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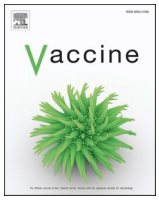

Short communication

Adaptive immune response to a wild boar-derived recombinant hepatitis e virus capsid protein challenge in pigs

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ABSTRACT

Hepatitis E virus genotype 3 (HEV-3) is a zoonotic pathogen capable of infecting human, porcine, and other animal hosts. Despite a broad host range and abundance of species that act as reservoirs for human infections, no commercially available animal vaccines against HEV-3 are currently available. In the present study, we tested the capacity of recombinant aa 112–608 wild boar-derived HEV-3 capsid protein (rORF2p) to induce an immune response in immunized pigs. Four 6 week old pigs were administered 1 ml of 200 μg/ml rORF2p, followed by booster administration after 14 days. Blood samples were collected until 28 days after initial immunization. Dominant cell phenotypes and anti-HEV IgG concentrations were determined. A significant anti-HEV IgG, monocyte/macrophage, B cell and T cell response has been detected in immunized pigs. In turn, our findings suggest the capacity of rORF2p to elicit an immune response in pigs, suggesting the potential for its use as a vaccine candidate.

1. Introduction

Hepatitis E virus (HEV) is a single-stranded positive sense RNA virus of the genus *Orthohepevirus* within the *Hepeviridae* family and a causative agent of hepatitis E. Five genotypes of *Orthohepevirus A* have been identified as capable of infecting multiple animal species, three of which (genotypes 3, 4 and 7) have been confirmed as zoonotic and capable of causing sporadic human cases [1]. HEV genotype 3 (HEV-3) is a zoonotic pathogen with a broad host range and geographical distribution, primarily affecting human and animal populations in developed countries. HEV-3 host species include both domestic (such as pigs, goats and sheep) and wild (such as wild boars, deer and hares) animals [2]. While HEV-3 infections are characterized as mostly mild or asymptomatic, HEV is still the main cause of acute hepatitis in some European countries with anti-HEV IgG prevalence rates surpassing 50 % in humans [3].

HEV genome contains four partially overlapping open reading frames (ORFs). ORF2, coding for major HEV viral capsid protein, shares over 85 % amino acid identity among human (HEV-1 and HEV-2) and zoonotic (HEV-3 and HEV-4) genotypes [4]. Numerous studies demonstrated the capacity of ORF2 protein to elicit neutralizing antibody response and described its potential as a vaccine candidate: the P domain of the ORF2 – a C-terminal portion spanning amino acids (aa) 459–607 – has been identified as an immunodominant subregion in humans [5]. While multiple truncated forms of ORF2 protein have previously been tested for recombinant vaccine development, only two candidates – namely genotype-1-derived HEV 239 and 56 kDA – have been carried to the human clinical trials [6].

Porcine species are considered one of the primary reservoirs for HEV-3. Considering the zoonotic potential of HEV-3 and the presence of viral RNA in slaughtered pigs, animal vaccination has been proposed as a desirable strategy for mitigation of HEV-3 infections in human and non-human animals [7]. However, no commercially available animal vaccines currently exist. While pigs have previously been employed as model animals for immune response to HEV research, no *in vivo* studies investigating immune response to recombinant vaccine candidates for pigs are currently available.

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In the present study, we used a recombinant wild boar-derived HEV-3 ORF2 capsid protein (rORF2p) generated in yeast [8] to test its immunogenicity in pigs. Following rORF2p challenge, changes in monocyte/macrophage, antibody-mediated and cell-mediated immune responses were evaluated.

2. Materials and methods

2.1. Experimental design

A summary of the experimental design is presented in Fig. 1A. Briefly, HEV-negative Lithuanian white pigs (6 week old, $n = 8$ pigs) were randomly divided into immunized and control groups of 4 pigs each (2 male and 2 female animals), receiving either intramuscular injection of 1 ml of 200 µg/ml of recombinant yeast-expressed HEV ORF2 capsid protein (prepared from isolate wbGER27 of wild-boar origin) [8] or 1 ml of phosphate buffered saline (PBS, 1×, pH 7.2; Gibco, Grand Island, NY, USA) on day 0 (D0)(prime injection) and D14 (boost injection). The total volume (1 ml) of HEV ORF2 capsid protein or PBS buffer was divided into two equal parts and administered in 0.5 ml doses intramuscularly on each side of the neck. Control and immunized group animals were kept in separate pens at a temperature of 25 ± 2 °C until the end of the experiment at D28. Blood samples were collected from the jugular vein of each pig on D0, D14 and D28 and immediately used for enzyme-linked immunosorbent assay (ELISA) and cell flow cytometry. Following termination of the experiment, all animals were euthanized, necropsied and investigated for presence of microscopic or macroscopic lesions. All experimental procedures were conducted in compliance with EU Directive 2010/63/EU for animal experiments and approval was issued by the State Food and Veterinary Service (Decision no. G2–123).

2.2. Anti-HEV antibody testing

Sera were generated from the pig blood samples and tested by a commercially available ID Screen Hepatitis E multi-species indirect ELISA (IDvet, France) for the presence of HEV-specific IgG antibodies according to manufacturer's instructions. All samples were tested in triplicates. The optical density (OD value) of each well was determined using a microplate reader set to 450 nm. The anti-HEV antibody levels in serum samples were represented as sample-to-positive control ratio (S/P %).

2.3. Cell flow cytometry and immune cell phenotyping

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood samples by Ficoll-Paque PLUS (GE Healthcare, Chicago, IL, USA) gradient centrifugation following the manufacturer's instructions. PBMCs were washed and suspended to a final concentration of 4×10^6 cells/ml in PBS. PBMCs were used for staining immediately with fluorochrome-conjugated antibodies (FITC-labeled anti-porcine CD4, APC-labeled anti-porcine CD8α, PE-labeled anti-porcine CD3, FITC labeled anti-porcine CD14, and AlexaFluor 674 labeled anti-porcine CD21) after isolation and analyzed using a flow cytometer (FACS Calibur, BD Biosciences, Franklin Lakes, NJ, USA), plotting at least 10,000 events for each sample, followed by cytometry data analysis using FlowJo v10 (Tree Star) software. All samples were tested in triplicates.

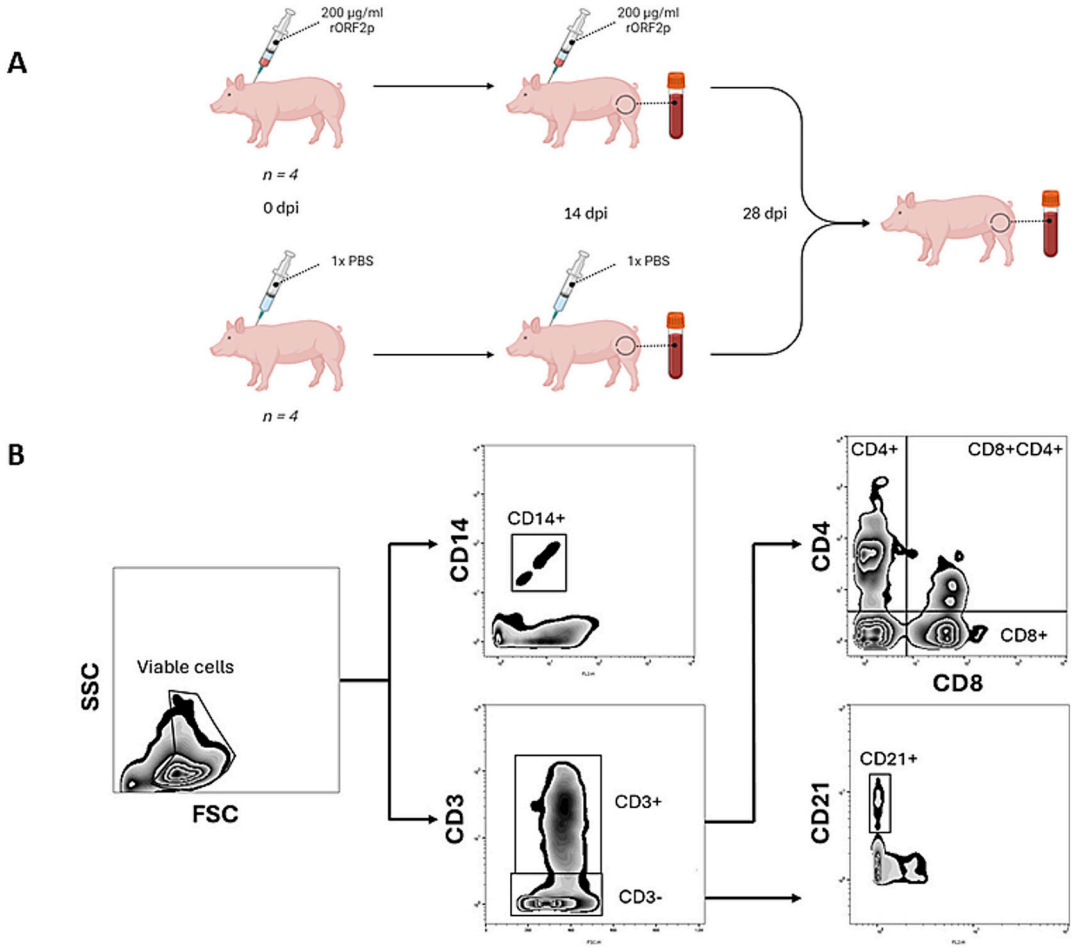

Fig. 1. Immunization of pigs with recombinant wild boar-derived HEV-3 ORF2 capsid protein. (**A**) Experiment workflow (image generated using BioRender). (**B**) Gating strategy of pig PBMCs for immune cell phenotyping. SSC – side scatter; FSC – forward scatter.

Summary of the gating strategy is presented in Fig. 1B. Briefly, $CD3^+$ and $CD3^-$ cell gates were established following the viable cell and single cell gating, whereas $CD4^-$ $CD8^+$, $CD4^+$ $CD8^{+low}$, and $CD4^+$ $CD8^-$ gates were established within the $CD3^+$ cell population. All T cell subpopulation sizes were expressed as proportions of CD3+ cells and total cells. $CD21^+$ and CD14+ gates were established within the $CD3^-$ and total cell populations, respectively.

2.4. Statistical analysis

All statistical analyses were performed using GraphPad Prism 10.1.0 software (GraphPad Software, San Diego, CA, USA). Differences between control and immunized groups were determined at each time point using repeated-measures two-way ANOVA followed by an uncorrected Fisher's least significant difference (LSD) test. $P < 0.05$ was considered statistically significant.

3. Results

Anti-HEV IgG antibody response in serum samples of pigs after prime (D0) and booster (D14) injections are summarized in Fig. 2. As expected, no statistically significant difference was recorded in S/P ratios of the control group pigs (all animals were anti-HEV IgG-negative) across all time points (Fig. 2A). A statistically significant ($p < 0.0001$) increase in serum anti-HEV IgG levels were observed in the immunized group on D14 [D14: control *vs* immunized: 15.95 % and 142.80 %] and D28 [D28: control *vs* immunized: 15.74 % and 227.30 %] (Fig. 2A). Moreover, a statistically significant ($p = 0.0005$) increase at D28 compared to D14 was observed within the immunized animal group [immunized: D14 *vs* D28: 142.80 % and 227.30 %] (Fig. 2A). A consistent trend was observed in B cell group ($CD3^-CD21^+$) proportion changes between D14 and D28 of immunized animals (D14 *vs* D28: 3.49 % and 5.80 %, $p = 0.0016$] (Fig. 2B).

A significant ($p = 0.0004$) increase in T cells ($CD3^+$) within the total tested cells of the immunized group of pigs between D14 and D28 [immunized: D14 *vs* D28: 30.59 % and 48.08 %] was observed. A similar tendency was also observed in the immunized group compared to the control pigs between D14 [D14: control *vs* immunized: 19.46 % and 30.59 %, $p = 0.0102$] and D28 [D28: control *vs.* immunized: 24.91 % and 48.08 %, $p < 0.0001$], respectively (Fig. 3A).

A statistically significant differences in frequencies of $CD4^+CD8^-$ T cells within total tested cells were determined in the control group, compared to the immunized group of pigs between D14 [D14: control *vs* immunized: 4.68 % and 8.85 %, $p = 0.0354$] and D28 [D28: control *vs* immunized: 8.24 % and 12.34 %, $p = 0.0385$] respectively (Fig. 3B), but not within T cell population (Fig. 3C).

No statistically significant differences in frequencies of $CD4^-CD8^+$ T cell (Fig. 3D), $CD4^-CD8^+$ T cell (Fig. 3E), NK cell ($CD3^-CD4^-CD8^{+low}$) (Fig. 3F), and $CD4^+CD8^{+low}$ T cell (Fig. 3G) subpopulations within total cells, and $CD4^+CD8^{+low}$ T cells within T cell population (Fig. 3H) were observed between control and immunized groups at both D14 and D28.

Furthermore, B cells ($CD3^-CD21^+$) cells demonstrated statistically significant differences between the control and immunized group of pigs on D14 [D14: control *vs* immunized: 1.22 % and 3.49 %, $p = 0.0018$ and D28 (D28: control *vs* immunized: 2.16 % and 5.80 %, $p < 0.0001$], respectively (Fig. 3I). Similarly, a statistically significant ($p = 0.0286$) increase of monocyte/macrophage ($CD14^+$) (Fig. 3J) cells within total tested cells of immunized group of pigs was fixed on D28 in comparison to control group of pigs [D28: control *vs* immunized: 1.87 % and 4.56 %].

4. Discussion

Domestic and wild pigs are considered to be one of the primary reservoir species for zoonotic HEV-3 and the main source of human infections. Multiple teams have established a link between human HEV infections and consumption of raw or insufficiently thermally treated meat or occupational risks associated with direct contact with reservoir animals [9,10]. A limited number of mitigation strategies currently exist with regards to HEV-3 control on a farm level and management of cross-species transmission [11]. More effective HEV-3 mitigation strategies at reservoir animal level would provide protection for both human and non-human hosts, especially in light of potentially novel ways of human-to-human transmission pathways, as highlighted by a recent study demonstrating presence of HEV-3 particles in the ejaculate of chronically infected patient [12]. One such strategy is the vaccination of pigs. Currently, there are no commercially available animal HEV vaccines. To our knowledge, the current study is the only one attempting to investigate the effect of a pig HEV vaccine candidate *in vivo*, albeit a few candidates have been tested in mice and rabbits in the past [13,14]. The benefit of reduced prevalence of HEV in pigs at slaughter as a result of a vaccination strategy has been established in a model study [15].

A rORF2p (aa 112–608) protein, derived from wild boar isolate wbGER27 (GenBank GU345042) and expressed in yeast [8] was used for

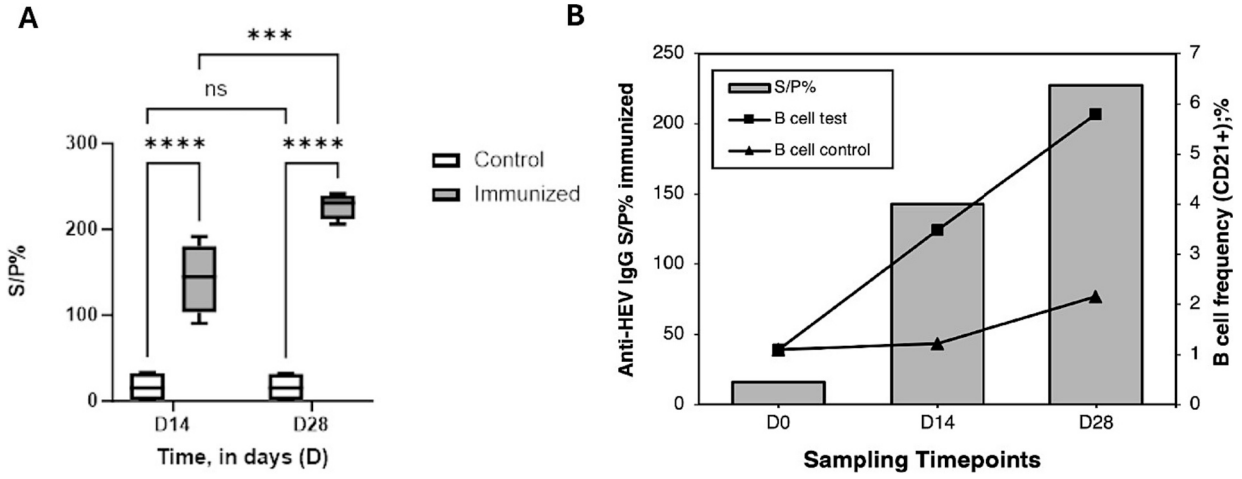

Fig. 2. Anti-HEV IgG and B cell (CD21+) changes in rORF2p-immunized pigs. (**A**) Box plot identifying differences in serum anti-HEV IgG levels at different sampling points (D14 and D28). Statistics are based on a two-way ANOVA comparison followed by uncorrected Fisher's LSD test for pairwise comparisons. Horizontal lines within boxes denote mean values of all animals ($n = 4$) in the group, boxes demonstrate interquartile range and whiskers represent the minimum and maximum values of the dataset. (**B**) B cell proportion change over time in reference to anti-HEV IgG ELISA S/P% ratios (bar plots). Each point represents mean B cell proportion values in all animals (n = 4) within the group. ***$p < 0.001$, ****$p < 0.0001$, ns – not significant.

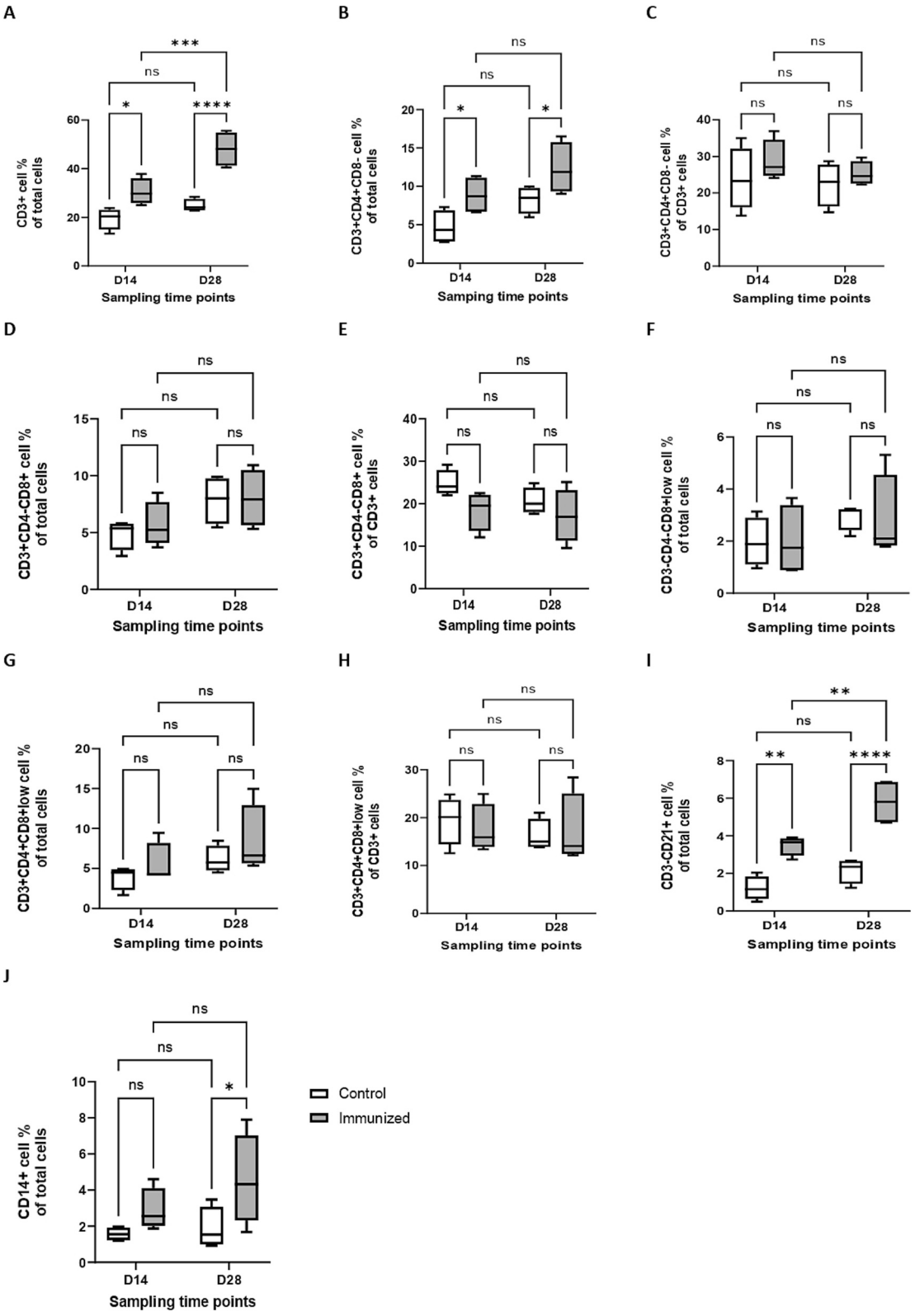

Fig. 3. The distribution pattern of immune cell subpopulations between two groups of pigs at two follow-up time points (D14 and D28). Statistics are based on a two-way ANOVA comparison followed by uncorrected Fisher's LSD test for pairwise comparisons. Horizontal lines within boxes denote mean values (percentage of $CD3^+$ T cells (**A**), $CD3^+CD4^+CD8^-$ proportion of total (**B**) and $CD3^+$ (**C**) cells, $CD3^+$ $CD4^-$ $CD8^+$ proportion of total (**D**) and $CD3^+$ (**E**) cells, percentage of NK cells($CD3^-$ $CD4^-$ $CD8^{+low}$) (**F**), $CD3^+$ $CD4^+$ $CD8^{+low}$ proportion of total (**G**) and $CD3^+$ (**H**) cells, percentage of B cells ($CD3^-$ $CD21^+$) (**I**), and percentage of monocytes/macrophages ($CD14^+$) (**J**)), boxes demonstrate interquartile range and whiskers represent the minimum and maximum values of the dataset. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns – not significant.

pig inoculations in the present study.

HEV ORF2 products of similar length, generated in other expression systems, have been shown to self-assemble into virus-like particles and contain the immunodominant region, capable of eliciting an antibody response, such as *E. coli*-expressed HEV p495 (aa 112–606) [16]. Characteristics of the p495 have been compared to previously described truncated aa 368–606 of ORF2, which has been used as the basis for HEV vaccine currently available in China [17]. Immunoreactivity of wild boar-derived aa 112–608 ORF2 protein has previously been established, demonstrating its capacity to react with HEV-infected serum samples and produce monoclonal antibodies when used as an immunogen in mice [8].

Although pigs as an animal model for HEV have previously been used to investigate pathobiology of HEV-3 and HEV-4, we are aware of only one other study that employs recombinant HEV capsid proteins to assess their immunoreactivity in experimentally challenged pigs [18]. Consistent with results in Sanford et al. 2012, we observed anti-HEV IgG response in experimentally immunized pigs at D14 and a further increase following a booster at D28, demonstrating that, together with pig, avian and rat, wild boar-derived HEV-3 capsid proteins are capable of eliciting an antibody response in pigs. Although all HEV genotypes are considered to constitute a single serotype, therefore suggesting cross-protective qualities of ORF2, the degree of cross-protection may differ greatly among zoonotic *Orthohepevirus A* genotypes [18] and has not yet been established in the case of recent findings associated with cross-species (including zoonotic) transmission of *Orthohepevirus C* [19,20]. It is important to note that a robust antibody response is not equivalent to total protection. Experiments involving challenges with infectious HEV strain following immunization should therefore be carried out to fully evaluate the protective quality of recombinant wild boar-derived HEV capsid protein in pigs.

Consistent with anti-HEV IgG response results, we have detected a significant increase in B cell proportion at D14 and a further increase following a booster at D28. CD21+ cells constitute two subpopulations of porcine B cells in the periphery – namely, naïve and activated B cells before maturation into antibody-producing cells. We are aware of one other study that investigated B cell response in HEV-immunized animals, however, the vaccine candidate used was not based on ORF2 [21]. A detailed frequency analysis of B cell subpopulations in response to recombinant HEV ORF2 protein stimulation in humans revealed minor changes in overall B cell frequency 72 h post stimulation, however, increase in immature B cells in acute hepatitis E patient PBMCs and consistent increase in mature and memory B cells in recovered patients and healthy controls were observed [22]. In combination, B cell data as a response to HEV suggests expansion of immature B cell subset soon following HEV ORF2 stimulation, without a noticeable change in total B cell frequency. However, data investigating cellular B cell response to vaccine candidates is scarce, constituting a few publications employing widely different models lacking in scope. As a result, a putative B cell response mechanism to HEV vaccine candidates was largely incomplete.

While porcine animal models have previously been used to investigate HEV-associated changes in the immune system [23], no studies have previously investigated changes in cell-mediated immunity associated with HEV vaccine candidate immunization. Most of the data on this topic has been accrued from primate studies [5]. Although in the present study the T cell subpopulation frequencies remained largely unchanged in the periphery of HEV ORF2-challenged pigs compared to controls, we have detected a marginal increase in CD4 + CD8- frequency and a significant increase in the overall number of CD3+ cells in immunized pigs, corresponding to a T cell response. This is consistent with findings from acute human HEV patients where an increased proportion of CD4+ but not CD8+ has been found in the periphery [24]. Although our findings revealed a CD3+ cell mobilization in the periphery following immunization with rORF2p, changes in specific CD4+ and CD8+ T cell subpopulations may be observed in later stages following immunization or in specific cytokine-producing T cells only, such as INFγ+ CD4 + CD8+ cells [23].

In combination, B and T cell data suggest that within the framework of adaptive immune response to rORF2p challenge, antibody-mediated immunity is primarily activated, as demonstrated not only by an increase in anti-HEV IgG production and B cell proportion, but a marginal increase in CD4 + CD8- cell proportion as well, corresponding to T-helper cell population, which may include T-helper 2 cells that play a role in B cell co-stimulation. While no significant change has been observed in cell-mediated immunity effector cells, such as CD4 + CD8+ T-cytotoxic cells, this is to be expected considering the use of viral subunit administration without adjuvant supplementation. While data on the principal modality of protection against HEV in pigs is lacking, data from humans and other animals suggest that antibody-mediated immunity protects individuals from HEV viremia, fecal shedding and hepatitis, while data on the necessity of T-cell response to control hepatitis E is inconclusive, suggesting that CD8+ cytotoxic cells may not be necessary to control acute HEV infection [25]. Further studies employing forms of vaccines typically associated with a more robust activation of cell-mediated immunity, such as polynucleotide vaccines of HEV ORF2 would elucidate on the capacity of the HEV capsid protein to activate both legs of the adaptive immunity.

We observed a marginal but significant increase in monocyte/macrophage population in HEV rORF2p challenged pigs at D28, but not at D14. Although data of monocyte/macrophage response to HEV is scarce, and no information is available about CD14+ response in porcine models, an increase in monocyte/macrophage proportion in the periphery of human acute hepatitis E patients has previously been observed [26]. Whether monocyte/macrophage response is important for vaccine-induced protection is yet to be established.

In the present study, we have for the first time tested a recombinant wild boar genotype 3-derived HEV capsid protein as a vaccine candidate in pigs. Although some general characteristics associated with immune response to HEV rORF2p in pigs have been established, the following limitations of the study need to be addressed. Firstly, although consistent with similar *in vivo* studies, sample size in this study was a potentially limiting factor, especially with regards to relatively wide ranges within certain immune cell subpopulation frequencies. Secondly, the experiment terminated after 28 days following rORF2p challenge. Some changes, especially those associated with adaptive immune response, are only observable several months after the challenge. We therefore could not distinctly demonstrate the memory cell response of both T and B cell origin and propose that changes in CD4+ and CD8+ cell frequencies may have been more readily pronounced at the later stages following the challenge. Finally, although we demonstrated immune response to HEV rORF2p as a vaccine candidate and its capacity to induce anti-HEV antibody response and changes in adaptive immune cell frequencies, we did not directly evaluate whether immunization was capable of protecting test subjects from the subsequent HEV infections. Limitations outlined above should therefore be taken into consideration when designing future experiments involving HEV vaccine candidates for pigs.

In conclusion, we demonstrated the capacity of a wild-boar-derived HEV-3 rORF2p comprising aa 112–608 to elicit an immune response in experimentally inoculated and booster-administered pigs, including anti-HEV IgG, monocyte/macrophage, B cell and T cell response. In turn, our findings suggest the potential of tested rORF2p as a candidate for porcine HEV vaccine development.

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CRediT authorship contribution statement

Juozas Grigas: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Conceptualization. **Ugne Spancerniene:** Writing – review & editing, Writing – original draft, Visualization, Formal analysis. **Martynas Simanavicius:** Writing – review & editing, Methodology. **Arnoldas Pautienius:** Writing – review & editing, Visualization, Validation, Investigation. **Rolandas Stankevicius:** Writing – review & editing, Project administration, Formal analysis. **Paulius Lukas Tamosiunas:** Writing – review & editing, Methodology. **Arunas Stankevicius:** Writing – review & editing, Writing – original draft, Supervision, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

All authors attest they meet the ICMJE criteria for authorship.

Data availability

The data that has been used is confidential.

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