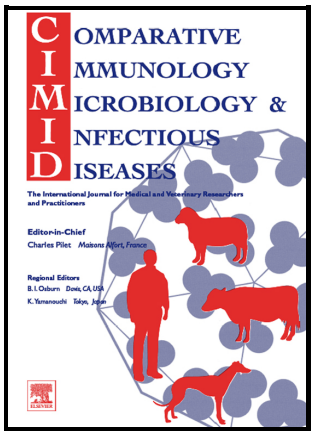

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UNRAVELING SWINE HEPATITIS E IN THE CENTRAL REGION OF ARGENTINA THROUGH ELISA DEVELOPMENT AND EPIDEMIOLOGICAL INSIGHTS

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ABSTRACT

Hepatitis E virus (HEV) is a public health concern globally, causing acute viral hepatitis in humans. Genotype-3 HEV (HEV-3), the most frequently genotype detected in South America, is zoonotic and the main reservoirs are the domestic pig and wild boar. Circulation of HEV-3 in Argentina

has been confirmed in humans as well as in pig herds, wild boar and environmental waters. However, data are scarce mainly due to the inaccessibility of serological assays in this country. In order to provide insights in the epidemiology of HEV in swine in Argentina, we developed an indirect ELISA based on the native recombinant protein ORF2 and conducted a serological survey to determine the prevalence of seropositive swine in small-scale pig farms in the central region of Argentina. The method was evaluated in a panel of 157 serum samples, resulting in relative sensitivity of 98.6% (95% CI 95%-100%) and relative specificity of 97.7% (95% CI 94%-100%) compared to a commercial test. An almost perfect agreement was obtained between the two tests (Kappa index of 0.961). A survey on 294 samples from 49 small-scale farms resulted in a seropositivity rate of 54%. Seropositive animals were found in 34 out of 49 (69.4%) farms. Most of the farms (70.6%) had over 50% of seropositive animals. The wide spreading of HEV in the swine population of Tandil, Argentina, underscore the need to better understand the epidemiology of HEV in the region, enabling the implementation of targeted interventions to mitigate the impact of this virus on public health.

Keywords

Hepatitis E virus (HEV), swine, seroprevalence, ELISA, ORF-2, Argentina

1. INTRODUCTION

Hepatitis E virus (HEV) is a cosmopolitan emergent virus, causing mostly acute self-limiting hepatitis in humans. In immunocompromised patients a chronic HEV infection may develop and lead to cirrhosis; and even extrahepatic manifestations, including a number of neurological

syndromes and renal injury. High mortality (20 to 25%) in pregnant women is particularly distinct of hyperendemic areas and infection with genotype 1, being unusual in most other regions [1].

HEV is a single-stranded positive-sense RNA virus classified within the genus *Hepevirus* in the family *Hepeviridae*. Eight HEV genotypes (1 to 8) have been recognized: genotypes 1 and 2 exclusively infect humans and cause large epidemics in developing countries due to poor sanitation and lack of secure drinking water. HEV-3 and 4 are zoonotic and have a broader host range being the domestic pig and wild boar the main reservoirs, while HEV-5 and -6 have been described in wild boar and HEV-7 and –8 only in camels [2].

HEV-3 is widely distributed in swine from many geographic areas, including all the continents, with seroprevalences estimated between 5 and 100%. There is consensus that pigs become infected at an early age, after the loss of maternal antibodies. Viral excretion in faeces peaks at 3 to 8 weeks after weaning, and decreases between 15 to 18 weeks of age, with the appearance of antibodies [3]. The antibodies elicited by infection are long lasting, therefore the detection of specific IgG in serum is a good indicator of past infection and can be conveniently used for monitoring HEV spread among pigs.

HEV infection has little impact on animal health, as the animals have no obvious symptoms. In both natural and experimental infections of pigs, histological signs of hepatitis are rarely observed. The virus replicates mainly in the liver but HEV can be found in other organs after experimental infection. At slaughterhouses, HEV is mainly detected in bile, faeces and liver [4]. Swine populations with high HEV prevalence pose a public health risk, especially in areas where pork consumption is widespread. In addition, the zoonotic potential of HEV in swine underscores the importance of understanding and implementing appropriate control measures in swine husbandry and food production [5].

In recent years, the epidemiology of HEV has gained increasing attention in Latin America, including Argentina. While hepatitis E was historically considered a disease predominantly affecting developing countries in Asia, the understanding of its global distribution has expanded. In Latin America, HEV is believed to be predominantly transmitted through zoonotic routes, with domestic pigs serving as a primary reservoir [6]. In Argentina, while data is still limited, seroprevalence surveys have suggested that HEV-3 is circulating in the country, with some areas and population groups exhibiting higher rates of infection compared to others [7-15].

Circulation of HEV-3 in Argentina has been confirmed in pig herds, wild boar and environmental waters; phylogenetic analysis showed a high degree of homology between animal and human strains [9, 10, 13, 16, 17], highlighting the role of these reservoirs in the transmission to humans. However, the scarce data on the seroprevalence of HEV in swine in Argentina shows 4 – 58% seropositivity, which widely differs from the high seroprevalence reported in swine herds from Brazil and Uruguay [18-20].

One important limitation to the research on the circulation of HEV in animal reservoirs, particularly in swine, is the lack of availability of serological assays in Argentina. ELISA kits for HEV antibodies must be imported, with the consequent high cost and low accessibility.

Argentina has experienced a notable increase in swine production and human consumption of pork meat and derivatives in recent years. More than 90% of the swine production in Argentina came from small-scale farms, which in general are less technical, with precarious facilities, scarce sanitary programs, presence of other species of domestic and wild animals (like wild boar) and rodents, and close contact of human with swine, its secretions, and excretions. This context may constitute a risk for the transmission of zoonotic pathogens, including HEV. Recently, Arce and colleagues developed an in-house indirect ELISA able to detect anti-HEV IgG in human serum using the recombinant ORF2 from HEV-3 expressed in *E. coli* as antigen, resulting in a highly sensitive and specific assay [8]. Therefore, our goal was to develop an ELISA to detect antibodies

in swine samples based on the native recombinant protein ORF2, and to investigate the prevalence of positive animals in small-scale farms from Tandil, Argentina.

2. MATERIAL AND METHODS

2.1. Expression of the recombinant truncated HEV ORF2 capsid protein in bacteria

The viral antigen used in the development of the in-house ELISA was a 66 kDa recombinant polypeptide comprising aa 112–608 of the capsid protein of HEV-3. The cloning and expression of the recombinant protein was described elsewhere [8]. Briefly, *E. coli* Rosetta (DE3) cells were transformed with pETG-A-His-N-ORF2 and protein expression was induced with 1 mM IPTG. The RGS-His5- tagged ORF2 protein was purified by NiNTA chromatography under native conditions. Expression and purity of the protein were analysed by SDS PAGE and Western blotting using an anti-His antibody and a HEV positive human serum. Protein concentration was determined by the Bradford method (BioRad). Aliquots of the purified recombinant protein were stored at -70°C.

2.2. Serum samples

Samples used for the optimization of the assay conditions and validation of the assay were obtained from a serum panel at the laboratory of Immunology of the Faculty of Veterinary Science (UNCPBA) (n=157).

Porcine serum samples were obtained from a local annual programme intended to give sanitary support to family-scale swine productions (both commercial and subsistence farms) in the county of Tandil, which is located in the south-east of the province of Buenos Aires, Argentina (Figure 1). This programme is implemented by the municipal government and the UNCPBA. Blood was obtained from 294 swine and 2 wild boar from 49 small-scale swine farms. Animals

sampled were 89% adults. Sows were sampled in all productions and boars when available. In some farms also gilts and/or barrows were included, which accounted for the remaining 11% of samples. Most of the farms had less than 20 sows, while 20% of them had between 20 and 60 sows. Blood samples were obtained by jugular vein puncture by veterinarians in vacutainer tubes and centrifuged. Serum was separated in vials and preserved at 4°C with the addition of sodium azide at 0.1 % final concentration. All procedures involving animals were approved by the Animal Welfare Committee (act 087/02) of the Faculty of Veterinary Science (Universidad Nacional del Centro de la Provincia de Buenos Aires, Tandil, Argentina).

2.3. ELISA procedure

Optimization of assay conditions included: concentration of viral antigen adsorbed onto the plate, blocking agents, type and dilution of conjugate, and dilution of swine serum. After all these variables were assayed, the final conditions for the assay were as follows: Jet Biofil ELISA plates (high binding) were coated by overnight incubation of the recombinant antigen at 1.2 µg/ml in carbonate buffer at 4°C. After two washes with PBS-T (phosphate buffer saline with 0.05% Tween 20) plates were blocked for 110 min at 37°C with a mixture of meat and casein peptone and meat extract (10% concentration, Cat B0212305 Britania Lab, Buenos Aires, Argentina) in PBS supplemented with 5% normal goat serum and 1% Tween 20. Serum samples were diluted 1:200 in sample diluent (same as blocking solution but supplemented with 1% normal goat serum instead of 5%) and incubated in the antigen coated plates for 1h at 37°C. After a series of 5 washes, bound antibodies were detected by incubation with peroxidase-conjugated goat anti-swine IgG (H+L) (Jackson ImmunoResearch Inc.) at 1:10,000 dilution. After 5 washes with PBS-T, the reaction was revealed by the addition of 3,3,5,5-tetramethylbenzidine (Life Technologies) and stopped, after 20 min incubation in the dark, by the addition of 4N H_2SO_4.

Absorbance was measured at 450 nm, using an automated microplate reader (DTX-880, Beckman Coulter).

A batch of plates was coated with the optimal concentration of antigen, and after overnight incubation and washing, was incubated at 37°C until completely dried. These plates were stored sealed in a bag with desiccant at 4°C and tested every 15 days in parallel with freshly prepared plates. Selected serum samples, including negative and positive samples both with weak and strong reactivity to HEV were tested in parallel plates.

2.4. Commercial ELISA test

The presence of anti-HEV specific antibodies in a panel of 157 porcine serum samples was determined by the commercial kit HEV Ab version Ultra (DIA.PRO, Italy), which was used as reference test. This is an antigen double sandwich ELISA that uses synthetic peptides encoding for conservative and immunodominant epitopes for coating and HRP-labeled peptides for detection. The procedure was followed as recommended by the manufacturers.

2.5. Statistical Analysis

A paired t test was conducted to compare ELISA absorbances obtained with different conjugates. For each sample, the ratio between mean absorbance of duplicate wells and mean absorbance of negative controls was obtained (A_{sample}/A_{NC} ratio). A receiver-operating characteristic (ROC) analysis was conducted using the free software Epitools Epidemiological Calculator, Ausvet (available in https://epitools.ausvet.com.au/roccurves) entering the A_{sample}/A_{NC} ratio value (test result) obtained for each sample. The software calculates sensitivity and specificity (with its corresponding estimates) for each possible cut-off point. Youden´s index (J statistic) was calculated in an Excell spreadsheet for each possible cut-off point. The cut-off point for the assay

was selected as the test result (A_{sample}/A_{NC} ratio) giving maximal Youden´s index. The agreement between the indirect and the commercial ELISA was determined by calculating the Kappa index using Epidat 3.1. According to Cohen's interpretation, the Kappa index can be understood in the following manner: values ≤ 0 indicate no agreement, 0.01–0.20 suggest none to slight agreement, 0.21–0.40 indicate fair agreement, 0.41–0.60 represent moderate agreement, 0.61–0.80 imply substantial agreement, and 0.81–1.00 suggest almost perfect agreement [21]. The intra-assay variability was assessed by calculating the coefficient of variation (CV) of strong positive, weak positive and negative samples tested in 8 replicates each within the same plate, while inter-assay variability was determined by evaluating the CV in 6 independent experiments. Association between HEV seropositivity and features of the swine productions were analysed by Chi square test or Fisher exact test (for qualitative variables) and Wilcoxon test (for quantitative variables), $p < 0.05\%$ was considered statistically significant.

3. RESULTS

The purification of RGS-His5-tagged ORF2 protein was conducted using NiNTA chromatography (ThermoFischer Scientific), resulting in higher yields under native conditions compared to denaturing conditions as described previously [8]. The expression of the truncated ORF2 protein was assessed through SDS-PAGE and Western blotting using an anti-RGS-His5 antibody and a HEV positive serum (data not shown). Under native conditions, the ORF2 protein formed dimers and trimers, with a yield of approximately 4.93 mg/l.

3.1. Optimization of the ELISA procedure

In order to find the optimal conditions for the assay, parameters such as antigen concentration adsorbed to the plate, serum dilution, blocking agents and type and dilution of conjugate were

assayed. For these experiments, we used previously characterised serum samples as positive or negative by a commercial ELISA. The optimal concentration of the recombinant protein used to coat the wells was determined after assaying strong and weak positive and negative porcine sera. As shown in Figure 2, maximal absorbance of positive samples with different degree of reactivity to HEV was obtained after coating the plate with 1.2 µg/ml of recombinant protein, while negative samples maintained low reactivity. Samples giving absorbance values above 1.0 were considered strong positive, while those resulting in absorbance below 1.0 were considered weak positive. Dilution of serum samples at 1:200 produced the highest positive/negative ratio (Figure 3). Bound antibodies could be detected both by the peroxidase-conjugated protein G and specific anti-swine IgG antibodies. As shown in Figure 4, absorbance of positive samples was higher ($p< 0.05$) using specific anti-swine IgG antibodies as detection reagent compared to peroxidase-conjugated protein G, hence the former method was used for further experiments. The implementation of skimmed milk as a plate-blocking agent led to an undesirable increase in background noise in negative samples and a decrease in absorbance levels in certain positive samples. A mixture of pluripeptone added with normal goat serum or isolated soy protein used as blocking agents resulted in maximal positive/negative ratios (Figure 5). Thus, we used pluripeptone added with 5% normal goat serum for blocking, and the same solution as sample diluent but supplemented with 1% normal goat serum instead of 5%.

Once the conditions of the assay were optimised, a panel of 157 characterised porcine serum samples was assayed. These samples were classified according to their reactivity in a highly sensitive commercial assay (HEV Ab ULTRA, DIA.PRO, Italy). For each sample, the ratio between mean absorbance of duplicate wells and mean absorbance of negative controls was obtained (A_{sample}/A_{NC} ratio). A receiver-operating characteristic (ROC) analysis was conducted in order to establish a cut-off value. As shown in Figure 6 the ability of the test to discriminate between positive and negative samples is demonstrated by a high area under curve (AUC) (value: 0.988, 95% CI 0.972-1). Maximal test efficiency and Youden´s Index were obtained with a cut-off point

of 1.78 (A_{sample}/A_{NC} ratio)(Figure 6). Therefore, this value was selected as cut-off for the analysis. A comparison of test results between our in-house indirect ELISA and the commercial test used as reference is presented in Table 1. At the selected cut-off value, a relative sensitivity of 98.6 % (95% CI 95%-100%) and relative specificity of 97.7% (95% CI 94%-100%) were calculated. Agreement between the two tests yielded a Kappa index of 0.961 which is indicative of a strong concordance. Intra-assay variability was 6.7, 4.9 and 12.7% for strong and weak positive and negative samples (8 replicates) respectively, while inter-assay variability was 7.0, 10.0 and 13.3 respectively (6 independent experiments). The storage of antigen coated plates at 4°C for at least 3 months did not affect the reactivity of selected serum samples tested in parallel plates.

Table 1. Performance of the in-house indirect ELISA in porcine serum samples classified according to their reactivity in the commercial HEV Ab ULTRA (DIA.PRO)

		Commercial ELISA		
		Positive	Negative	Total
In-house indirect ELISA	Positive	68	2	70
	Negative	1	86	87
	Total	69	88	157
Relative sensitivity	0.986 (95% CI 0.95-1.00)			
Relative specificity	0.977 (95% CI 0.94-1.00)			
Kappa index	0.961 (CI 95% 0.9179-1.00)			

3.2. Detection of specific anti-HEV antibodies in pigs from small-scale farms

Our indirect ELISA was used to investigate the presence of anti-HEV antibodies in samples of swine collected in small-scale farms in the suburban region of Tandil. Anti-HEV antibodies were detected in 159 from 294 samples, giving a seropositivity rate of 54% at the individual level. Seropositive animals were found in 34 out of 49 farms, resulting in a seropositivity rate of 69.4% at farm level. The rate of positive animals in each farm was variable (median: 70.7% and 35 - 90.7 for first and third quartils), but most (70.6%) of the farms had more than 50% of seropositive animals. Farms were classified as positive or negative according to the presence of seropositive animals. Positive farms had a bigger stock of sows and gilts ($p< 0.05$, Wilcoxon test for independent data). The frequency of commercial farms was higher in the group of positive farms ($p< 0.05$), while there were more subsistence farms in the group of negative farms. Rodents were present in most (84%) of the farms, independent of the HEV status, while wild boars were observed unfrequently, only in 12% of the holdings.

4. DISCUSSION

HEV is an emergent pathogen responsible for epidemics and endemics of acute hepatitis in humans, mainly through waterborne, foodborne, and zoonotic transmission routes [22]. In the past decade it has become clear that certain genotypes are zoonotic and that swine, and more generally Suidae, are the main reservoirs [3]. Although HEV is barely pathogen in the swine, this species poses a risk to humans and environment. In fact, naturally or experimentally infected swine show a mild to moderate subclinical hepatitis, with normal levels of liver enzymes in serum and without any other effect on production or health [23-26]. HEV RNA can be detected in infected pigs from infected farms in serum, bile, liver, mesenteric lymph nodes and faeces with peak values at 12 to 15 weeks of age [23]. Virus shedding in faeces can last up to 7 weeks, with maximal values in the first month of fattening, around 13 to 16 weeks of age [24, 27].

Specific antibodies appear early after infection; while IgM and IgA last for 4 - 7 weeks, IgG is long lasting, eventually until the time of slaughter [23].

The most widely used assay for the detection of HEV antibodies is the enzyme immunoassay, in which either recombinant HEV proteins or synthetic HEV peptides corresponding to antigen epitopes of the structural HEV proteins, such as ORF2 and ORF3, are used as target antigens. Among the two peptides, recombinant ORF2, which includes both linear and conformational epitopes, was proven to be a more sensitive and specific antigen than ORF3 [28, 29].

We developed an indirect ELISA using recombinant truncated ORF2 expressed in bacteria. After conditions were optimized a satisfactory segregation between negative and positive sera was observed. The goat serum added to the blocking solution was a key element which aided in obtaining very low background absorbance values in negative samples, while maintaining high values in both strong and weak positive samples (Figure 5). This may be due mostly because the secondary antibody used in our assay was developed in goats. Infection by HEV has been demonstrated in goats [30], which may be considered a potential reservoir of HEV. Studies showing evidence of HEV infection in goats are mainly from China and Europe [31] and there is no evidence of HEV seropositivity in goats from South America yet. However, we have been careful in ensuring the absence of HEV antibodies in the normal goat serum used as blocking agent. Alternatively, we obtained similar results by blocking the plates with soy protein (Figure 5). The evaluation of the assay by comparison of a panel of selected pig sera with a commercial ELISA showed an excellent agreement between the two tests, as determined by the high AUC in the ROC analysis, a Kappa index of 0.961 and relative sensitivity and specificity above 97%. Arce and colleagues [8] have previously used the same recombinant antigen in an in-house ELISA aimed at detecting specific IgG in human samples, also with excellent agreement towards a commercial licensed ELISA. Similar in-house indirect ELISAs have also been developed to detect antibodies in swine, using ORF2 expressed in bacteria [32], in insect cells [33-35] or in plant cell

systems [36, 37]. The performance of our assay is similar to that obtained by others using recombinant ORF2 expressed in bacteria [32] or in plant cells [37]. It has been reported that the convalescent immune response of pigs to HEV is mainly directed to a conformational epitope present in truncated ORF2 but not in overlapping peptides covering the ORF2 sequence [38]. The fact that the recombinant antigen used in our assay, purified under native conditions, contain both linear and conformational epitopes, may account for the good performance of the test.

Commercial ELISAs developed to detect specific antibodies in swine are available from limited sources (specially in peripheral countries like Argentina). In most cases antibodies in swine have been detected by use of commercial human HEV immunoassays, where the secondary antibody was replaced by antisera specific for the porcine IgG. A double sandwich ELISA (like the one used as reference in the present study), employing recombinant ORF2 or synthetic peptides both as coating antigen and as detection reagent (by conjugating it with peroxidase) is also commercially available. This latter format, initially developed for human diagnosis, has the advantage of detecting antibodies independently of species and isotype and has been used to detect specific antibodies in swine, wild boar and deer [39, 40]. The ELISA developed herein fulfils the needs for diagnostics and epidemiological purposes. Preliminary data (not shown) indicate that our assay is also capable of detecting anti-HEV IgG in wild boar, although with low sensitivity. Optimization of some of the assay conditions in order to obtain higher sensitivity, and testing of a greater number of samples may be needed.

Argentina is a country well known because of traditional beef consumption. However, in the last two decades both the production and the consumption of pork meat have experienced a huge rise (119% increase in pork meat production and 96% increase in annual consumption per capita)[41]. In face of this massive increase, data on the extent of circulation of HEV in swine herds in Argentina are scarce. The first report on swine HEV in Argentina dates from 2006, with

variable seroprevalence according to the region studied [16]. HEV RNA has been also detected in swine stool samples with variable infection rates according to the region [17, 42].

We focused our study in the county of Tandil, where the pork industry is an important component of its economy. In particular, the salami produced in Tandil contributes to the identity of the city, and has gained the certification of guarantee of origin, thanks to the climate conditions prevailing in the region. Our data showing that 69% of the farms had seropositive animals with an overall prevalence of 54% indicates that HEV is widely spread in the swine population of Tandil, particularly in small-scale farms. The situation may differ in farms with a higher scale of production. Our results agree with those obtained in a previous study where seropositive pigs were found in all of 5 farms studied in different regions of Argentina [16]. We found a high HEV seroprevalence rate in small-scale pig farms, however, lower risk was observed in those with a lower amount of sows and in subsistence farms compared to commercial ones. This result agrees with that obtained in Thailand, where higher seroprevalence rate was observed in medium sized compared to small size farms [43].

HEV spreading in swine poses a public health risk by different ways. The most common transmission routes of zoonotic HEV are occupational exposure (pig farmers, veterinarians, slaughterhouse workers, hunters) and consumption of contaminated undercooked meat and meat-derived products (specially containing liver) from infected pigs or wild boar. Thereon, a case of acute hepatitis E was recently reported in the central region of Argentina in a man with occupational exposure to pigs [44], highlighting the relevance of this transmission route. HEV shed by infected animals can contaminate water courses, and thereby the consumption of contaminated water or food other than animal products may also be sources of foodborne transmission [3, 45]. These risks may be particularly relevant in small-scale farms, where there is a close contact between humans and domestic animals and some of the pigs are slaughtered and consumed without any sanitary control.

In conclusion, HEV represents a significant health hazard. Increased awareness, surveillance, and research efforts are vital to better understand the epidemiology of HEV in the region, enabling the implementation of targeted interventions to mitigate the impact of this virus on public health. Vaccination strategies targeting HEV in pigs are being explored as a means to reduce viral shedding and prevent transmission.

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REFERENCES

[1] N. Kamar, J. Izopet, N. Pavio, R. Aggarwal, A. Labrique, H. Wedemeyer, H.R. Dalton, Hepatitis E virus infection, Nat Rev Dis Primers 3 (2017) 17086.

[2] B. Wang, X.J. Meng, Hepatitis E virus: host tropism and zoonotic infection, Curr Opin Microbiol 59 (2021) 8-15.

[3] N. Pavio, V. Doceul, E. Bagdassarian, R. Johne, Recent knowledge on hepatitis E virus in Suidae reservoirs and transmission routes to human, Vet Res 48(1) (2017) 78.

[4] I. Di Bartolo, E. Ponterio, L. Castellini, F. Ostanello, F.M. Ruggeri, Viral and antibody HEV prevalence in swine at slaughterhouse in Italy, Vet Microbiol 149(3-4) (2011) 330-8.

[5] J. Denner, Hepatitis E virus (HEV)-The Future, Viruses 11(3) (2019).

[6] G. Di Cola, A.C. Fantilli, M.B. Pisano, V.E. Ré, Foodborne transmission of hepatitis A and hepatitis E viruses: A literature review, Int J Food Microbiol 338 (2021) 108986.

[7] J.M. Echevarría, J.E. González, L.L. Lewis-Ximenez, D.R. Dos Santos, M.S. Munné, M.A. Pinto, F.H. Pujol, L.A. Rodríguez-Lay, Hepatitis E virus infection in Latin America: a review, J Med Virol 85(6) (2013) 1037-45.

[8] L.P. Arce, M.F. Müller, A. Martinez, A. Baiker, G. Marranzino, F. Agote, M.G. Vizoso-Pinto, A Novel In-House Enzyme-Linked Immunosorbent Assay for Genotype 3 Hepatitis E Virus Reveals High Seroprevalence in Blood Donors in Northern Argentina, Front Microbiol 10 (2019) 2481.

[9] I. Lo Castro, C. Espul, V.S. de Paula, N.R. Altabert, J.E. Gonzalez, B.V. Lago, L.M. Villar, High prevalence of hepatitis A and E viruses in environmental and clinical samples from West Argentina, Braz J Infect Dis 27(2) (2023) 102738.

[10] M.G. Martínez Wassaf, M.B. Pisano, P.A. Barril, O.C. Elbarcha, M.A. Pinto, J. Mendes de Oliveira, P. DiGiusto, S.V. Nates, V.E. Ré, First detection of hepatitis E virus in Central Argentina: environmental and serological survey, J Clin Virol 61(3) (2014) 334-9.

[11] M.B. Pisano, D. Balderramo, M.M. Wassaf, M. Lotto, Y. Carlino, V.E. Ré, J.D. Debes, Hepatitis E virus infection in patients on dialysis and in solid organ transplant recipients in Argentina: exploring associated risk factors, Arch Virol 162(3) (2017) 787-792.

[12] M.B. Pisano, S. Blanco, G. Di Cola, A.C. Fantilli, M.G. Martinez Wassaf, L.H. Carrizo, V.E. Re, S.V. Gallego, Hepatitis E virus in blood donors from Argentina: A possible source of viral infection?, Travel Med Infect Dis 48 (2022) 102355.

[13] M.B. Pisano, M. Winter, N. Raimondo, M.G. Martínez-Wassaf, S.D. Abate, V.E. Ré, New pieces in the transmission cycle of the hepatitis E virus in South America: first viral detection in wild boars from Argentina, Trans R Soc Trop Med Hyg 113(8) (2019) 497-499.

[14] M.B. Pisano, B.C. Lugo, R. Poma, H.A. Cristóbal, V. Raskovsky, M.G. Martínez Wassaf, V.B. Rajal, V.E. Ré, Environmental hepatitis E virus detection supported by serological evidence in the northwest of Argentina, Trans R Soc Trop Med Hyg 112(4) (2018) 181-187.

[15] G. Tissera, M.C. Lardizabal, S.B. Torres, A.C. Fantilli, M.G. Martinez Wassaf, F. Venezuela, R. Capra, D.C. Balderramo, C. Travella, V.E. Re, M.B. Pisano, Hepatitis E virus infection in pregnant women, Argentina, BMC Infect Dis 20(1) (2020) 368.

[16] M.S. Munne, S. Vladimirsky, L. Otegui, R. Castro, L. Brajterman, S. Soto, E. Guarnera, V. Molina, M. Monfellano, G.G. Schlauder, J.E. Gonzalez, Identification of the first strain of swine hepatitis E virus in South America and prevalence of anti-HEV antibodies in swine in Argentina, J Med Virol 78(12) (2006) 1579-83.

[17] F. Marziali, J. Acosta, E. Bolatti, S. Mirazo, P. Skejich, P. Silva, J. Brassard, A. Costaguta, D. Gardiol, A.L. Cavatorta, Detection of HEV in naturally infected swine from central Argentina by an optimized HEV/MS2 duplex RT-qPCR, Zoonoses Public Health 66(7) (2019) 729-38.

[18] M.S. da Silva, S. Silveira, V.S. Caron, A.C.S. Mósena, M.N. Weber, S.P. Cibulski, A.A.R. Medeiros, G.S. Silva, L.G. Corbellini, R. Klein, L.C. Kreutz, R. Frandoloso, C.W. Canal, Backyard pigs are a reservoir of zoonotic hepatitis E virus in southern Brazil, Trans R Soc Trop Med Hyg 112(1) (2018) 14-21.

[19] C.L. Vitral, M.A. Pinto, L.L. Lewis-Ximenez, Y.E. Khudyakov, D.R. dos Santos, A.M. Gaspar, Serological evidence of hepatitis E virus infection in different animal species from the Southeast of Brazil, Mem Inst Oswaldo Cruz 100(2) (2005) 117-22.

[20] S. Mirazo, N.R. Gardinali, D. Cecilia, L. Verger, F. Ottonelli, N. Ramos, G. Castro, M.A. Pinto, V. Re, B. Pisano, A. Lozano, J.M. de Oliveira, J. Arbiza, Serological and virological survey of hepatitis E virus (HEV) in animal reservoirs from Uruguay reveals elevated prevalences and a

very close phylogenetic relationship between swine and human strains, Vet Microbiol 213 (2018) 21-27.

[21] M.L. McHugh, Interrater reliability: the kappa statistic, Biochem Med (Zagreb) 22(3) (2012) 276-82.

[22] D.M. Yugo, X.J. Meng, Hepatitis E virus: foodborne, waterborne and zoonotic transmission, Int J Environ Res Public Health 10(10) (2013) 4507-33.

[23] N. de Deus, M. Casas, B. Peralta, M. Nofrarías, S. Pina, M. Martín, J. Segalés, Hepatitis E virus infection dynamics and organic distribution in naturally infected pigs in a farrow-to-finish farm, Vet Microbiol 132(1-2) (2008) 19-28.

[24] X.J. Meng, P.G. Halbur, J.S. Haynes, T.S. Tsareva, J.D. Bruna, R.L. Royer, R.H. Purcell, S.U. Emerson, Experimental infection of pigs with the newly identified swine hepatitis E virus (swine HEV), but not with human strains of HEV, Arch Virol 143(7) (1998) 1405-15.

[25] C. Kasorndorkbua, B.J. Thacker, P.G. Halbur, D.K. Guenette, R.M. Buitenwerf, R.L. Royer, X.J. Meng, Experimental infection of pregnant gilts with swine hepatitis E virus, Can J Vet Res 67(4) (2003) 303-6.

[26] P.G. Halbur, C. Kasorndorkbua, C. Gilbert, D. Guenette, M.B. Potters, R.H. Purcell, S.U. Emerson, T.E. Toth, X.J. Meng, Comparative pathogenesis of infection of pigs with hepatitis E viruses recovered from a pig and a human, J Clin Microbiol 39(3) (2001) 918-23.

[27] S. Fernández-Barredo, C. Galiana, A. García, S. Vega, M.T. Gómez, M.T. Pérez-Gracia, Detection of hepatitis E virus shedding in feces of pigs at different stages of production using reverse transcription-polymerase chain reaction, J Vet Diagn Invest 18(5) (2006) 462-5.

[28] I. Di Bartolo, F. Ostanello, F.M. Ruggeri, Hepatitis E Virus, in: D. Liu (Ed.), Molecular Detection of Animal Viral Pathogens, Taylor and Francis Group2016, pp. 111-119.

[29] H. Wang, W. Zhang, H. Gu, W. Chen, M. Zeng, C. Ji, R. Song, G. Zhang, Identification and characterization of two linear epitope motifs in hepatitis E virus ORF2 protein, PLoS One 12(9) (2017) e0184947.

[30] F. Long, W. Yu, C. Yang, J. Wang, Y. Li, Y. Li, F. Huang, High prevalence of hepatitis E virus infection in goats, J Med Virol 89(11) (2017) 1981-1987.

[31] S. Santos-Silva, P. López-López, H.M.R. Gonçalves, A. Rivero-Juarez, W.H.M. Van der Poel, M.S.J. Nascimento, J.R. Mesquita, A Systematic Review and Meta-Analysis on Hepatitis E Virus Detection in Farmed Ruminants, Pathogens 12(4) (2023).

[32] W.J. Lee, M.K. Shin, S.B. Cha, H.S. Yoo, Development of a novel enzyme-linked immunosorbent assay to detect anti-IgG against swine hepatitis E virus, J Vet Sci 14(4) (2013) 467-72.

[33] W.H. van der Poel, N. Pavio, J. van der Goot, M. van Es, M. Martin, B. Engel, Development and validation of a genotype 3 recombinant protein-based immunoassay for hepatitis E virus serology in swine, Braz J Med Biol Res 47(4) (2014) 334-9.

[34] B. Peralta, M. Casas, N. de Deus, M. Martín, A. Ortuño, E. Pérez-Martín, S. Pina, E. Mateu, Anti-HEV antibodies in domestic animal species and rodents from Spain using a genotype 3-based ELISA, Vet Microbiol 137(1-2) (2009) 66-73.

[35] E. Ponterio, I. Di Bartolo, G. Orrù, M. Liciardi, F. Ostanello, F.M. Ruggeri, Detection of serum antibodies to hepatitis E virus in domestic pigs in Italy using a recombinant swine HEV capsid protein, BMC Vet Res 10 (2014) 133.

[36] M. Mazalovska, N. Varadinov, T. Koynarski, I. Minkov, P. Teoharov, G.P. Lomonossoff, G. Zahmanova, Detection of Serum Antibodies to Hepatitis E Virus Based on HEV Genotype 3 ORF2 Capsid Protein Expressed in Nicotiana benthamiana, Ann Lab Med 37(4) (2017) 313-319.

[37] K. Takova, T. Koynarski, G. Minkov, V. Toneva, E. Mardanova, N. Ravin, G.L. Lukov, G. Zahmanova, Development and Optimization of an Enzyme Immunoassay to Detect Serum Antibodies against the Hepatitis E Virus in Pigs, Using Plant-Derived ORF2 Recombinant Protein, Vaccines (Basel) 9(9) (2021).

[38] J.D. Chandler, M.A. Riddell, F. Li, R.J. Love, D.A. Anderson, Serological evidence for swine hepatitis E virus infection in Australian pig herds, Vet Microbiol 68(1-2) (1999) 95-105.

[39] W.P. Hu, Y. Lu, N.A. Precioso, H.Y. Chen, T. Howard, D. Anderson, M. Guan, Double-antigen enzyme-linked immunosorbent assay for detection of hepatitis E virus-specific antibodies in human or swine sera, Clin Vaccine Immunol 15(8) (2008) 1151-7.

[40] S.A. Rutjes, F. Lodder-Verschoor, W.J. Lodder, J. van der Giessen, H. Reesink, M. Bouwknegt, A.M. de Roda Husman, Seroprevalence and molecular detection of hepatitis E virus in wild boar and red deer in The Netherlands, J Virol Methods 168(1-2) (2010) 197-206.

[41] M.d.E.d.l.R. Argentina, Evolution of annual indicators. Swine sector., in: G.y.P. Secretaría de Agricultura (Ed.) 2022.

[42] M.S. Munne, N.R. Altabert, M.L. Otegui, S.N. Vladimirsky, R. Moreiro, M.P. Espul, C. Espul, A. Manzur, S.S. Soto, L.S. Brajterman, J.E. Gonzalez, Updating the knowledge of hepatitis E: new variants and higher prevalence of anti-HEV in Argentina, Ann Hepatol 13(5) (2014) 496-502.

[43] S. Hinjoy, K.E. Nelson, R.V. Gibbons, R.G. Jarman, P. Chinnawirotpisan, S. Fernandez, P. Tablerk, A.B. Labrique, P. Patchanee, A cross-sectional study of hepatitis E virus infection in pigs in different-sized farms in northern Thailand, Foodborne Pathog Dis 10(8) (2013) 698-704.

[44] J. Acosta, A. Galimberti, F. Marziali, A. Costaguta, F. Bessone, H. Tanno, D. Gardiol, M.V. Reggiardo, A.L. Cavatorta, Zoonotic transmission of hepatitis E virus in a pig farmer from Argentina: A case report, Zoonoses Public Health 69(3) (2022) 235-241.

[45] H.R. Dalton, J. Izopet, Transmission and Epidemiology of Hepatitis E Virus Genotype 3 and 4 Infections, Cold Spring Harb Perspect Med 8(11) (2018).

FIGURE LEGENDS

Figure 1. Map of Argentina depicting the location of the county of Tandil (A), in the south-east of the province of Buenos Aires (B), in the central region of Argentina. Samples were obtained from 49 small-scale farms in the periurban and rural region of Tandil county.

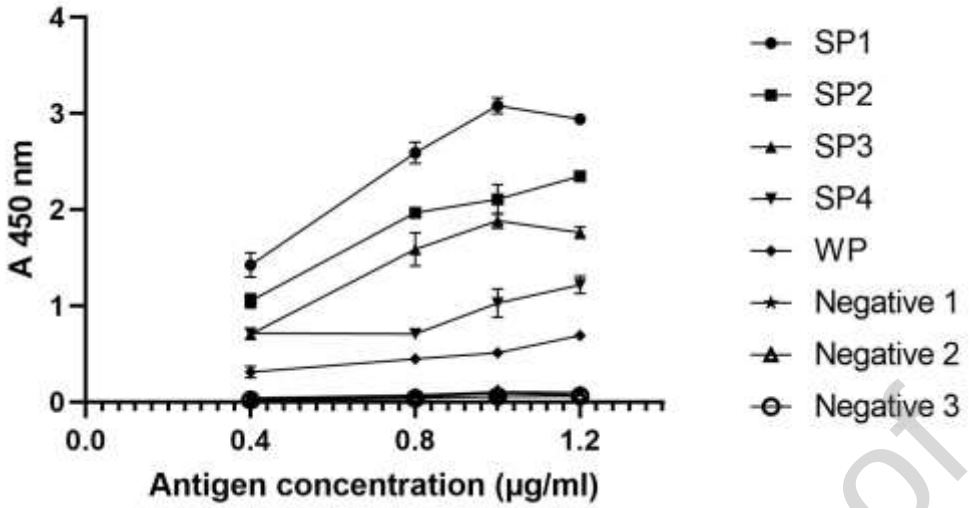


Figure 2. Effect of concentration of recombinant ORF2 on reactivity (OD 450nm) of strong positive (SP), weak positive (WP) and negative serum samples.

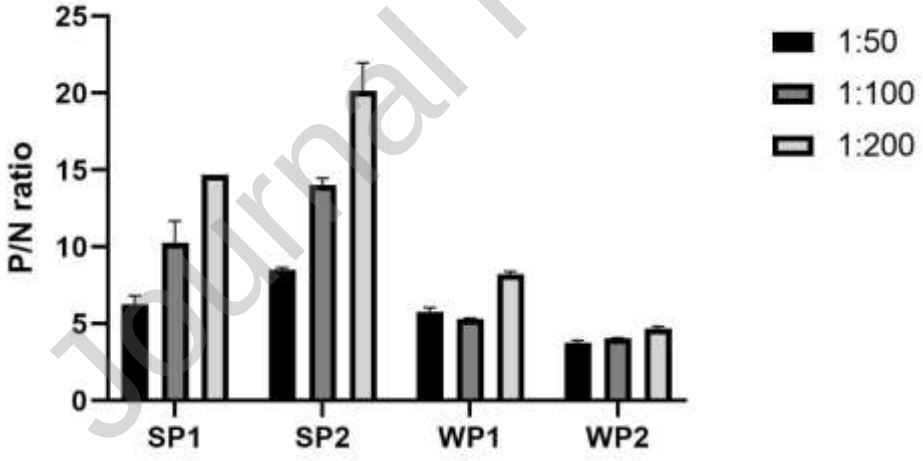


Figure 3. Effect of serum dilution on positive/negative (P/N) ratio. Bars represent mean ± SD of strong positive (n= 2) and weak positive (n= 2) serum samples tested in duplicate wells.

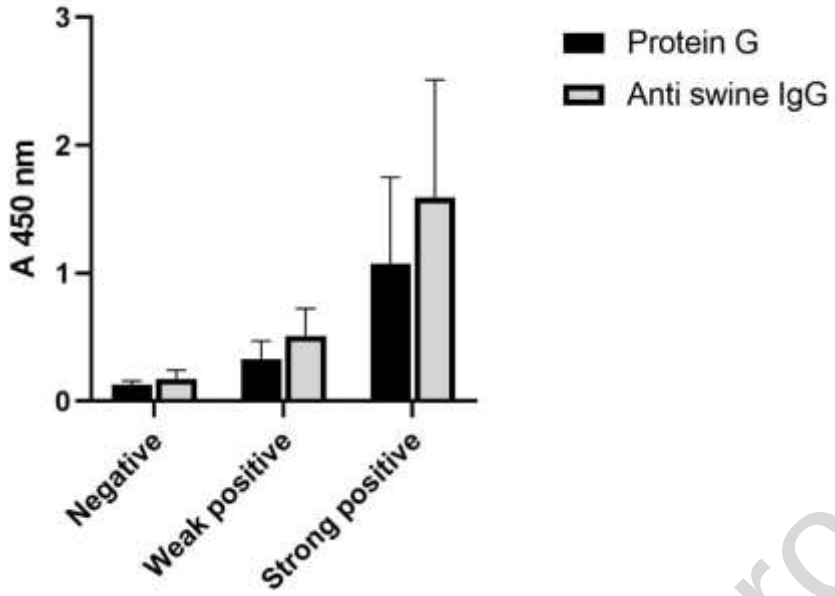


Figure 4. Comparison of two peroxidase conjugates for the detection of specific bound antibodies. Bound antibodies were detected with peroxidase-conjugated protein G or anti-swine IgG at optimal dilution. Bars represent mean ± SD of negative (n=12), weak positive (n= 11) and strong positive (n=6) serum samples tested in duplicate wells.

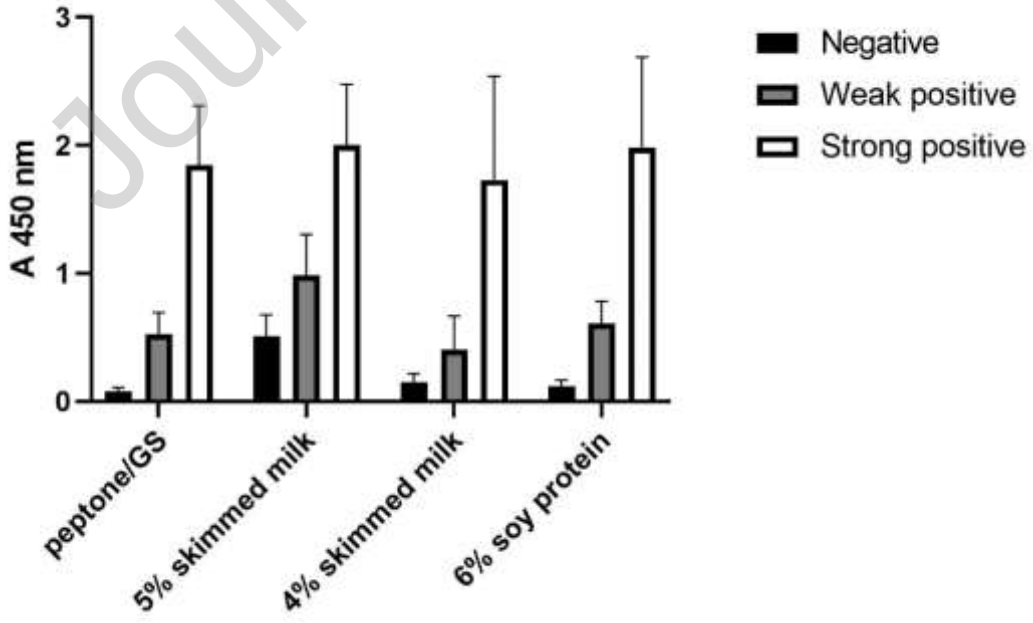
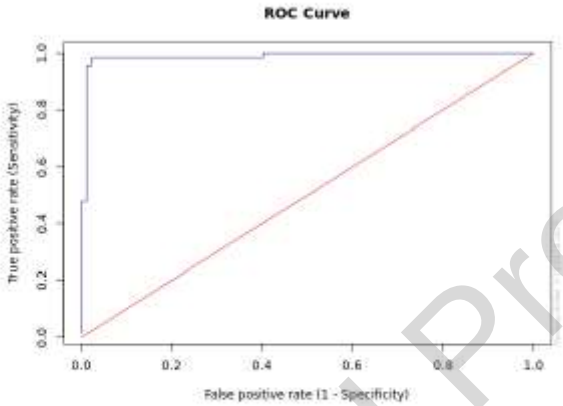
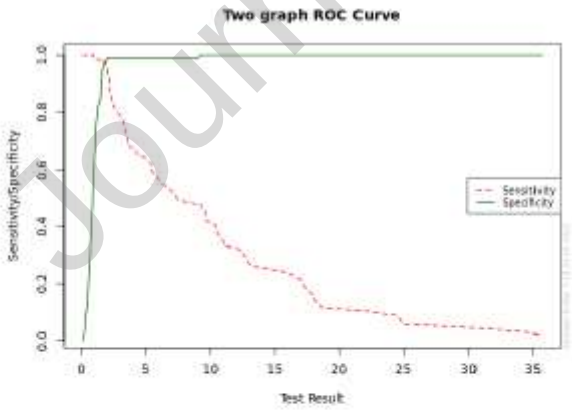


Figure 5. Effect of different blocking agents on reactivity of negative and positive samples. Plates were coated with 1.2 µg/ml of recombinant ORF2 and followed by a blocking step during 110 min at 37°C. Peroxidase-conjugated anti-swine IgG was used to detect bound antibodies. Bars represent mean ± SD of negative (n=4), weak positive (n=3) and strong positive serum samples (n=3) tested in duplicate wells. GS: goat serum.

A



B



C

Test Result (A_{sample}/A_{NC})	Sensitivity	Specificity	Youden's index
0.97	1	0.598	0.598
1.78	**0.986**	**0.977**	**0.963**
1.99	0.957	0.985	0.942

Figure 6. Receiver-operating characteristic (ROC) analysis was conducted with results (A_{sample}/NC ratio) from 157 serum samples categorized as positive or negative by a commercial ELISA. A. Area under ROC curve (blue line) is 0.988, showing the ability of the test to differentiate positive and negative samples. B. Sensitivity and specificity of the test as function of cut-off value. C. The table shows cut-point results for target cut-off points close to the intersection of sensitivity and specificity curves.

Declaration of Competing Interest

The manuscript has been read and approved by all named authors and there are no other persons who satisfied the criteria for authorship but are not listed. I further confirm that the order of authors listed in the manuscript has been approved by all of us.

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The authors declare that they have no conflict of interest.

HIGHLIGHTS

- Development of a sensitive indirect ELISA for detection of HEV antibodies in swine
- High spreading of HEV in small-scale pig farms from Tandil, Argentina
- Need to mitigate the impact of HEV on public health