRTGA-3′ and the probe (20 µM) 5'-FAM-TGAYTCYCARCCCTTCGC-TAMRA-3′. Both primers and probe were obtained by aligning all whole genome sequences of *Paslahepevirus balayani* available in GenBank. Reactions were conducted using 10 µl of template and the QIAGEN One-step PCR Kit (QIAGEN). The thermal profile was 50 °C for 30 min and 95 °C for 15 min, followed by 45 cycles of 94 °C for 10 s, 51 °C for 30 s and 60 °C for 20 s. All samples that tested positive by real-time RT-PCR were extracted again and analyzed to confirm the positivity. Prior to analysis, the extraction control assay was refined using different known concentrations of the WHO standard *Paslahepevirus balayani* strain (consistent with genotype 3a) supplied by the Paul-Ehrlich Institute (code 6329/10). A second extraction control test was performed using a serum sample in our laboratory from a patient diagnosed with *Paslahepevirus balayani* infection, carrying genotype 3f (Genbank accession number: MN628563) with a viral load of 420.000 IU/µl.

Samples that tested positive in the two independent runs of sample testing were then sequenced by nested RT-PCR targeting the ORF2 region, according to a previously described procedure [35] (no sample showed discrepancies in the two independent runs). Subtype assignment and phylogenetic analyses were performed using the HEVnet genotyping tool (https://www.rivm.nl/mpf/typingtool/hev/) and confirmed by BLAST. Sequence alignments were generated by the MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. Phylogenetic trees were constructed by the maximum likelihood method, using the *Paslahepevirus balayani* genotype/subtype standard reference strains reported by Smith et al. [36]. The final tree was obtained with MEGA Software (Version 6) using the bootstrap method (1500 bootstrap replicates).

2.5. Statistical analysis

The overall prevalence of *Paslahepevirus balayani* was calculated for all samples included in the study. The detection limit was determined by probit analysis (at the 95% confidence level). The prevalence of *Paslahepevirus balayani* was also calculated for each food group (I, II, III and IV) and each specific type of food within each group (group I: pork and wild boar; group II: *salchichón* and chorizo). For prevalence, a two-sided, 95% confidence interval (95% CI) was calculated, based on exact binomial distributions. Analyses were carried out with SPSS statistical software, version 25.0 (IBM Corporation, Somers, NY, USA).

3. Results

3.1. Sampling

A total of 1265 samples were included in the study, distributed by groups as follows: group I, 389 samples (30.7%); group II, 391 (30.9%); group III, 219 (17.3%); and group IV, 266 (21.0%). Table 2 shows the distribution by product type.

3.2. Detection limit of Paslahepevirus balayani for each matrix group

The results obtained on the detection limits of HEV for each food group are described in Table 1. HEV was detected for each group from the 1/1 eluate (concentration 100,000 IU/mL), as well as for the 1/10 (10,000 IU/mL) and 1/100 (1000 IU/mL) dilutions. No replicates were detected for the 1/1000 (100 IU/mL) and 1/10,000 (10 IU/mL) dilutions. The detection limit was 10^3 IU/mL for blade bone, *salchichón*, chorizo, and blood sausage, and 10^2 IU/mL for cured ham, respectively. Furthermore, detection limits were calculated by probit analysis (Table 1), expressing this limit in relation to the number of replicates detected per dilution.

3.3. Prevalence of Paslahepevirus balayani in pork and wild boar products

A total of five samples were positive for *Paslahepevirus balayani* (overall prevalence: 0.4%; 95% CI: 0.17% - 0.92%). All positive samples belonged to group I, giving a prevalence in this group of 1.3% (95% CI: 0.42% - 3.44%). Of these five positive samples, two were from Iberian pigs (1.1%; 95% CI: 0.13%–3.81%) and three from wild boar (1.5%; 95% CI: 0.31%–4.28%). *Paslahepevirus balayani* RNA was not detected in samples included in groups II, III and IV (Table 2).

Of the five samples detected as positive for *Paslahepevirus balayani*, only one sample from wild boar had sufficient viral load to be sequenced. This corresponded to genotype 3f (Genbank accession number: OQ291368). BLAST analysis of this sequence showed 99% homology with other sequences detected in humans from Spain and France (MZ289128, MZ289106, MW355310, MF444073, MN646692). Finally, the phylogenetic tree showed our detected sequence clustered within genotype 3f, together with sequences downloaded from BLAST (Fig. 2).

Table 2
Prevalence of *Paslahepevirus balayani* RNA according to different types of pig-derived food products (none of the products contained pork liver).

Food group	Type of food	N	Positive *Paslahepevirus balayani*	Prevalence (%)	95% CI
I	Pork	187	2*	1.1	0.13–3.81
	Wild boar	202	3**	1.5	0.31–4.28
	Total	389	5	1.3	0.42–3.44
II	*Salchichón*	155	0	0	0.00–2.35
	Chorizo	236	0	0	0.00–1.55
	Total	391	0	0	0.00–0.94
III	Cured ham	219	0	0	0.00–1.67
IV	Blood sausage	266	0	0	0.00–1.38
Total products		**1265**	**5**	**0.4**	**0.17–0.92**

Legend: number of samples (N); 95% confidence interval (95% CI).
* PCR Ct value: Sample 13 (39.58) and sample 54 (38.38).
** PCR Ct value: Sample 22 (33.09), sample 153 (37.19) and sample 180 (39.11).

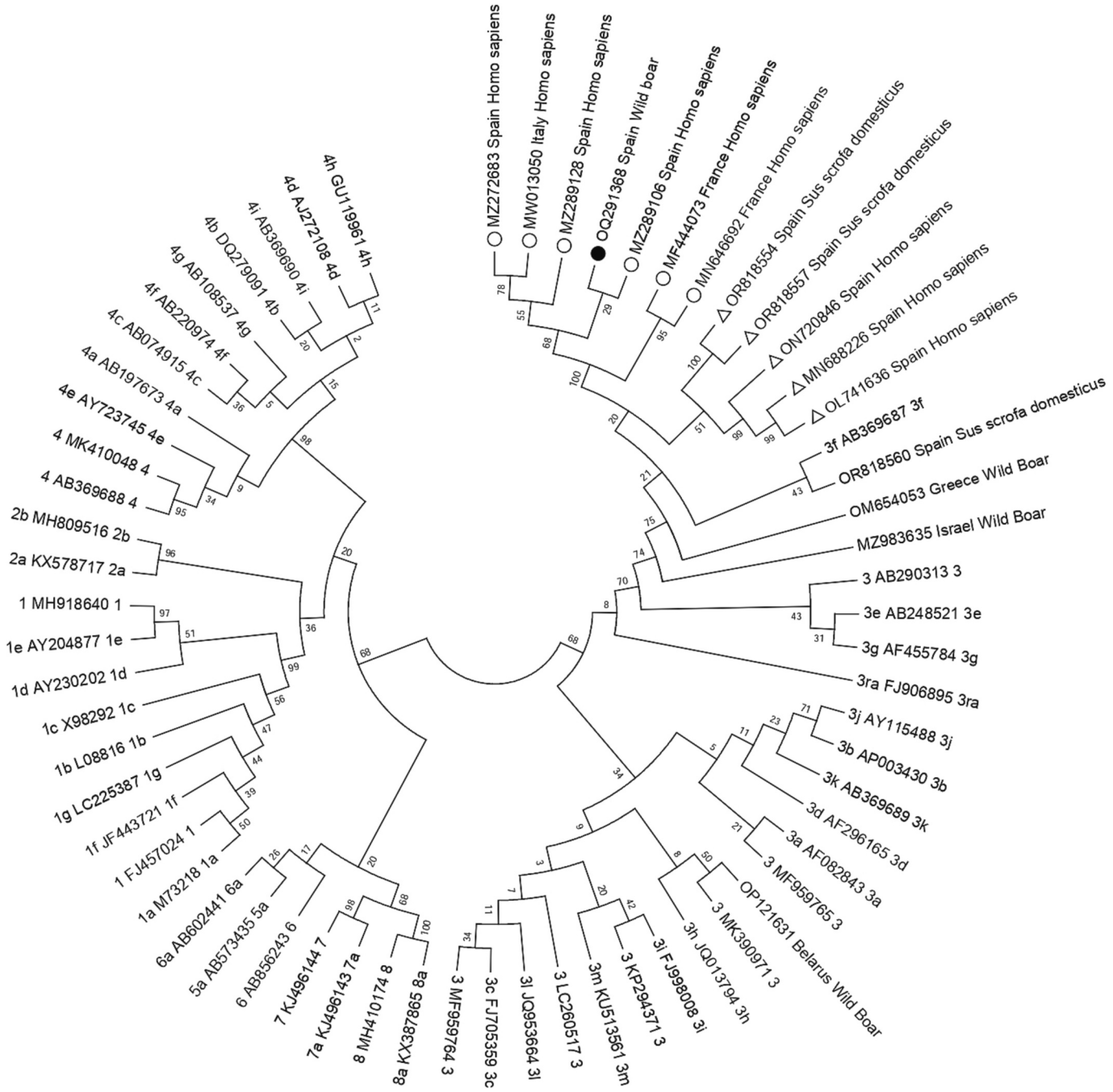

Fig. 2. Molecular Phylogenetic analysis by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model [37]. The bootstrap consensus tree inferred from 1500 replicates [38] is taken to represent the evolutionary history of the taxa analyzed [38]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.5442)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 55.3211% sites). The analysis involved 65 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 285 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [39].
Legend: All sequences recognized as standard for each HEV genotype and subtype have been added to the phylogenetic analysis. The isolated HEV sequence identified in our study is identified with ●. In addition, sequences identified by BLAST analysis with homology higher than 97% (○), as well as HEV sequences identified by our laboratory (Δ) have been added.

4. Discussion

This is the first study to evaluate a wide range of Spanish commercial pork products for the presence of *Paslahepevirus balayani*. Our results show that the prevalence of the virus in four types of pig food products was low compared to the prevalence previously detected in serum samples from pigs and wild boars in the same area [9,31,32]. The prevalence of active infection has been found to be as high as 20% in both pig and wild boar populations [31,32]. Given that pigs are the main reservoir of the virus [40,41] and in view of the numerous reports of

foodborne infections in humans in different European countries [20,23,42,43], the prevalence of *Paslahepevirus balayani* in pork foods was expected to be higher. However, it is possible that the different processing procedures that each type of food undergoes affects the integration of the virus in them.

The prevalence value of *Paslahepevirus balayani* detected in unprocessed meat frozen at −20 °C from Iberian pigs and wild boar (1.3%) was very similar to a previous study carried out in Italy, with a prevalence of 1.4% of viral RNA in muscle samples [44], but lower than those detected in pig and wild boar sera in the same area as ours [31,32]. This could be related to the viremic status of the animal [44], as experimental studies have confirmed that *Paslahepevirus balayani* can be detected in muscle tissue only in animals with very high titers of virus in blood [45]. Another study that tested for the presence of *Paslahepevirus balayani* in different pork samples from Thailandfound no positive samples in unprocessed meat, just one in liver (2%), while 27.7% were reported in feces [46]. A Spanish study conducted in 2017 analyzed different samples of pig tissue (serum, feces, liver, kidney, heart, and diaphragm) from different slaughterhouses [47]. In that study, only 1 out of 45 (2.2%) pigs tested positive for *Paslahepevirus balayani* RNAin the diaphragm, a highly vascularized muscle compared to the other visceral organs tested, in which significantly higher prevalences of positive samples were found, ranging from 8.9% to 15.6% [47]. Even so, the data shown in these studies together with our own results indicate that, while unlikely, unprocessed meat consumption cannot be ruled out as a major route of transmission.

The 3f genotype found is the most prevalent in both human and animal populations in Spain [48]. Studies in which *Paslahepevirus balayani* transmission was linked to pork consumption were associated with genotype 3f [3]. Thus, in Spain, in 2017, a family outbreak affecting nine members was identified as due to consumption of wild boar meat, and confirmed by phylogenetic analysis [21]. Similarly, in 2013, an outbreak of *Paslahepevirus balayani* transmission was reported at a wedding in France after guests ate spit-roasted piglet with an undercooked stuffing [20]. In another case identified in Japan, evidence of *Paslahepevirus balayani* genotype 3 transmission was found after consumption of wild boar meat [49]. Meanwhile, in France, a case of direct *Paslahepevirus balayani* transmission and infection with genotype 3f was identified in a woman who regularly ateraw pork liver sausage, *figatelli* [23]. Therefore, the high homology between our sequence and sequences detected by BLAST analysis, as well as the results reported in studies, strongly suggest that consumption of raw or undercooked meat favors *Paslahepevirus balayani* transmission.

Paslahepevirus balayani was not detected in any of the dry cured foods (*salchichón* and chorizo) analyzed. These products are widely consumed in Spain and are also exported internationally, with Spain ranking as the fourth largest international exporter in Europe [25]. The absence of virus in these products could suggest a very low risk of acquiring infection via this route, in contrast to other pork products produced in the European Union, such as *figatelli* [50] or liver sausages [51]. The composition of group II products such as *salchichón* and chorizo, which are produced with meat only, is different from these Corsican and German sausages, respectively, which are made with meat and liver, the main target organ of *Paslahepevirus balayani* [52]. In contrast, studies found high prevalences of HEV in 20% of the raw sausages from Germany [51], and in other pork products such as salami (18.5%), *cervelaat* (10.8%), *metworst* (26.1%) or *snijworst* (16.3%) from Netherlands [53]. Considering the low prevalence of *Paslahepevirus balayani* in meat found in our study, the viral load detected in these products from Spain would be expected to be low, and the risk of acquiring infection from consumption of these products would likewise be low. However, this controversy needs to be studied in order to assess the risk of infection by consumption of these products. We also tested dry cured and salted foods (group III), without finding positive results, which may imply that the lack of positive samples is also related to the process used for conservation. In connection with this, Wolff et al. (2020) analyzed sausages inoculated with *Paslahepevirus balayani* that were exposed to high salt concentrations to evaluate the viral stability in different buffer solutions at different incubation times and temperatures [54]. In one experiment, the sausages were exposed to salt buffer solution for 6 days at 22 °C, and in the next, for 8 weeks at 16 °C [54]. The results obtained in the two experiments were not significant, and the authors concluded that the salting process was not effective in inactivating *Paslahepevirus balayani* [54]. There were two major differences between the latter experiment and our study. First, in our study, the hams were not submerged in salt buffer solutions, they were coated with salt and cured for 12 months, and second, the curing process combined salting and drying. This combination of drying and salting may mean inactivation of the virus. Other studies have evaluated the inactivation of other microorganisms such as *Toxoplasma gondii*. Studies that have examined whether salting and drying processes can affect the viability of this parasite found that high salt concentrations [55,56] and drying for more than 12 months [33,57] can inactivate the parasite. In spite of the controversy over which processing method is more effective, studies suggest that the two are complementary [29,55,56]. Furthermore, a study assessed the stability of HEV after drying on various surfaces, including steel, wood, plastics, and ceramics, and concluded that HEV exhibits considerable resistance to drying, suggesting its potential for prolonged storage on surfaces [58]. However, it is important to note that these findings pertain to surfaces, not food, where the procedures differ. Therefore, additional studies are required to substantiate this hypothesis.

We also tested blood sausage (group IV), another typical Spanish sausage, in which we did not detect the virus either. This pork product, composed mainly of blood, is boiled for 20 min in hot water at more than 100 °C before drying [34]. This cooking procedure may possibly affect the integrity of the virus present in the blood of slaughtered animals [34,59,60].

In addition, in our study, we performed the evaluation of the detection limits of HEV RNA in each tested product. We performed 6 replicates, in contrast to other studies from Germany and the Netherlands, which performed 3 and 4 replicates, respectively [51,53]. The results obtained indicated that the detection limit of our technique was close to those described above. Nevertheless, certain limitations should be considered for the correct interpretation of the data. The methodologies for detecting limit calculation differ. In our study, we inoculated a food sample with a known virus concentration, and after extraction, dilutions of the RNA extract were performed. In contrast, the previously referenced studies inoculated food samples with serial dilutions of the virus [51,53]. Furthermore, these two studies calculated the detection limit for 2-g or 5-g portions of food [51,53], while we used the specific weight of tissue required for extraction (0.05 g). Additionally, it is necessary to consider that we used IU, in contrast to the German study that calculated the detection limit using genome copies [51]. The calculated detection limits for chorizo and ham were higher in our study compared to previous studies, suggesting the use of TRI Reagent in extractions to improve sensibility for detection of low viral loads in products with high fat percentages [53]. Finally, we did not compare *Paslahepevirus balayani* detected in the meat of group I animals with its detection in serum, liver, or feces, and are therefore unable to rule out the possibility of other animals being positive for the virus that was not detected in the meat. Our study does not evaluate whether food processing influences the absence of *Paslahepevirus balayani*, for which specific studies should be developed to evaluate the effect of the production process, such as salting or curing procedures, on the viability of the virus.

5. Conclusions

The prevalence of *Paslahepevirus balayani* observed in unprocessed pork food products from Spain is low; however, its detection implies that there is a real risk of viral transmission. The methodology used in this study demonstrates that HEV-RNA can be detected in processed pork

products; however, very low amounts of HEV-RNA cannot be detected, so the risk of these food types cannot be assessed in detail and improvements for future studies should be made. The absence of viral detection in cured, salted, and boiled pork products intended for human consumption indicates that the risk of transmission to humans through consumption of these foods should be considered minimal, but cannot be completely ruled out. Nevertheless, transmission of this zoonotic virus by eating such pork products should be more seriously considered, being necessary future studies to determine the viability and infectivity of the virus in efficient cell culture methods.

Author's contributions

Dra. Risalde had full access to all the data in the study and assumes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: MAR, AR, ARJ, JCGV.

Sample collection and procedures: PLL, JCG, MCJ, AMG, JMB.

Analysis and interpretation of the data: PLL, MAR, ARJ, IA, MF.

Drafting of the manuscript: PLL, MAR, ARJ.

Critical revision of the manuscript for important intellectual content: All authors.

Statistical analysis: PLL, IGB.

Obtained funding: MAR, AR.

Declaration of competing interest

The author(s) declare(s) that there are no conflicts of interest regarding the publication of this paper. The funding or other payments received by public institution, as well as the commercial affiliation of two of the authors with Sociedad Cooperativa Andaluza Ganadera del Valle de los Pedroches (COVAP) did not affect to the neutrality or objectivity of the work or its assessment.

Data availability

Data will be made available on request.

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