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Research paper

Acute phase protein concentrations in colostrum-deprived pigs immunized with subunit and commercial vaccines against Glässer's disease

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ABSTRACT

Haemophilus parasuis is the etiological agent of Glässer's disease, which is characterized by fibrinous polyserositis, polyarthritis and meningitis in pigs. This study was focused on the characterization of the acute-phase response after immunization and infection of colostrum-deprived pigs with *H. parasuis* serovar 5, by measuring serum concentrations of three positive acute-phase proteins (APPs) (pig major acute-phase protein pig, MAP; haptoglobin, HPG; C-reactive protein, CRP) and one negative APP (apolipoprotein A-I, ApoA-I). Six experimental groups were established: a non-immunized but infected control group (CTL); two groups immunized with either a recombinant transferrin-binding protein (Tbp) A or TbpB fragment from *H. parasuis* Nagasaki strain (rTbpA and rTbpB, respectively); two groups immunized with native outer membrane proteins with affinity to porcine transferrin (NPAPT), one of them inoculated intramuscularly (NPAPTim) and the other intratracheally (NPAPTit), and the last group receiving a commercially available bacterin (PG). The greatest concentrations of the three positive APPs and the lowest concentration of the negative APP were detected in CTL group, as well as in those animals belonging to rTbpA or rTbpB groups that died in response to challenge. Significant differences ($P < 0.005$) were found in these groups when comparing challenge with the following days after it. However, no significant differences were seen for the remaining vaccinated groups (NPAPTim, NPAPTit and PG), which were effectively protected against Glässer's disease. Therefore, APPs could be used as useful biomarkers for both evaluating disease progression and determining vaccination effectiveness.

1. Introduction

Glässer's disease, whose main symptoms are serofibrinous to fibrinopurulent polyarthritis, polyserositis, and meningitis, as well as septicaemia, pneumonia or myositis of the masseter muscles, is a swine disorder caused by a pleomorphic Gram-negative rod of the family *Pasteurellaceae* known as *Haemophilus parasuis* (Oliveira and Pijoan, 2004). Although this organism is also considered as an early colonizer of the upper respiratory tract of pigs and, therefore, it can be isolated from this location of healthy animals (Møller and Kilian, 1990), some strains can disseminate

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to produce the severe systemic disease described above. In recent years, there has been a significant increase in the morbidity and mortality rates in pig of all ages due to Glässer's disease, mainly in herds with high sanitary standards (Oliveira and Pijoan, 2004).

H. parasuis contains several potential virulence factors (lipooligosaccharide, outer membrane proteins – OMPs, capsule, fimbriae, neuraminidase and iron acquisition systems – Oliveira and Pijoan, 2004), having been described some of them as potent inducers of an acute-phase response in some domestic species (Henderson and Wilson, 1996). In addition, the infection caused by *H. parasuis* results in tissue injury (Vahle et al., 1995), which could aid to the development of an acute-phase reaction. This sort of response is an innate, non-specific early response of the organism to a variety of stimuli, such as infection, neoplastic growth, tissue damage or immunological disorders (Kuschner, 1998; Heegaard et al., 1998). The acute-phase response is triggered by the synthesis of proinflammatory cytokines (interleukins – ILs – 1 and 6, and tumor necrosis factor α – TNF α) at the local site of the injury and involves local and systemic effects, such as fever, increase in muscle catabolism or hormonal alterations, also resulting in changes in the concentrations of the proteins called acute-phase proteins (APPs) (Gabay and Kushner, 1999; Murata et al., 2004). Hepatocytes produce most of these APPs, which have been classified as positive or negative APPs depending on the increase or reduction of their serum concentration, respectively, within hours or days under those conditions cited above (Petersen et al., 2004). Pig major acute-phase protein (pig MAP), haptoglobin (HPG), C-reactive protein (CRP), and serum amyloid A represent the main positive APPs in pigs (Eckersall et al., 1996; Heegaard et al., 1998; Petersen et al., 2004; Gómez Laguna et al., 2010), while albumin, apolipoprotein A-I (ApoA-I) and transthyretin are the main negative APPs (Campbell et al., 2005; Carpintero et al., 2005). Recently, the baseline concentrations of pig MAP, HPG, CRP and transthyretin have been studied in large populations of health pigs (Piñeiro et al., 2009; Diack et al., 2011).

APPs have been proposed in veterinary medicine as suitable biomarkers for monitoring stress (Marco Ramell et al., 2011), discriminating respiratory lesions in swine herds at slaughter (Saco et al., in press), diagnosing inflammation and infection, as well as for assessing the efficacy of different vaccines (Carroll et al., 2004; Petersen et al., 2004; Carpintero et al., 2007). To our knowledge, apart from a recent investigation by us (Martín de la Fuente et al., 2010), there are no reports regarding APP responses in pigs after *H. parasuis* infection. Therefore, the purpose of this study was to contribute to the characterization of this response in naïve and previously immunized piglets following experimental infection with *H. parasuis* serovar 5 (a highly virulent serovar of worldwide prevalence – Oliveira and Pijoan, 2004) and to establish the usefulness of pig MAP, HPG, CRP, and ApoA-I measurements as biomarkers for the evaluation of the severity and propagation of Glässer's disease. As animal model, colostrum-deprived piglets (Blanco et al., 2008) were used in order to avoid the colonization of upper respiratory tract of suckling piglets being in contact with their sows.

2. Materials and methods

2.1. Vaccines

Five vaccines were compared. rTbpA vaccine contained 400 μg of rTbpA antigen, consisting of a recombinant TbpA fragment from *H. parasuis* Nagasaki strain, cloned in *Escherichia coli* TOP10 and expressed in *E. coli* LMG194 (Martínez et al., 2010). This antigen was adjuvanted with Montanide IMS 225 VG PR (Seppic, Inc., Paris, France) in a 1:4 ratio. rTbpB vaccine contained 400 μg of rTbpB antigen, consisting of a recombinant N-terminal fragment from the same strain, cloned and expressed in *E. coli* TOP10 (Del Río et al., 2005). This antigen was also adjuvanted as described for the rTbpA vaccine. NPAPTim vaccine contained 400 μg of NPAPT antigen, consisting of a mixture of nine outer membrane proteins from Nagasaki strain with affinity to porcine transferrin, which were purified by gel filtration on a fast-protein liquid chromatography-CN-Br-activated Sepharose 4B (GE Healthcare) column (Frandoloso et al., 2011). This antigen was also adjuvanted with Montanide IMS 225 VG PR in a 1:4 ratio. NPAPTit vaccine contained 400 μg of the same NPAPT antigen, but now potentiated with neuraminidase from *Clostridium perfringens* (type VI) at a concentration of 100 mU/ml of vaccine. Finally, PG vaccine consisted of a commercially available bacterin (Porcilis Glässer, Intervet), composed of *H. parasuis* cells belonging to serovar 5, strain 4800. The vaccines designed by us were developed following high aseptic conditions and strict sterility controls. A 1-ml aliquot of each of these vaccines was grown in tryptic soy broth and fluid thioglycolate medium at 37 and 24 °C, respectively, for 14 days in order to confirm absence of growth after these incubation conditions.

2.2. Animals and experimental design

A total of 33 colostrum-deprived Large White × Pietrain, 4 week-old piglets, coming from a farm with a high sanitary status and no previous clinical history of infections by *H. parasuis* and *Actinobacillus pleuropneumoniae* were reared artificially and fed a pasteurized bovine colostrum for 7 days, a porridge mixture (bovine colostrum plus Startrite 100, SCA Ibérica, Spain) from 7 to 14 days, and a piglet dry meal formula (Startrite 100) for the rest of the study. Nasal and tonsillar swabs were obtained from each animal and determined to be negative for *H. parasuis* by PCR (Angen et al., 2007). Additionally, the piglets were found to be serologically negative for porcine reproductive and respiratory syndrome virus (PRRSV) and *A. pleuropneumoniae* by enzyme-linked immunosorbent assay, and for circovirus by PCR. The piglets were randomly assigned to one control (CTL) and five test groups. The rTbpA ($n = 6$), rTbpB ($n = 5$) and NPAPTim ($n = 6$) test groups received 2 ml of rTbpA, rTbpB or NPAPTim vaccines, respectively, by intramuscular injection at 28 and 49 days of age. The NPAPTit test group ($n = 6$) received 2 ml of NPAPTit vaccine by intratracheal injection at the same times. The PG group ($n = 6$) received 2 ml of PG vaccine intrasmuscularly at 28 and 42 days of age, as recommended by the manufacturer. CTL group ($n = 4$) received 2 ml of phosphate-buffered saline (pH 7.4)

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intramuscularly at the same times that rTbpA, rTbpB and NPAPT vaccines were given.

At 63 days of age, all groups were challenged intratracheally with a lethal dose (3×10^8 CFU) of *H. parasuis* Nagasaki strain, suspended in 2 ml of RPMI 1640. Animals with severe signs of distress (swollen joints, limb uncoordination, dyspnea) were immediately euthanized in order to avoid unnecessary pain, and necropsied. The experiment was ended 15 days post-challenge (dpc) and surviving piglets were humanely euthanized with an intracardiac sodium pentobarbital overdose. All the animal handling and procedures were conducted in accordance with the guidelines of the University of León Ethical Committee and the Spanish Government.

2.3. Clinical and pathological examinations

Survival, rectal temperatures, as well as other clinical signs were assessed every 12 h during the first 7 dpc and then, once a day until 15 dpc. All piglets were necropsied, and macroscopic lesions were recorded. Blood samples were collected by venipuncture from the jugular vein at 0 (challenge), 1, 2, 3, 4, 5, 6, 7, 10, 13 and 15 dpc. Serum was obtained after clotting by centrifugation at $2000 \times g$ and room temperature for 15 min and was stored at −20 °C until use.

2.4. Acute-phase protein assays

The concentrations of pig MAP, HPG, CRP and ApoA-I in serum were determined by radial immunodiffusion in 1% agarose gel containing specific rabbit polyclonal antisera raised by subcutaneous injection of the purified proteins, according to previous reports (Carpintero et al., 2005; Martín de la Fuente et al., 2010). Serum calibrated with the purified proteins was used as standard. Plates were incubated for 2–3 days in a moist chamber at room temperature, stained with Coomassie blue, and their halo diameter was measured.

2.5. Statistical analysis

APP concentrations in serum were analysed using the SPSS statistical program (version 17.0). Normality of the data was studied using the Kolmogorov–Smirnov test. A paired *T* test (one-tail) was used for comparison of APP levels between challenge (day 0) and different times after it. A Tukey–Kramer test was used for comparison of APP concentrations between different treatment groups at 2 dpc. *P* values of <0.05 were considered significant.

3. Results

3.1. Survival, clinical signs and lesions

Two piglets from rTbpA group and one from each of CTL and rTbpB groups died between 1 and 2 dpc. Eight more animals died the following two days: three belonging to each of CTL and rTbpB groups, and two belonging to rTbpA group. The piglets in these three groups showed increased rectal temperatures until death (until 3 °C above those measured at challenge, $P < 0.005$). Clinical signs suspicious of Glässer's disease (swollen joints, limb uncoordination, severe dyspnea) were seen, more evident in CTL group and milder in piglets vaccinated with rTbpA or rTbpB antigens. Among the macroscopic lesions observed, severe fibrinous polyserositis was found in the pericardial, pleural and peritoneal cavities, together with a moderate fibrinous polyarthritis, located mainly in carpal and hock joints.

The remaining animals, included all those belonging to NPAPTim, NPAPTit and PG groups, survived the challenge with the lethal dose of Nagasaki strain until the end of the experiment, and no rise in rectal temperature was found. No appreciable clinical signs or lesions were recorded, except for the appearance in some piglets of a mild fibrinous peritonitis (NPAPTim and NPAPTit groups) or polyarthritis (PG group). In addition, no adverse reactions to any of the vaccines compared were detected.

3.2. Pig MAP response

The mean values of the serum pig MAP concentration in each experimental group are summarized in Table 1. There was a significant increase ($P < 0.005$) in CTL piglets, from 0.48 mg/ml measured at challenge to 2.62 mg/ml recorded two days after it, when the highest level was reached. Pig MAP mean values in rTbp groups also increased sharply, reaching significant maximum levels at 2 dpc for rTbpA group (with a concentration being almost four times higher than that found at challenge), or at 3 dpc for rTbpB group (with a value more than 14 times that encountered at infection) ($P < 0.005$ in both cases). It must be taken into account in rTbp groups that the surviving piglets showed small and almost constant pig MAP levels from challenge to the end of the experiment: until 0.64 mg/ml for rTbpA group at 7 dpc and until 0.54 mg/ml for the only surviving rTbpB piglet at 13 dpc. Similar kinetics to those of the surviving animals in rTbp groups were seen for the three other vaccinated (NPAPTim, NPAPTit and PG) groups, and consequently, no significant differences were seen between challenge and different times after it. Globally, pig MAP response was significantly higher in CTL than in the five vaccinated groups, and in rTbp groups than in PG piglets ($P < 0.005$ in all cases).

3.3. HPG response

The mean values at challenge varied between 0.28 mg/ml for rTbpB and 0.38 mg/ml for CTL group (Table 2). The highest increases (with almost four times the values measured at challenge) were found two days after infection for CTL (1.26 mg/ml) and rTbpB groups (1.09 mg/ml), resulting in significant differences ($P < 0.005$ in both cases). A peak of less than thrice that measured at challenge (without significant differences) was seen at 2 dpc for rTbpA. This elevation was even smaller for NPAPTim, NPAPTit and PG groups, with mean values varying between 0.07 and 0.37 mg/ml throughout the experiment. Individually, a piglet belonging to NPAPTim group showed a maximum level of 0.71 mg/ml at 5 dpc, but this value was reduced sharply almost one-third from this day to the end of the study. No significant differences were obtained when comparing challenge and days after

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Table 1
Mean ± standard deviation of the serum concentration of pig MAP in each experimental group after infection with 3×10^8 CFU of *H. parasuis*, serovar 5, Nagasaki strain.

Day post-challenge (dpc)	Concentration (mg/ml) pig MAP in each treatment group					
	CTL	rTbpA	rTbpB	NPAPTim	NPAPTit	PG
Challenge	0.48 ± 0.07 (*n* = 4)	0.38 ± 0.04 (*n* = 6)	0.27 ± 0.08 (*n* = 5)	0.45 ± 0.21 (*n* = 6)	0.46 ± 0.05 (*n* = 6)	0.42 ± 0.05 (*n* = 6)
1 dpc	1.05 ± 0.26 (*n* = 4)	0.87 ± 0.78 (*n* = 6)	0.59 ± 0.22 (*n* = 5)	0.47 ± 0.10 (*n* = 6)	0.61 ± 0.07 (*n* = 6)	0.45 ± 0.08 (*n* = 6)
2 dpc	2.62 ± 1.22 (*n* = 3)	1.37 ± 1.46 (*n* = 4)	1.92 ± 1.04 (*n* = 4)	0.57 ± 0.10 (*n* = 6)	0.50 ± 0.06 (*n* = 6)	0.60 ± 0.11 (*n* = 6)
3 dpc	(*n* = 0)	1.05 ± 0.58 (*n* = 3)	3.92 ± 4.89 (*n* = 2)	0.50 ± 0.10 (*n* = 6)	0.42 ± 0.16 (*n* = 6)	0.54 ± 0.09 (*n* = 6)
4 dpc	(*n* = 0)	0.42 ± 0.06 (*n* = 2)	0.30 (*n* = 1)	0.32 ± 0.07 (*n* = 6)	0.37 ± 0.23 (*n* = 6)	0.34 ± 0.04 (*n* = 6)
5 dpc	(*n* = 0)	0.45 ± 0.02 (*n* = 2)	0.30 (*n* = 1)	0.27 ± 0.06 (*n* = 6)	0.42 ± 0.24 (*n* = 6)	0.30 ± 0.03 (*n* = 6)
6 dpc	(*n* = 0)	0.41 ± 0.14 (*n* = 2)	0.23 (*n* = 1)	0.37 ± 0.07 (*n* = 6)	0.42 ± 0.26 (*n* = 6)	0.37 ± 0.07 (*n* = 6)
7 dpc	(*n* = 0)	0.64 ± 0.11 (*n* = 2)	0.38 (*n* = 1)	0.42 ± 0.12 (*n* = 6)	0.49 ± 0.24 (*n* = 6)	0.39 ± 0.10 (*n* = 6)
10 dpc	(*n* = 0)	0.44 ± 0.08 (*n* = 2)	0.32 (*n* = 1)	0.34 ± 0.07 (*n* = 6)	0.50 ± 0.24 (*n* = 6)	0.37 ± 0.10 (*n* = 6)
13 dpc	(*n* = 0)	0.55 ± 0.27 (*n* = 2)	0.54 (*n* = 1)	0.41 ± 0.08 (*n* = 6)	0.61 ± 0.20 (*n* = 6)	0.43 ± 0.08 (*n* = 6)
15 dpc	(*n* = 0)	0.50 ± 0.08 (*n* = 2)	0.50 (*n* = 1)	0.63 ± 0.12 (*n* = 6)	0.75 ± 0.18 (*n* = 6)	0.57 ± 0.23 (*n* = 6)

n: number of surviving piglets per day in each group.
CTL: control, non-immunized group; rTbpA: group immunized with a recombinant TbpA fragment; rTbpB: group immunized with a recombinant TbpB fragment; NPAPTim: group immunized with native proteins with affinity to porcine transferrin administered intramuscularly; NPAPTit: group immunized with native proteins with affinity to porcine transferrin administered intratracheally; PG: group immunized with Porcilis-Glässer (Intervet, Spain).

it in any of NPAPTim, NPAPTit and PG groups. As for pig MAP, HPG response was significantly greater in CTL than in any of the five immunized animals ($P < 0.001$ compared to PG group, and $P < 0.005$ for the remaining ones).

3.4. CRP response

Once again, results were quite similar for CTL and rTbpB animals (Table 3). An increase about 6–7 times was seen at 2 or 3 dpc in both groups, resulting in significant differences compared to challenge ($P < 0.005$ in both cases). More concretely, a rise of more than 15 times the value of CRP measured at challenge could be detected for one of the rTbpB piglets that died after 3 dpc. However, the concentrations seen for the only surviving animal in this group were quite constant throughout the study. In addition, rather comparable values (comprised between 10 and 20 μg/ml) were achieved for NPAPTim, NPAPTit and PG groups (without significant differences). The only exception was showed by one NPAPTit piglet, whose CRP value was of 210 μg/ml at 2 dpc, this level being more than 20 times higher than that seen for the remaining five NPAPTit piglets at the same day. Globally, CRP response was significantly higher in CTL than in rTbp, NPAPT and PG groups ($P < 0.005$ in all comparisons).

3.5. ApoA-I response

The mean levels of this negative APP varied at challenge between 2.77 mg/ml for CTL and 3.52 mg/ml for rTbpA animals (Table 4). From then, a considerable reduction was observed during the following days, especially among the groups in which all or some of their piglets died as a consequence of the infection. So, almost one-third of the measurement at challenge was seen for CTL group at 2 dpc, and of 2.5 or 1.7 times smaller for rTbpB or rTbpA groups, respectively, at this same day ($P < 0.005$ in the three cases). There was a considerably minor decrease in NPAPT groups until 3 dpc, or even a slight increase, as it was seen in PG group at 3 dpc. After this day, ApoA-I values remained between 3 and 4 mg/ml until 13 dpc, and then, they increased in all groups at 15 dpc. No significant differ-

Table 2
Mean ± standard deviation of the serum concentration of haptoglobin (HPG) in each experimental group after infection with 3×10^8 CFU of *H. parasuis*, serovar 5, Nagasaki strain.

Day post-challenge (dpc)	Concentration (mg/ml) haptoglobin in each treatment group					
	CTL	rTbpA	rTbpB	NPAPTim	NPAPTit	PG
Challenge	0.38 ± 0.18 (*n* = 4)	0.30 ± 0.01 (*n* = 6)	0.28 ± 0.01 (*n* = 5)	0.32 ± 0.02 (*n* = 6)	0.30 ± 0.02 (*n* = 6)	0.30 ± 0.01 (*n* = 6)
1 dpc	1.23 ± 0.31 (*n* = 4)	0.72 ± 0.28 (*n* = 6)	0.55 ± 0.17 (*n* = 5)	0.35 ± 0.06 (*n* = 6)	0.36 ± 0.04 (*n* = 6)	0.37 ± 0.03 (*n* = 6)
2 dpc	1.26 ± 0.57 (*n* = 3)	0.82 ± 0.55 (*n* = 4)	1.09 ± 0.73 (*n* = 4)	0.16 ± 0.18 (*n* = 6)	0.11 ± 0.10 (*n* = 6)	0.19 ± 0.21 (*n* = 6)
3 dpc	(*n* = 0)	0.77 ± 0.33 (*n* = 3)	0.99 ± 0.93 (*n* = 2)	0.34 ± 0.02 (*n* = 6)	0.21 ± 0.20 (*n* = 6)	0.32 ± 0.16 (*n* = 6)
4 dpc	(*n* = 0)	0.41 ± 0.34 (*n* = 2)	0.48 (*n* = 1)	0.14 ± 0.11 (*n* = 6)	0.11 ± 0.08 (*n* = 6)	0.26 ± 0.18 (*n* = 6)
5 dpc	(*n* = 0)	0.51 ± 0.15 (*n* = 2)	0.61 (*n* = 1)	0.21 ± 0.26 (*n* = 6)	0.18 ± 0.14 (*n* = 6)	0.29 ± 0.19 (*n* = 6)
6 dpc	(*n* = 0)	0.49 ± 0.13 (*n* = 2)	0.64 (*n* = 1)	0.09 ± 0.02 (*n* = 6)	0.07 ± 0.03 (*n* = 6)	0.08 ± 0.01 (*n* = 6)
7 dpc	(*n* = 0)	0.26 ± 0.05 (*n* = 2)	0.39 (*n* = 1)	0.17 ± 0.08 (*n* = 6)	0.13 ± 0.07 (*n* = 6)	0.18 ± 0.11 (*n* = 6)
10 dpc	(*n* = 0)	0.18 ± 0.12 (*n* = 2)	0.10 (*n* = 1)	0.11 ± 0.03 (*n* = 6)	0.25 ± 0.09 (*n* = 6)	0.09 ± 0.01 (*n* = 6)
13 dpc	(*n* = 0)	0.22 ± 0.05 (*n* = 2)	0.21 (*n* = 1)	0.19 ± 0.07 (*n* = 6)	0.29 ± 0.08 (*n* = 6)	0.11 ± 0.05 (*n* = 6)
15 dpc	(*n* = 0)	0.16 ± 0.13 (*n* = 2)	0.28 (*n* = 1)	0.20 ± 0.09 (*n* = 6)	0.20 ± 0.06 (*n* = 6)	0.08 ± 0.02 (*n* = 6)

n: number of surviving piglets per day in each group.
CTL: control, non-immunized group; rTbpA: group immunized with a recombinant TbpA fragment; rTbpB: group immunized with a recombinant TbpB fragment; NPAPTim: group immunized with native proteins with affinity to porcine transferrin administered intramuscularly; NPAPTit: group immunized with native proteins with affinity to porcine transferrin administered intratracheally; PG: group immunized with Porcilis-Glässer (Intervet, Spain).

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Table 3
Mean ± standard deviation of the serum concentration of C-reactive protein (CRP) in each experimental group after infection with 3×10^8 CFU of *H. parasuis*, serovar 5, Nagasaki strain.

Day post-challenge (dpc)	Concentration (mg/ml) C-reactive protein in each treatment group					
	CTL	rTbpA	rTbpB	NPAPTim	NPAPTit	PG
Challenge	0.01 ± 0 (*n* = 4)	0.08 ± 0 (*n* = 6)	0.01 ± 0.01 (*n* = 5)	0.01 ± 0 (*n* = 6)	0.01 ± 0 (*n* = 6)	0.01 ± 0 (*n* = 6)
1 dpc	0.05 ± 0.01 (*n* = 4)	0.04 ± 0.14 (*n* = 6)	0.02 ± 0.01 (*n* = 5)	0.01 ± 0 (*n* = 6)	0.01 ± 0 (*n* = 6)	0.02 ± 0.01 (*n* = 6)
2 dpc	0.07 ± 0.03 (*n* = 3)	0.05 ± 0.03 (*n* = 4)	0.05 ± 0.03 (*n* = 4)	0.01 ± 0 (*n* = 6)	0.04 ± 0.08 (*n* = 6)	0.02 ± 0.02 (*n* = 6)
3 dpc	(*n* = 0)	0.06 ± 0.03 (*n* = 3)	0.06 ± 0.05 (*n* = 2)	0.01 ± 0 (*n* = 6)	0.01 ± 0 (*n* = 6)	0.01 ± 0 (*n* = 6)
4 dpc	(*n* = 0)	0.04 ± 0.11 (*n* = 2)	0.01 (*n* = 1)	0.01 ± 0.01 (*n* = 6)	0.01 ± 0 (*n* = 6)	0.02 ± 0 (*n* = 6)
5 dpc	(*n* = 0)	0.04 ± 0.01 (*n* = 2)	0.01 (*n* = 1)	0.02 ± 0 (*n* = 6)	0.02 ± 0 (*n* = 6)	0.02 ± 0.01 (*n* = 6)
6 dpc	(*n* = 0)	0.04 ± 0 (*n* = 2)	0.01 (*n* = 1)	0.01 ± 0 (*n* = 6)	0.02 ± 0 (*n* = 6)	0.02 ± 0 (*n* = 6)
7 dpc	(*n* = 0)	0.03 ± 0.01 (*n* = 2)	0.02 (*n* = 1)	0.01 ± 0 (*n* = 6)	0.02 ± 0.01 (*n* = 6)	0.02 ± 0 (*n* = 6)
10 dpc	(*n* = 0)	0.02 ± 0.01 (*n* = 2)	0.01 (*n* = 1)	0.01 ± 0 (*n* = 6)	0.02 ± 0.01 (*n* = 6)	0.02 ± 0.01 (*n* = 6)
13 dpc	(*n* = 0)	0.02 ± 0.01 (*n* = 2)	0.02 (*n* = 1)	0.02 ± 0 (*n* = 6)	0.02 ± 0.01 (*n* = 6)	0.02 ± 0.01 (*n* = 6)
15 dpc	(*n* = 0)	0.02 ± 0.01 (*n* = 2)	0.02 (*n* = 1)	0.02 ± 0 (*n* = 6)	0.02 ± 0.01 (*n* = 6)	0.02 ± 0.01 (*n* = 6)

n: number of surviving piglets per day in each group.
CTL: control, non-immunized group; rTbpA: group immunized with a recombinant TbpA fragment; rTbpB: group immunized with a recombinant TbpB fragment; NPAPTim: group immunized with native proteins with affinity to porcine transferrin administered intramuscularly; NPAPTit: group immunized with native proteins with affinity to porcine transferrin administered intratracheally; PG: group immunized with Porcilis-Glässer (Intervet, Spain).

Table 4
Mean ± standard deviation of the serum concentration of apolipoprotein A-I (ApoA-I) in each experimental group after infection with 3×10^8 CFU of *H. parasuis*, serovar 5, Nagasaki strain.

Day post-challenge (dpc)	Concentration (mg/ml) apolipoprotein A-I in each treatment group					
	CTL	rTbpA	rTbpB	NPAPTim	NPAPTit	PG
Challenge	2.77 ± 0.11 (*n* = 4)	3.52 ± 0.32 (*n* = 6)	3.39 ± 0.53 (*n* = 5)	3.49 ± 0.58 (*n* = 6)	3.22 ± 0.29 (*n* = 6)	3.33 ± 0.24 (*n* = 6)
1 dpc	2.12 ± 0.55 (*n* = 4)	2.81 ± 0.78 (*n* = 6)	3.15 ± 0.25 (*n* = 5)	2.98 ± 0.26 (*n* = 6)	2.90 ± 0.41 (*n* = 6)	3.13 ± 0.21 (*n* = 6)
2 dpc	1.09 ± 0.26 (*n* = 3)	2.03 ± 1.10 (*n* = 4)	1.39 ± 0.95 (*n* = 4)	3.45 ± 0.23 (*n* = 6)	3.16 ± 0.12 (*n* = 6)	3.58 ± 0.37 (*n* = 6)
3 dpc	(*n* = 0)	2.31 ± 0.76 (*n* = 3)	1.35 ± 1.86 (*n* = 2)	2.95 ± 0.45 (*n* = 6)	3.44 ± 0.45 (*n* = 6)	3.33 ± 0.32 (*n* = 6)
4 dpc	(*n* = 0)	4.44 ± 0.58 (*n* = 2)	3.82 (*n* = 1)	3.52 ± 0.33 (*n* = 6)	3.45 ± 0.46 (*n* = 6)	3.47 ± 0.24 (*n* = 6)
5 dpc	(*n* = 0)	3.56 ± 0.37 (*n* = 2)	4.03 (*n* = 1)	3.55 ± 0.38 (*n* = 6)	3.47 ± 0.30 (*n* = 6)	3.39 ± 0.22 (*n* = 6)
6 dpc	(*n* = 0)	3.66 ± 0.23 (*n* = 2)	4.72 (*n* = 1)	3.77 ± 0.22 (*n* = 6)	3.32 ± 0.40 (*n* = 6)	3.71 ± 0.16 (*n* = 6)
7 dpc	(*n* = 0)	3.30 ± 0 (*n* = 2)	3.50 (*n* = 1)	3.41 ± 0.44 (*n* = 6)	3.05 ± 0.38 (*n* = 6)	3.34 ± 0.25 (*n* = 6)
10 dpc	(*n* = 0)	3.98 ± 0.38 (*n* = 2)	3.30 (*n* = 1)	4.26 ± 0.26 (*n* = 6)	4.39 ± 0.61 (*n* = 6)	3.77 ± 0.31 (*n* = 6)
13 dpc	(*n* = 0)	3.73 ± 1.26 (*n* = 2)	4.78 (*n* = 1)	4.54 ± 0.28 (*n* = 6)	3.92 ± 1.32 (*n* = 6)	3.48 ± 0.62 (*n* = 6)
15 dpc	(*n* = 0)	4.16 ± 0.88 (*n* = 2)	5.11 (*n* = 1)	5.11 ± 1.96 (*n* = 6)	7.39 ± 2.05 (*n* = 6)	4.90 ± 0.53 (*n* = 6)

n: number of surviving piglets per day in each group.
CTL: control, non-immunized group; rTbpA: group immunized with a recombinant TbpA fragment; rTbpB: group immunized with a recombinant TbpB fragment; NPAPTim: group immunized with native proteins with affinity to porcine transferrin administered intramuscularly; NPAPTit: group immunized with native proteins with affinity to porcine transferrin administered intratracheally; PG: group immunized with Porcilis-Glässer (Intervet, Spain).

ences were recorded for NPAPTim, NPAPTit and PG groups. The response produced by ApoA-I was significantly lower in CTL than in the remaining five vaccinated groups, as well as in rTbp than in NPAPTit and PG piglets ($P < 0.005$ in all cases).

4. Discussion

APP response has widely been used for monitoring the progression of several infectious diseases, as well as for diagnosis of them, evaluation of antibiotic therapy and determination of vaccination effectiveness (Carroll et al., 2004; Petersen et al., 2004). More concretely, a correlation between disease and increased values of positive APPs (Hall et al., 1992; Francisco et al., 1996; Heegaard et al., 1998; Carpintero et al., 2007; Grau-Roma et al., 2009; Skovgaard et al., 2009; Gómez Laguna et al., 2010; Saco et al., in press) or reduced values of negative APPs (Campbell et al., 2005; Sorensen et al., 2006; Carpintero et al., 2007) has been published in porcine infectious diseases, but only one investigation on the kinetics of acute phase response to Glässer's disease, also conducted by our investigation group, has been reported until now (Martín de la Fuente et al., 2010). The characterization of the acute-phase reaction developed by an experimental *H. parasuis* infection has been improved in the present study, and the response induced by different subunit vaccines based on OMPs during a longer time period has been compared, recording a higher number of measurements of pig MAP, HPG, CRP and ApoA-I.

The APP levels observed in our study at challenge day (that is, before *H. parasuis* infection) for pig MAP, HPG and CRP were in agreement with those recently published (Diack et al., 2011) as baseline concentrations in a high health boar population, with reference ranges of 0.32–2.9 mg/ml for pig MAP, 0.01–1.31 mg/ml for HPG, and 0.0036–0.183 mg/ml for CRP; ApoA-I was not measured in that investigation. Even so, the values obtained by us for pig MAP and HPG (Tables 1 and 2) were substantially lower than those calculated for growing 8-week pigs (0.94 and 0.85 mg/ml, respectively) (Piñeiro et al., 2009), or than that reported for pig MAP in 7- to 8-month boars (median of

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1.1 mg/ml) (Diack et al., 2011). These differences could be attributed to several causes, such as age, sex, breed, housing and/or management conditions (Piñeiro et al., 2009; Saco et al., in press; Diack et al., 2011). In this way, APP concentration differences between lines are considered to be genetic and related to polymorphisms in genes controlling APP production, either the genes themselves or those involved in control mechanisms such as the cytokine genes (Diack et al., 2011).

A noticeable increase or decrease (depending on the APP) of the acute-phase response was clearly observed among the piglets infected with *H. parasuis*, but not previously immunized, during the two following days after challenge compared to it, and until death, thus rendering evident the severity of the experimental infection. This variation was comparable to that found for HPG and CRP in a report (Martín de la Fuente et al., 2010) in which a higher *H. parasuis* concentration (5×10^9 CFU instead of 3×10^8 CFU) of Nagasaki strain was inoculated to colostrum-deprived, 13-week-old pigs; however, the variation was considerably greater in the previous study (Martín de la Fuente et al., 2010) for pig MAP (until 27 times higher the mean level detected at 3 dpc compared to that of challenge) and ApoA-I (about four times lower the mean value measured at 1 dpc in comparison to that of infection).

The protection afforded by the two rTbp formulations was partial (probably because of the short length of the protein fragments selected and/or the scarce exposition of their epitopes to the immune response – Frandoloso et al., 2011), and four of the piglets in each group died four days after challenge. Even so, the APP response in the surviving animals in both groups was rather comparable to that of the completely protected groups, while that of dead piglets was in accordance to that found in CTL animals. This different result, depending on the survival or death of the piglets in these groups, matched with that seen for colostrum-deprived pigs, first immunized with an OMP-based vaccine or inoculated with a sublethal dose of *H. parasuis* Nagasaki strain and then challenged with 5×10^9 CFU of the same strain (Martín de la Fuente et al., 2010). However, the pig MAP, CRP and ApoA-I concentrations measured 2 dpc showed significant differences compared to those of infection in the previous report (Martín de la Fuente et al., 2010), while they were already detected in the present study the following day after challenge.

The three groups vaccinated that resulted in an effective protection against Glässer's disease (NPAPTim, NPAPTit and PG) clearly showed a reduction in the duration and intensity of clinical signs, macroscopic lesions and APP response, which was in close agreement with the results obtained formerly (Martín de la Fuente et al., 2010) for pigs vaccinated with commercial or non-commercial bacterins. The immunity against *H. parasuis* conferred by NPAPT antigen (irrespective of the inoculation route) or PG commercial bacterin (Martínez, 2011) would have triggered a faster beginning of the adaptive response to this infection, mediated by memory cells, with the further elimination of *H. parasuis*, thus reducing tissue damage, which is one of the main inducers of the APP response. The abnormally elevated concentrations calculated for HPG in one NPAPTim piglet and for CRP in other NPAPTit animal could be explained on the basis of inter-individual differences or the effect of some stressors (Piñeiro et al., 2009) having affected especially to these two piglets, probably during the bleeding management.

CRP is considered as a type I APP, characterized by a faster reaction in response to tissue injury and infection, and mediated by IL 1 and IL 6 (Ramadori and Christ, 1999), but both HPG and pig MAP belong to the type II APPs, resulting in later but longer-lasting responses, which are specifically induced by IL 6, but not by IL 1 or TNF α (González Ramón et al., 2000). However, these differences in APP reaction could not be observed in our study, because the highest concentrations of these three APPs were found 2–3 dpc irrespective of the experimental group.

Taken together our results, the APP response evidences a parallel evolution with that of experimentally induced Glässer's disease, because the duration of changes in APP concentrations and their magnitude correlated to the severity of clinical signs and macroscopic lesions, in according to the previous report published by our group (Martín de la Fuente et al., 2010). In addition, the greatest changes in APPs were measured in the piglets that died because of the infection/challenge with the lethal dose of Nagasaki strain. However, the APP reaction was mainly measured the following days after challenge in surviving piglets, matching with the minor clinical signs detected, and then returned to basal values, while in CTL group and in those rTbp piglets that died both APP response and clinical signs went on until death. A strong correlation between disease ongoing and APP kinetics was previously reported for other bacterial infections, like those induced by Gram-negative (*A. pleuropneumoniae* – Hall et al., 1992; Hulten et al., 2003; Skovgaard et al., 2009, *Pasteurella multocida* – Francisco et al., 1996, or *Yersinia enterocolitica* – Platt-Samoraj et al., 2009) or Gram-positive organisms (*Streptococcus suis* – Sorensen et al., 2006), and also by viral infections, like African swine fever, Aujeszky's disease (Carpintero et al., 2007), PRRS (Gómez Laguna et al., 2010), or the postweaning multisystemic wasting syndrome induced by porcine circovirus type 2 (Grau-Roma et al., 2009).

5. Conclusion

The present results indicate that the serum concentration of the four APPs here studied may be useful, along with other clinical parameters, for monitoring Glässer's disease status. In addition, pig MAP, HPG, CRP and ApoA-I seem to be useful and rapid biomarkers (because they can be tested the following days after challenge) for the evaluation of the efficacy of the vaccines developed against Glässer's disease caused by *H. parasuis* Nagasaki strain.

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