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Immunoproteomic analysis of the protective response obtained with subunit and commercial vaccines against Glässer's disease in pigs

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ABSTRACT

An immunoproteomic analysis of the protective response of subunit and commercial vaccines in colostrum-deprived pigs against Glässer's disease was carried out. A mixture of proteins with affinity to porcine transferrin (PAPT) from *Haemophilus parasuis* Nagasaki strain (serovar 5) was inoculated intramuscularly ($PAPT_M$) and intratracheally ($PAPT_{Cp}$), along with a commercial bacterin. PAPT were separated using 2 dimensional electrophoresis (2DE) gels and with them, 2DE Western blots were carried out. A total of 17 spots were identified as positive with sera of pigs from any of the three vaccinated groups, the highest number of immunoreactive proteins being detected in those having received $PAPT_{Cp}$. Among them, six proteins (FKBP-type peptidyl-prolyl cis-trans isomerase, neuraminidase exo-α-sialidase, xanthine-guanine phosphoribosyl transferase, CMP-N-acetylneuraminic acid synthetase, phenylalanyl-tRNA synthetase and glyceraldehyde 3-phosphate dehydrogenase) were found to be novel immunogens in *H. parasuis*. These proteins showed a high potential as candidates in future subunit vaccines against Glässer's disease. The three experimental groups developed specific systemic total IgG (IgGt), IgG1, IgG2 and IgM antibodies after immunizations. In addition, those receiving $PAPT_{Cp}$ yielded a serum IgA response.

1. Introduction

Haemophilus parasuis, a gram-negative bacillus classified in the *Pasteurellaceae* family, is considered a commensal organism of the upper respiratory tract of healthy pigs, but also the etiological agent of porcine polyserositis, meningitis and arthritis syndrome known as Glässer's disease (Amano et al., 1994). *H. parasuis* has also been isolated from cases of acute pneumonia without polyserositis and more rarely from myositis of the masseter muscles. Historically, Glässer's disease was a sporadic disorder of stressed or immunosupressed pigs. However, with the recent changes in production methods, *H. parasuis* has become in the last few years one of the main causes of nursery morbidity and mortality in modern swine husbandry, resulting in significant economic losses worldwide and in a detriment of porcine welfare (Oliveira and Pijoan, 2004).

Fifteen serovars of this organism have been described thus far by means of an immunodiffusion test (Kielstein and Rapp-Gabrielson, 1992). However, a large number of non-typable isolates are frequently reported (Raffie and Blackall, 2000; del Río et al., 2003). Though serovar was initially used as indicator of virulence, no clear correlation has been demonstrated because considerable genetic heterogeneity can be detected, not only between but also within serovars (Oliveira and Pijoan, 2004). Inconsistent

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cross-protection among serovars is one of the major problems for the control of Glässer's disease by means of bacterins (Nielsen, 1993; Rapp-Gabrielson et al., 1997; Bak and Riising, 2002). On the other hand, several *H. parasuis* proteins, and especially outer membrane proteins (Omps), have resulted in a good immunogenicity with diverse rates of protection, and their potentials to be vaccine candidates have been proposed (Martín de la Fuente et al., 2009b; Zhang et al., 2009; Zhou et al., 2009; Olvera et al., 2011). In this respect, the role of the antibody response developed against Omps after immunization has been considered a critical factor to control Glässer's disease (Miniats et al., 1991; Martín de la Fuente et al., 2009b; Olvera et al., 2011).

A revolution in the design of vaccines has emerged from the use of postgenomic technologies (Serruto and Rappuoli, 2006). A lot of information about immunogenic components can be derived from proteomics coupled to Western blotting, namely immunoproteomics, which has been successfully applied for the discovery of antigens from various bacterial pathogens, such as *Francisella tularensis* (Havlasová et al., 2002), *Streptococcus pyogenes* (Rodríguez-Ortega et al., 2006) or *Neisseria meningitidis* (Hsu et al., 2008).

In order to adapt to the iron-restricted environment of the host, *H. parasuis* organisms have iron acquisition pathways mediated by surface receptors that specifically bind transferrin from the host: transferrin-binding protein A (TbpA) and TbpB (del Río et al., 2005). These Tbps play a crucial role in virulence (Gray-Owen and Schryvers, 1996) and, for this reason, their potential utility as vaccine immunogens has been demonstrated in relevant human or animal pathogens, such as *H. influenzae* (Loosmore et al., 1996) or *A. pleuropneumoniae* (Rossi-Campos et al., 1992) respectively, both belonging, as *H. parasuis*, to the *Pasteurellaceae* family. In an earlier study (Frandoloso et al., 2011), the immunoprotective effect of recombinant fragments designed in our laboratory from Tbp A (Martínez et al., 2010) and TbpB (del Río et al., 2005) obtained from *H. parasuis* serovar 5 was examined, but only a partial protection was observed. However, a strong protection was achieved when native proteins with affinity to porcine transferrin (PAPT) from this same serovar were used as immunogen (Frandoloso et al., 2011).

As a continuance of that report (Frandoloso et al., 2011), the immunoproteomic analysis of the protective response developed by these vaccines based on PAPT has been studied and it has been compared to the response afforded by one commercial bacterin also based on serovar 5.

2. Materials and methods

2.1. Vaccines and immunization schedule

$PAPT_M$ vaccine, containing 400 μg of a mixture of proteins from Nagasaki strain (serovar 5) with affinity to porcine transferrin (PAPT), was inoculated intramuscularly in the neck region. This antigen was adjuvanted with Montanide IMS 2215 VG PR (a mineral oil which has been successfully used as adjuvant for potentiating the immune response induced by proteins inoculated intramuscularly -Martín de la Fuente et al., 2009a-) in a 1:4 ratio. $PAPT_{Cp}$ vaccine, also containing 400 μg of the same antigen, was potentiated with neuraminidase from *Clostridium perfringens* type VI (100 μ/ml vaccine -Frandoloso et al., 2011-) and inoculated intratracheally. This glycoprotein was used as potentiator because it attracts antigen-presenting cells to the inoculation site (Stamatos et al., 2003; Kuroiwa et al., 2009). Finally, PG vaccine consisted of a commercial bacterin (Porcilis Glässer, Intervet), composed of inactivated *H. parasuis*, strain 4800 (serovar 5), which was inoculated intramuscularly in the neck region.

A total of 22 colostrum-deprived piglets (this animal model was used in order to avoid the colonization of upper respiratory tract of suckling piglets being in contact with their sows, Blanco et al., 2004) were randomly assigned to three groups of six animals each and the remaining four animals were maintained as infection control group. The $PAPT_M$, $PAPT_{Cp}$ and PG groups received $PAPT_M$, $PAPT_{Cp}$ or PG vaccines respectively. All groups were challenged intratracheally with 3×10^8 CFU of *H. parasuis* Nagasaki strain. The immunization and infection schedule is shown in Fig. 1. Blood samples were collected by venipuncture from the jugular vein before the first immunization (day 28) and the second one (day 36 for PG group or day 49 for PAPT groups), before challenge (day 63), and ten days after it

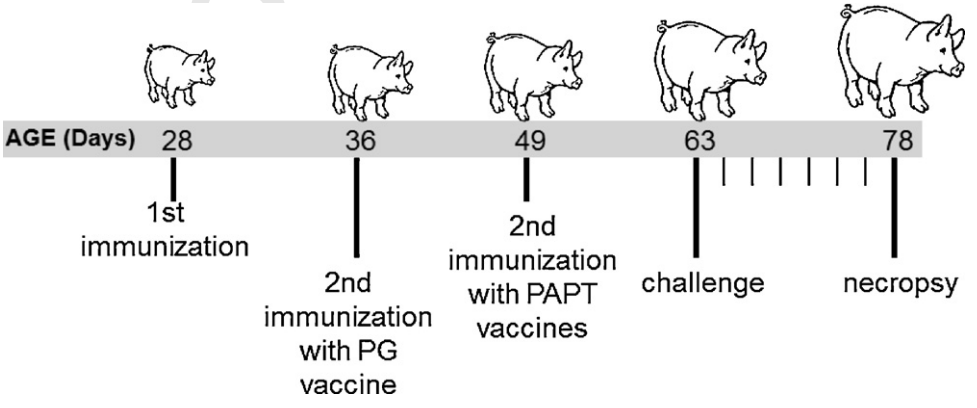

Fig. 1. Immunization, infection and necropsy schedule for colostrum-deprived pigs before and after challenge with *H. parasuis*.

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(day 73), and serum was obtained. The sera from the animals in each group were pooled at each collection time and used for the identification of immunogenic *H. parasuis* proteins in Western blotting of 2DE gels. In ELISAs, the sera from each animal and group were used individually at each time.

2.2. Antigen extract and 2D gel electrophoresis

The PAPT extract was purified by gel filtration on a fast-protein liquid chromatography-CN-Br-activated Sepharose 4B (GE Healthcare) column. Once purified as described previously (Frandoloso et al., 2011), the PAPT extract was subjected to 2D gel electrophoresis. Isoelectric focusing (IEF) was conducted using commercial nonlinear Immobiline Drystrips (18 cm, pH 3–10, GE Healthcare) and the IPGphor unit (GE Healthcare). The immobilized pH gradient Drystrips were rehydrated overnight (passive rehydration) by applying 350 μl of rehydration buffer containing 250 μg of protein. The following voltage profile was used for IEF: 500 V for 3 h; 500–1000 V for 6 h; 1000–8000 V for 3 h, and 8000 V for 10.5 h. After equilibration of IPG strips, the proteins were resolved in the second dimension by electrophoresis in homogenous 10% T, 1.6% C (piperazine diacrylamide was used as a crosslinker) polyacrylamide gels (1.5 mm thick). Electrophoresis was conducted overnight at 1 W/gel constant electrophoresis ETAM system DALT (Amersham). To visualize the separated proteins, gels were stained with colloidal Coomassie blue and were scanned with a GS-690 Imaging Densitometer (Bio-Rad).

2.3. Western blotting of 2DE gels

Proteins from the twin, non-stained gels, were transferred to a nitrocellulose membrane (Amersham) for 3 h at 250 mA at 4 °C in Towbin buffer (Towbin et al., 1979) by using Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). After transfer, the membrane was blocked for 2 h with 5% skim milk in TBS solution. After rinsing with 0.05% Tween-20 in TBS (TTBS), the membrane was incubated with serum at a dilution of 1:100 in TTBS containing 1% skim milk for 1 h at room temperature on a gentle shaker. The membrane was then rinsed and incubated with rabbit anti-pig IgG-peroxidase (Sigma), at a dilution of 1:10,000 in TTBS containing 1% skim milk for 1 h. The membrane was then washed with TBS and developed with substrate (AP Conjugate Substrate Kit, Bio-Rad) until optimum color was developed. Each membrane was stripped and reused (up to two times) with the following buffer: 100 mM 2-mercaptoethanol, 2% (w/v) SDS, 62.5 mM Tris-HCl pH 6.7. The immunoreactivity of each spot that gave positive signal in Western blot analysis was verified through their excision from 2D gels followed by dot-blotting. In each group, Western blot analyses were carried out in duplicate.

Western blot analyses were carried out for each of the three vaccinated groups comparing four times: day 28, day 36 for PG group or day 49 for the $PAPT_M$ and $PAPT_{Cp}$ groups, day 63 and day 73.

2.4. Protein identification

Protein identification was done at the Proteomic Service Facility from the University Complutense of Madrid, a member of ProteoRed Network. The protein spots of interest were manually excised from preparative Coomassie gels by biopsy punches, placed in Eppendorf tubes, and washed twice with double-distilled water. Proteins for analysis were in-gel reduced, alkylated, and digested with bovine trypsin (12.5 ng/μl, sequencing grade; Roche), as described previously (Sechi and Chait, 1998). After digestion, the supernatant was collected and 1 μl was spotted on a MALDI target plate and allowed to air dry for 10 min at room temperature. Subsequently, 0.5 μl of matrix (3 mg/ml CHCA, Sigma) diluted in 0.1% TFA-CAN/H_2O (1:1 v/v) was added to the dried peptide digest spots and allowed to air dry for another 5 min at room temperature (Martínez López et al., 2008).

The samples were analysed with a 4800 Plus MALDI-TOF/TOF Analyzer mass spectrometer (Applied Biosystems). All mass spectra were internally calibrated by using peptides from the autodigestion of trypsin. The analysis by MALDI-TOF-TOF mass spectrometry produced peptide mass fingerprints, and the peptides observed can be collated and represented as a list of monoisotopic molecular weights. For the protein identification, a house database with the entries of *H. parasuis* from NCBI was made. This database, *H. parasuis* 100118, containing 10,314 sequences and the mass spectra, were automatically searched by using a local license of Mascot 2.1 from Matrix Science through the Global Protein Server v3.5 (Applied Biosystems). The search parameters for peptide mass fingerprints were set as follows: fixed and variable modifications were considered (carbamidomethyl cystein and oxidized methionine), allowing for one missed cleavage site; precursor tolerance 50 ppm; peptide charge 1. For combined analysis, Mascot search parameters were the same described above except for peptide tolerance, 100 ppm and MS/MS tolerance, 0.3 Da. In all protein identification, the probability scores were greater than the score fixed by Mascot as significant with a P value <0.05.

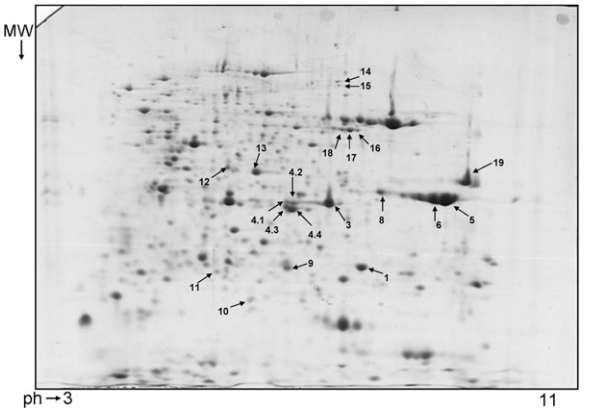

Fig. 2. Coomassie stained 2-DE gel of proteins with affinity to porcine transferrin from *H. parasuis*, Nagasaki strain. All the proteins indicated with arrows are those detected as immunogenic in the different Western blots and represent spots identified by MS/MS analysis (Table 2).

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2.5. Antigen preparation and ELISAs

Three antigens were compared: (a) whole-cells of *H. parasuis* Nagasaki strain (Solano-Aguilar et al., 1999; Martín de la Fuente et al., 2009b); (b) Omps, which were extracted from cultures of the same strain grown under iron restrictive conditions, and consisted of the cell-free culture supernatant obtained after treatment with 0.075% sodium deoxycholate (Goethe et al., 2001; Martín de la Fuente et al., 2009b), and (c) PAPT extract (Frandoloso et al., 2011).

Total IgG (IgGt), IgG1, IgG2, IgM and IgA responses were measured in serum. All ELISA steps were carried out at 37 °C, and reaction volume was of 100 μl per well. The plates were coated with these antigens diluted in carbonate buffer (10 μg/well). Sera (1:100) of each pig from each experimental group were incubated for 60 min, and peroxidase-conjugated, goat anti-swine IgM (Serotec), IgGt or IgA (Sigma–Aldrich), or mouse anti-swine IgG1 or IgG2 (Sigma–Aldrich) were diluted according to manufacturer's instructions, and incubated for 60 min. The remaining ELISA steps were described earlier (Martín de la Fuente et al., 2009b). A serum was considered positive when its optical density (OD) was twice as high as that of mean at day 28 (before the first immunization) ± SD. For each group, a cut-off [(mean of all sera at day 28 ± SD) × 2] was also established. Serum samples at different collection times were run in triplicate. ODs were analysed by the SPSS statistical program, and normality of the data was confirmed using the Kolmogorov–Smirnov test. A Tukey-Kramer test was used for comparison between ODs, at before and after the first immunization, after the first and second immunizations, and after the second immunization and ten days post-challenge. Significance was set at $P \leq 0.005$.

3. Results

3.1. Clinical signs

Two piglets from control group died between 1 and 2 days post-challenge (dpc), and the two others died between 2 and 3 dpc. These animals showed increased recta temperatures (until 3 °C above those measured at challenge). Clinical signs suspicious of Glässer's disease (swollen joints, limb uncoordination, dyspnea) were seen. The piglets belonging to $PAPT_M$, $PAPT_{Cp}$ and PG groups survived the challenge until the end of the experiment. No appreciable clinical signs were seen in these three vaccinated groups, and no adverse reactions were detected. Clinical signs and pathological findings have been also described broadly (Frandoloso et al., 2011).

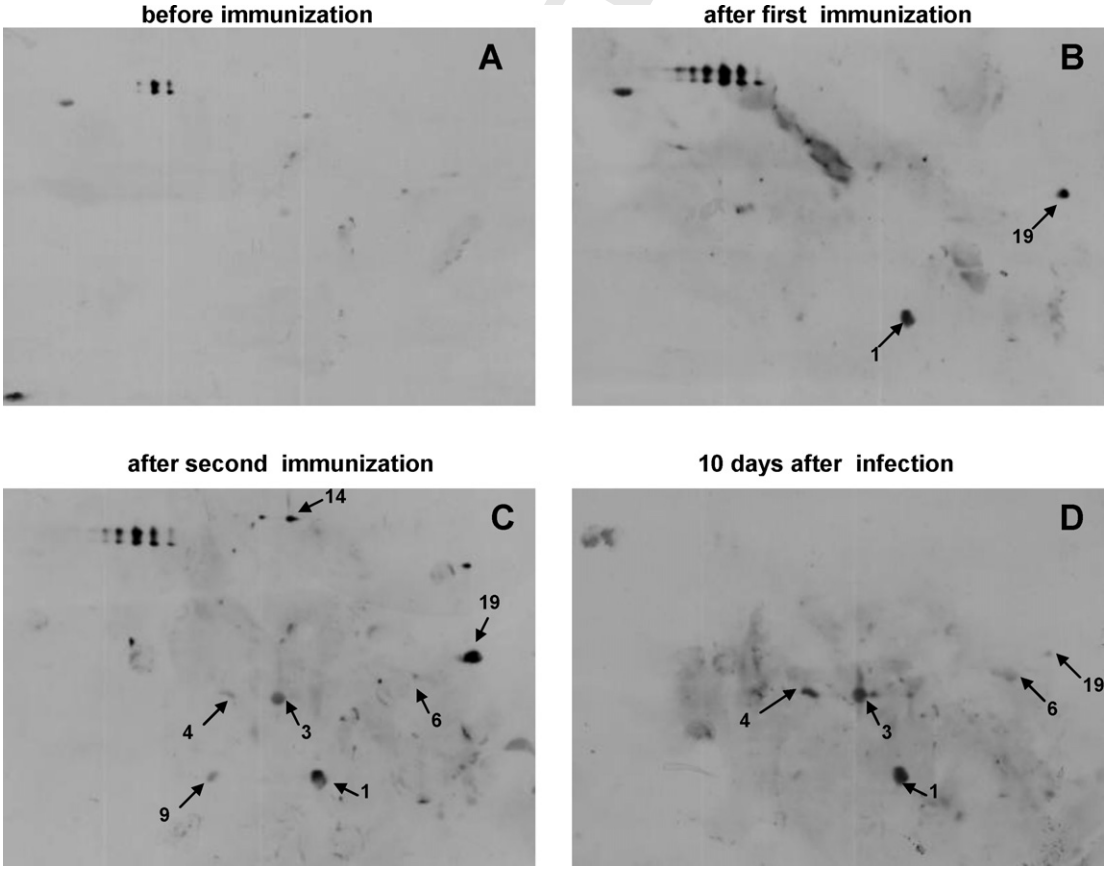

Fig. 3. 2-DE Western blots with the sera of the animals immunized with proteins with affinity to porcine transferrin administered intramuscularly ($PAPT_M$ group).

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3.2. Detection and identification of immunoreactive proteins

Native Omps with affinity to porcine transferrin from *H. parasuis*, Nagasaki strain, were previously separated on a 1DE gel, and nine proteins were identified (Frandoloso et al., 2011). The present investigation describes this same sample, but now separated in a 2DE gel, in which the presence of about 200 protein spots could be detected (Fig. 2). This 2DE gel served as a reference map in order to identify the immunogenic proteins observed in the 2DE Western blots.

The Western blots of the immunized groups at different times are shown in Figs. 3–5. The immunogenic protein spots identified by MALDI-TOF-TOF MS are indicated by arrows in these figures and the differences in the detection of protein spots are listed in Table 1. A total of 19 spots could be detected and identified as immunogenic in the different immunoblots. They were named from 1 to 19, but spots 2 and 7 are missing because they could not be identified, and spot 4 was seen as one sole spot on the 2DE Western blots, but four isoforms were identified on the 2DE gel (Fig. 2). Therefore, 17 different proteins were identified (Table 2 and Fig. 2). When the 2DE Western blots were compared irrespective of the vaccinated group, an increase of immunoreactive proteins could be seen after the first (Figs. 3–5B), but especially after the second immunization (Figs. 3–5C). However, a lesser or similar number of spots were detected ten days after challenge (Figs. 3–5D).

3.2.1. $PAPT_M$ group

In the 2DE Western blots belonging to $PAPT_M$ group (Fig. 3), FKBP-type peptidyl-prolyl cis-trans isomerase, chelated ABC-transporter periplasmic binding protein, periplasmic iron-binding protein, protective surface antigen D15 precursor, and Omp2 were detected as immunogenic (Table 2). Omp2 exhibited the most intense reaction, concretely after the second immunization (Fig. 3C). The antibodies present in sera after the second immunization were those reacting with the highest number of proteins, while some of them could not be identified with the sera collected ten days after challenge (Table 1 and Fig. 3C and D).

3.2.2. $PAPT_{Cp}$ group

The greatest number of immunoreactive proteins was detected in this group (Table 1 and Fig. 4). Some of the isoforms here observed (periplasmic iron-binding protein,

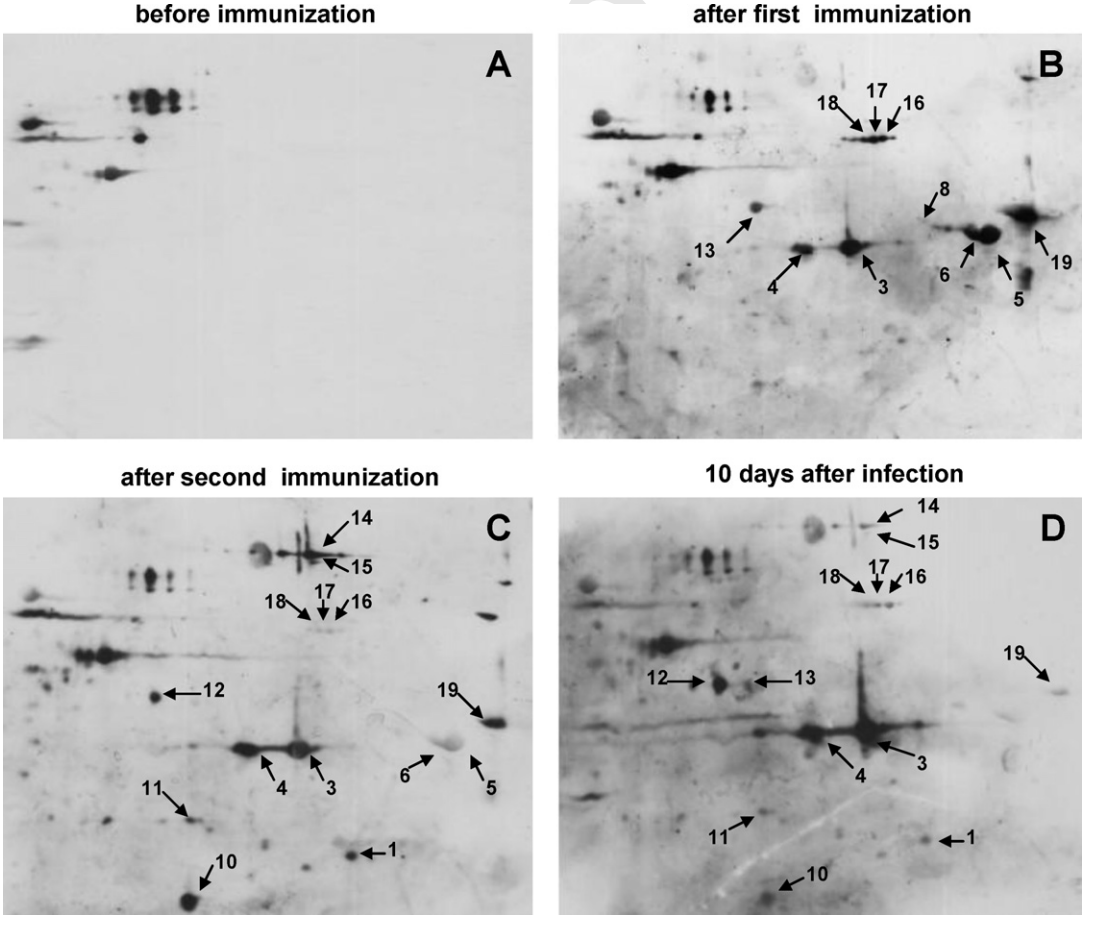

Fig. 4. 2-DE Western blots with the sera of the animals immunized with proteins with affinity to porcine transferrin administered intratracheally ($PAPT_{Cp}$ group).

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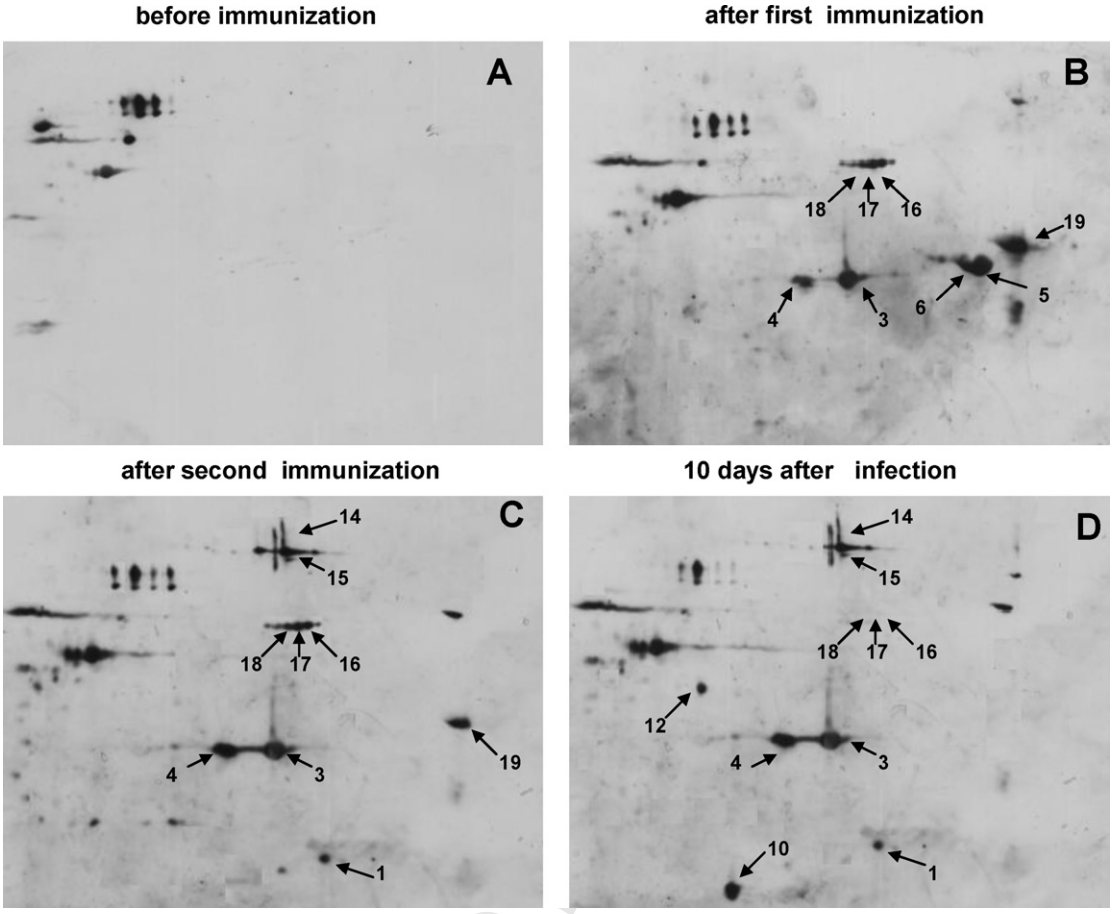

Fig. 5. 2-DE Western blots with the sera of the animals immunized intramuscularly with the commercial bacterin (PG group).

chelated ABC-transporter and catalase) had been already detected in the PAPT extract (Frandoloso et al., 2011). However, other isoforms which did not appear in it (surely because of their scarce concentration), such as FKBP-type peptidyl-prolyl cis-trans isomerase, protective surface antigen D15 precursor, neuraminidase exo-α-sialidase, protective surface antigen D15 precursor and Omp2 were identified in $PAPT_{Cp}$ and $PAPT_{M}$ groups. In addition, other proteins were detected exclusively in $PAPT_{Cp}$ group: CMP-N-acetylneuraminic acid syntethase and glyceraldehyde 3-phosphate dehydrogenase (Tables 1 and 2). Chelated ABC-transporter, periplasmic binding protein exhibited the highest reaction at the three times compared (Fig. 4B–D and Table 1).

3.2.3. PG group and comparison between groups

There were less immunogenic proteins detected from PG group than from $PAPT_{Cp}$ group, but more than from $PAPT_{M}$ group (Table 1). Concretely, FKBP-type peptidyl-prolyl cis-trans isomerase, chelated ABC-transporter periplasmic binding protein, periplasmic iron-binding protein, xanthine-guanine phosphoribosyl transferase, phenylalanyl tRNA synthetase subunit α, protective surface antigen D15 precursor, neuraminidase exo-α-sialidase, catalase and Omp2 were found in PG group. As for the $PAPT_{Cp}$ group, chelated ABC-transporter, periplasmic binding protein was that rendering the highest reaction (Fig. 5B–D). It was followed by catalase and Omp2, this latter giving a negative result ten days after infection (Fig. 5D).

Comparing $PAPT_{Cp}$, $PAPT_{M}$ and PG groups, the intratracheal challenge did not boost the response to the intramuscular vaccinated pigs ($PAPT_{M}$), but did so in the PG group. Intratracheal priming and booster ($PAPT_{Cp}$) seemed most efficient for a proper response to intratracheal challenge.

3.3. Sera titration by ELISAs

The ODs for a group inoculated with Montanide instead of vaccines and with RPMI 1640 instead of *H. parasuis* (negative control group) were always under 0.05. Antibody response after challenge could not be measured in the infection control group because all pigs died between 24 and 48 h after it.

The ODs before the first immunization were below 0.1 in the three vaccinated groups (Figs. 6–8). $PAPT_{M}$ group became seropositive for IgGt and IgM just before the second immunization (Fig. 6A and D). Significant differences ($P \leq 0.005$) compared to before the first vaccination were observed with any of the antigens for all isotypes.

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Table 1
Detection of immunogenic proteins from *H. parasuis*, Nagasaki strain, at different times in the three vaccinated groups.

Spot number	Protein	Experimental group											
		$PAPT_M$[a]				$PAPT_{Cp}$				PG			
		0[b]	1	2	inf	0	1	2	inf	0	1	2	inf
1	FKBP-type peptidyl-prolyl cis-trans isomerase		+	+	+			+	+			+	+
9	FKBP-type peptidyl-prolyl cis-trans isomerase			±									
3	Chelated ABC-transporter, periplasmic binding protein			+	+		+++	+++	+++		+++	+++	+++
4	Chelated ABC-transporter, periplasmic binding protein			±	+		+++	+++	+++		+++	+++	+++
5	Periplasmic iron-binding protein						+++	±			+++		
6	Periplasmic iron-binding protein			±	±		++	±			++		
8	Periplasmic iron-binding protein						±						
10	Xanthine-guanine phosphoribosyl transferase							+++	+				+++
11	CMP-N-acetylneuraminic acid synthetase							+	+				
12	Phenylalanyl tRNA synthetase subunit α [c]							++	+++				++
13	Glyceraldehyde 3-phosphate dehydrogenase						++		+				
14	Protective surface antigen D15 precursor			+				++	±			++	++
15	Neuraminidase exo-α-sialidase nanH [c]							+++	±			+++	+++
16	Catalase						+++	±	+		+++	+++	±
17	Catalase						+++	±	+		+++	+++	±
18	Catalase						+++	±	+		+++	+++	±
19	Outer membrane protein 2		+	++	±		+++	++	±		+++	+++	

±, +, ++, +++, indicate relative intensity -low to high- of the spots in Western blots.
[a] $PAPT_M$, group immunized with a mixture of proteins with affinity to porcine transferrin administered intramuscularly; $PAPT_{Cp}$, group immunized with a mixture of proteins with affinity to porcine transferrin administered intratracheally; PG, group immunized with Porcilis-Glässer (Intervet, Spain).
[b] 0, before immunization; 1, after the first immunization; 2, after the second immunization; inf, ten days after infection.

These values suffered a significant increase ($P < 0.005$) after the second immunization, but after challenge, the ODs remained in similar values as before infection or even decreased slightly. Responses were similar for IgG1 and IgG2, and negative ODs were recorded for IgA (<0.04, Fig. 6E).

IgGt, IgG1, IgG2 and IgM measured in $PAPT_{Cp}$ group resulted in significant increases ($P < 0.005$) after the first and second immunizations (Fig. 7). The highest values for IgGt, IgG1 and IgG2 were reached at day 63, and these ODs maintained or mostly reduced ten days after challenge. However, the greatest value for IgM was measured at day 73 (0.50 ± 0.08, Fig. 7D). The piglets became seropositive for IgA after the first immunization, and resulted in the greatest levels after the second one. Then, the ODs fell at day 73 until levels slightly higher than those of the first vaccination. Significant increases were only detected after the first immunization ($P < 0.005$).

In PG group, values significantly greater ($P < 0.005$) were measured for IgGt, IgG1 and IgG2 after the first immunization; antibody response increased after the second vaccination and then, ODs slightly reduced after challenge (Fig. 8A–C). However, the IgM levels increased throughout the study, with significant differences after the first and second vaccinations ($P < 0.005$, Fig. 8D). Finally, seronegative results were found for IgA (Fig. 8E).

4. Discussion

In a previous report (Frandoloso et al., 2011), we developed subunit vaccines based on recombinant Tbp A or B fragments from *H. parasuis*, Nagasaki strain, but the protection afforded by these two molecules was partial, with death of some of animals after challenge, clinical signs, gross and microscopic lesions, and bacterial invasion. In contrast, strain Nagasaki proteins with affinity to porcine transferrin conferred a strong protection against challenge, with only mild histopathological changes and no recovery of *H. parasuis* from any organic location. In the present study, we assessed the immunoproteomic analysis of the protective response produced by this mixture of proteins and we identified for the first time new immunogenic molecules contained in this pool.

The Omp2 is the most abundant protein in the outer membrane of *H. parasuis*. Although it was scarcely detected in the PAPT extract (Frandoloso et al., 2011), this molecule rendered a high response in the immunoproteomic analyses of the three vaccinated groups, as evidenced by spot intensity. This is in agreement with that previously reported for this porin in *H. parasuis* SH0165 strain (Zhou et al., 2009), also belonging to serovar 5. In addition, Zhou et al. (2009) evidenced that Omp2 was expressed *in vivo* and could elicit an immune response. Similarly, other proteins hardly detected in our PAPT immunogen (as, for instance, chelated ABC-transporter, periplasmic binding protein) also rendered a great response in our immunoproteomic study. Furthermore, catalase enzyme was identified both in the PAPT extract which was inoculated to piglets and in the 2-DE Western blots, as three well-identified spots in $PAPT_{Cp}$ and PG groups. This result supports the idea that catalase could play an immunoprotective role in *H. parasuis*.

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Table 2
Immunogenic proteins from *H. parasuis*, Nagasaki strain, with affinity to porcine transferrin identified by MALDI-TOF-TOF mass spectrometer in the different vaccinated groups.

Spot number	Protein	Accession number[a]	Molecular weight (kDa)[b]	Theoretical isoelectric point[b]	Matched/searched peptides[b]	Protein coverage (%)[b]	Score[b]	Antibody response detected in groups[c]
1	FKBP-type peptidyl-prolyl cis-trans isomerase	gi/219691880	26,316	8.74	21/65	57	187	$PAPT_M$, $PAPT_{Cp}$, PG
3	Chelated ABC-transporter, periplasmic binding protein	gi/219691491	32,632	7.74	25/65	87	286	$PAPT_M$, $PAPT_{Cp}$, PG
4.1	Chelated ABC-transporter, periplasmic binding protein	gi/219691491	32,632	7.74	18/49	68	199	$PAPT_M$, $PAPT_{Cp}$, PG
4.2	Chelated ABC-transporter, periplasmic binding protein	gi/219691491	32,632	7.74	20/65	70	207	$PAPT_M$, $PAPT_{Cp}$, PG
4.3	Chelated ABC-transporter, periplasmic binding protein	gi/219691491	32,632	7.74	17/65	62	160	$PAPT_M$, $PAPT_{Cp}$, PG
4.4	Chelated ABC-transporter, periplasmic binding protein	gi/219691491	32,632	7.74	16/65	64	155	$PAPT_M$, $PAPT_{Cp}$, PG
5	Periplasmic iron-binding protein	gi/219691519	37,755	8.80	31/65	68	374	$PAPT_{Cp}$, PG
6	Periplasmic iron-binding protein	gi/219691519	37,755	8.80	33/65	81	385	$PAPT_M$, $PAPT_{Cp}$, PG
8	Periplasmic iron-binding protein	gi/219691519	37,755	8.80	6/18	81	69	$PAPT_{Cp}$
9	FKBP-type peptidyl-prolyl cis-trans isomerase	gi/219691880	26,316	8.74	14/49	48	123	$PAPT_M$
10	Xanthine-guanine phosphoribosyl transferase	gi/219691806	17,910	5.64	7/65	52	64	$PAPT_{Cp}$, PG
11	CMP-N-acetylneuraminic acid synthetase	gi/219690522	24,144	5.58	17/65	76	166	$PAPT_{Cp}$
12	Phenylalanyl tRNA synthetase subunit α[d]	gi/21969114	37,350	5.49	9/70 R.ADHDTFWFDAER.L (62)	22	107	$PAPT_{Cp}$, PG
13	Glyceraldehyde 3-phosphate dehydrogenase	gi/219690484	36,380	5.82	23/65	69	240	$PAPT_{Cp}$
14	Protective surface antigen D15 precursor	gi/167854834	90,241	7.04	34/65	46	310	$PAPT_M$, $PAPT_{Cp}$, PG
15	Neuraminidase exo-α-sialidase nanH[d]	gi/219691444	74,913	7.25	8/71 K.YQLDNHQIAGFIR.Y (3) K.HQDFIER.N (35)	9	53	$PAPT_{Cp}$, PG
16	Catalase	gi/219692447	54,954	6.50	26/65	50	244	$PAPT_{Cp}$, PG
17	Catalase	gi/219692447	54,954	6.50	31/65	50	244	$PAPT_{Cp}$, PG
18	Catalase	gi/219692447	54,954	6.50	13/65	50	244	$PAPT_{Cp}$, PG
19	Outer membrane protein 2	gi/224177693	38,595	9.25	27/78	59	283	$PAPT_M$, $PAPT_{Cp}$, PG

[a] From NCBI protein database.
[b] Molecular weight, isoelectric point, matched peptides, protein coverage and protein score are derived from Mascot result page.
[c] $PAPT_M$, group immunized with a mixture of proteins with affinity to porcine transferrin administered intramuscularly; $PAPT_{Cp}$, group immunized with a mixture of proteins with affinity to porcine transferrin administered intratracheally; PG, group immunized with Porcilis-Glässer (Intervet, Spain).
[d] Proteins identified by PMF combined with fragmentation analysis of peptides. The sequences of the peptides that were identified by TOF/TOF analysis are shown in the matched peptides column and the individual ion score of the fragmented peptides are shown in brackets.

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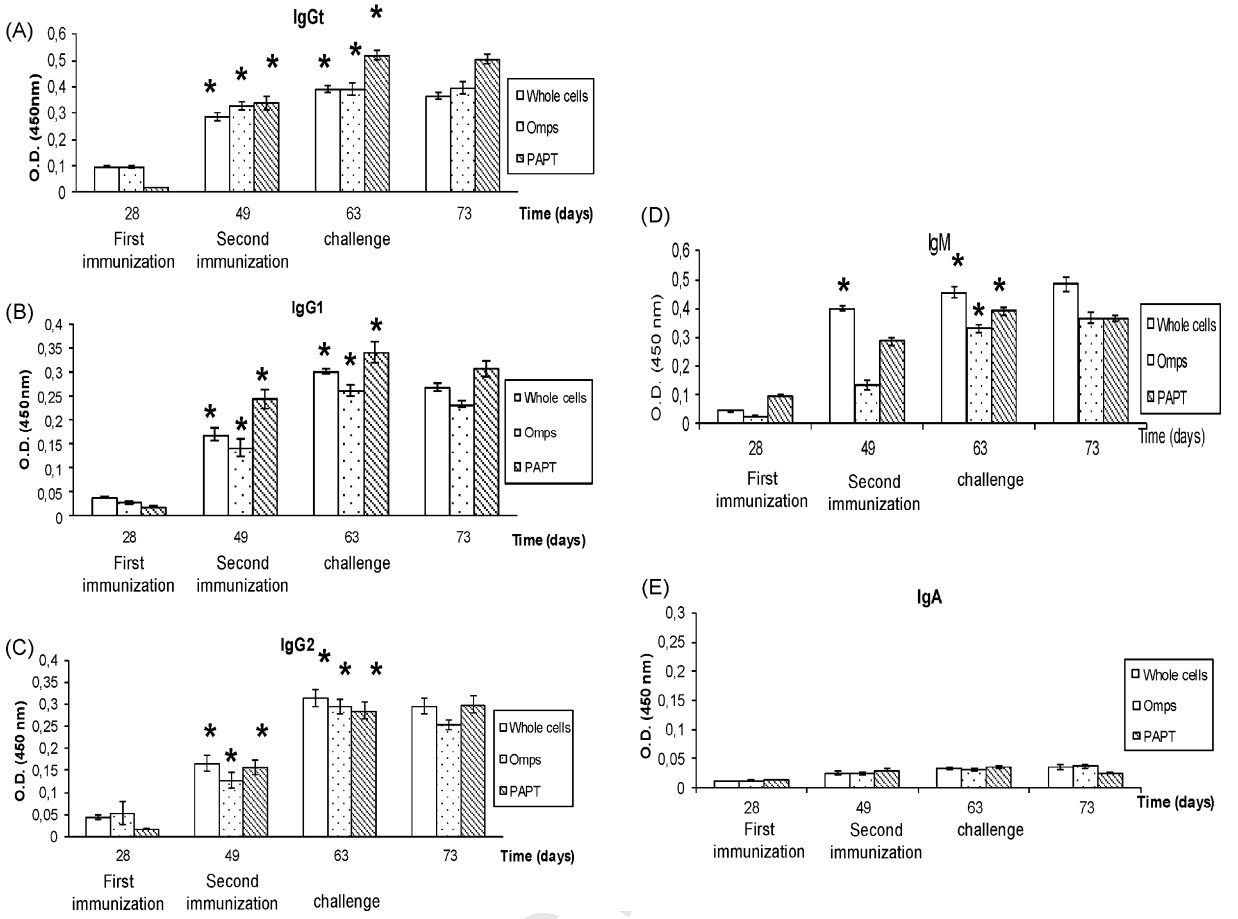

Fig. 6. Levels of immunoglobulins in the animals immunized with proteins with affinity to porcine transferrin administered intramuscularly ($PAPT_M$ group): Mean optical densities at 450 nm ± standard deviation of serum total IgG (IgGt) (A), IgG1 (B), IgG2 (C), IgM (D) and IgA (E) measured at different times for whole-cell, Omp and PAPT antigens. (*) Significant differences ($P < 0.005$) compared to the time before.

Other proteins not being found in this PAPT extract, probably because of their low concentration (such as FKBP-type peptidyl-prolyl cis-trans isomerase, protective surface antigen D15 precursor, neuraminidase exo-α-sialidase, xanthine-guanine phosporibosyl transferase, CMP-N-acetylneuraminic acid synthetase, phenylalanyl-tRNA synthetase and glyceraldehyde 3-phosphate dehydrogenase), also were able to elicit a high immunoreactivity as evidenced by 2-DE Western blots. Therefore, these proteins must be considered as good immunogens, especially the two former ones, because the antibodies developed against them were found simultaneously in the three vaccinated groups. Consequently, after purification and recombination, they might be suitable candidates to prevent Glässer's disease by means of subunit vaccines. Anyway, further studies are required to demonstrate this hypothesis. In this respect, protective surface antigen D15 precursor has been proposed as a good candidate for inclusion in vaccines, not only against *H. parasuis* (Zhou et al., 2009), but also against other *Pasteurellaceae*, for instance, *H. influenzae* (Thomas et al., 2001a), *H. ducreyi* or *Pasteurella multocida* (Thomas et al., 2001b).

Just the opposite, Tbps A and B; chelated ABC-transporter, periplasmic binding protein and elongation factor Tu, which were detected in the PAPT extract (Frandoloso et al., 2011) could not be identified in any of the three vaccinated groups. This result seems to indicate that these molecules would not behave as good immunogens, at least under the conditions in our study, and it could justify the scarce protection obtained by Tbps in our previous investigation (Frandoloso et al., 2011). However, in an earlier report, the cytoplasmic membrane protein ABC-transporter was also identified as immunogen for the first time in *H. parasuis*, along with 14 other proteins from a fraction enriched in Omps; nevertheless, this protein was not selected for further studies about its potential to be vaccine candidate (Zhou et al., 2009). Additionally, ABC-transporter has been previously reported as immunogenic protein in other organisms, such as *Mannheimia haemolytica* (Ayalew et al., 2010), *N. meningitidis* (Williams et al., 2009) or *S. suis* (Zhang et al., 2008). To our knowledge, this is the first time that antigenic properties have been described for the following proteins of *H. parasuis*: FKBP-type peptidyl-prolyl cis-trans isomerase, neuraminidase exo-α-sialidase, xanthine-guanine phosphoribosyl transferase, CMP-N-acetylneuraminic acid synthetase, phenylalanyl-tRNA synthetase and glyceraldehyde 3-phosphate dehydrogenase.

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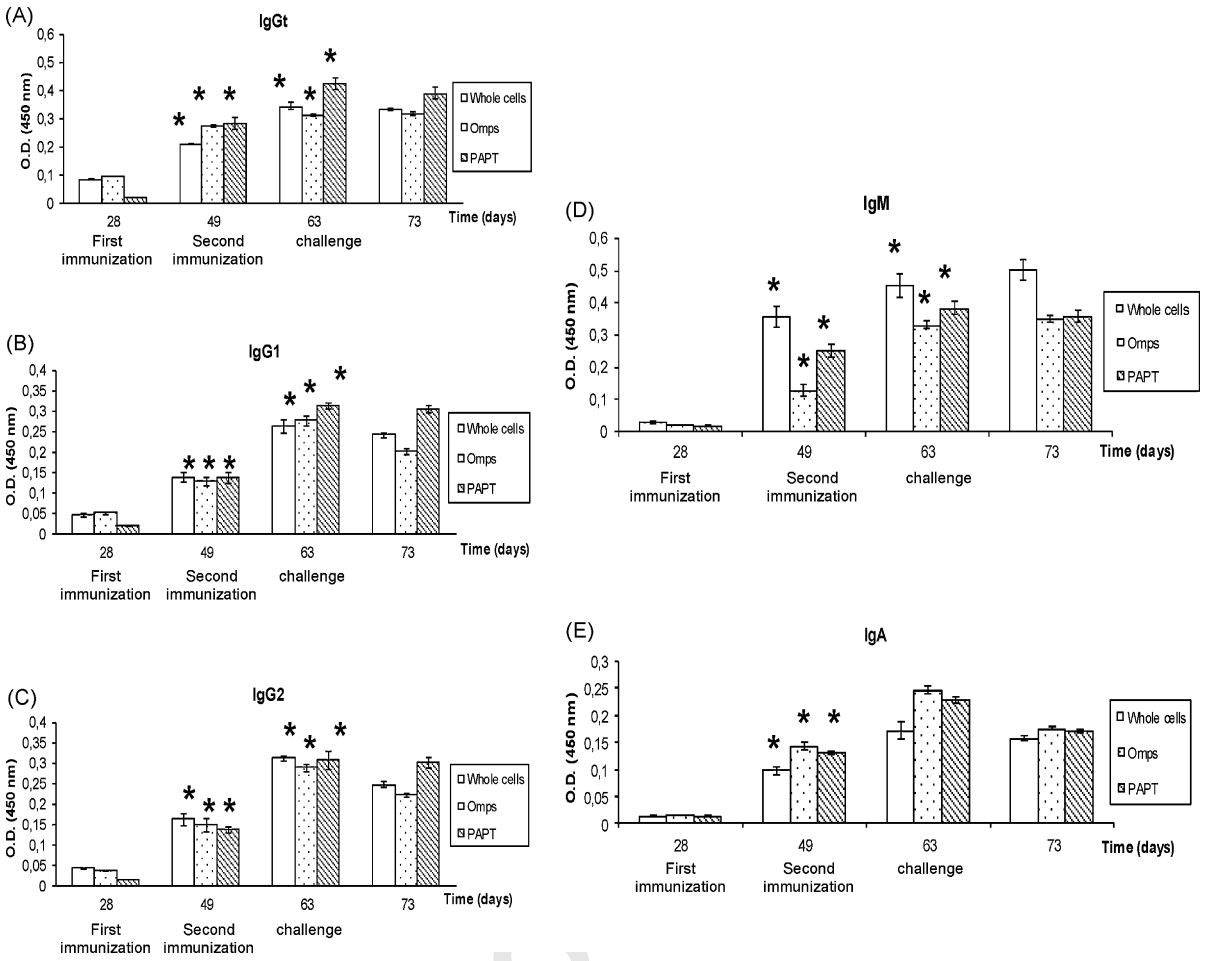

Fig. 7. Levels of immunoglobulins in the animals immunized with proteins with affinity to porcine transferrin administered intratracheally ($PAPT_{Cp}$ group): Mean optical densities at 450 nm ± standard deviation of serum total IgG (IgGt) (A), IgG1 (B), IgG2 (C), IgM (D) and IgA (E) measured at different times for whole-cell, Omp and PAPT antigens. (*) Significant differences ($P < 0.005$) compared to the time before.

The clinical results clearly suggested that both PAPT immunogen and commercial bacterin were fully effective in preventing experimental *H. parasuis* infection in the conditions of our study (Frandoloso et al., 2011). Furthermore, the IgG, IgM and IgA responses developed to *H. parasuis* whole-cells, Omps and PAPT after the two immunizations and challenge were characterized in order to elucidate their protective role. Irrespective to the group they belonged to, all vaccinated animals developed IgGt, IgG1, IgG2 and IgM levels, and all were protected against challenge with a so high inoculum as 3×10^8 CFU of Nagasaki strain. However, in a recent study in which only partial protection was achieved with the recombinant virulence-associated trimeric autotransporters (Vta), all Vta vaccinated animals developed high antibody titers (Olvera et al., 2011). Therefore, it could be speculated that the strong antibody response developed could be enough to effectively prevent Glässer's disease. This finding was already obtained by our investigation group for a mixture of Omps from *H. parasuis* (Martín de la Fuente et al., 2009b). Nevertheless, the significant increases in IgGt or IgM isotypes could not be observed until after the second immunization or even after challenge in that report (Martín de la Fuente et al., 2009b), while in the present study significant rises were already measured after the first immunization, a finding also corroborated by immunoproteomic analysis. For this reason, it can be stated that PAPT immunogen resulted in a stronger and more rapid humoral response than the mixture of Omps previously tested by us (Martín de la Fuente et al., 2009b) or than that developed using bacterin-based vaccines (Takahashi et al., 2001). In addition, the response against the PG bacterin vaccine was similar to that against PAPT, not particularly stronger or more intense. The cell-mediated response of PAPT extract has been recently characterized, inducing a stronger response of $CD21^+\alpha IgM^+$ B and helper T cells in comparison to PG bacterin (Frandoloso et al., 2012), thus confirming the potential of this extract in future vaccines against Glässer's disease.

Of the three vaccinated groups, the piglets receiving PAPT intratracheally were those that developed a stronger immune response, being directed against a larger number of proteins in such a manner that CMP-N-acetylneuraminic acid synthetase and glyceraldehyde 3-phosphate dehydrogenase were exclusively detected in $PAPT_{Cp}$ group

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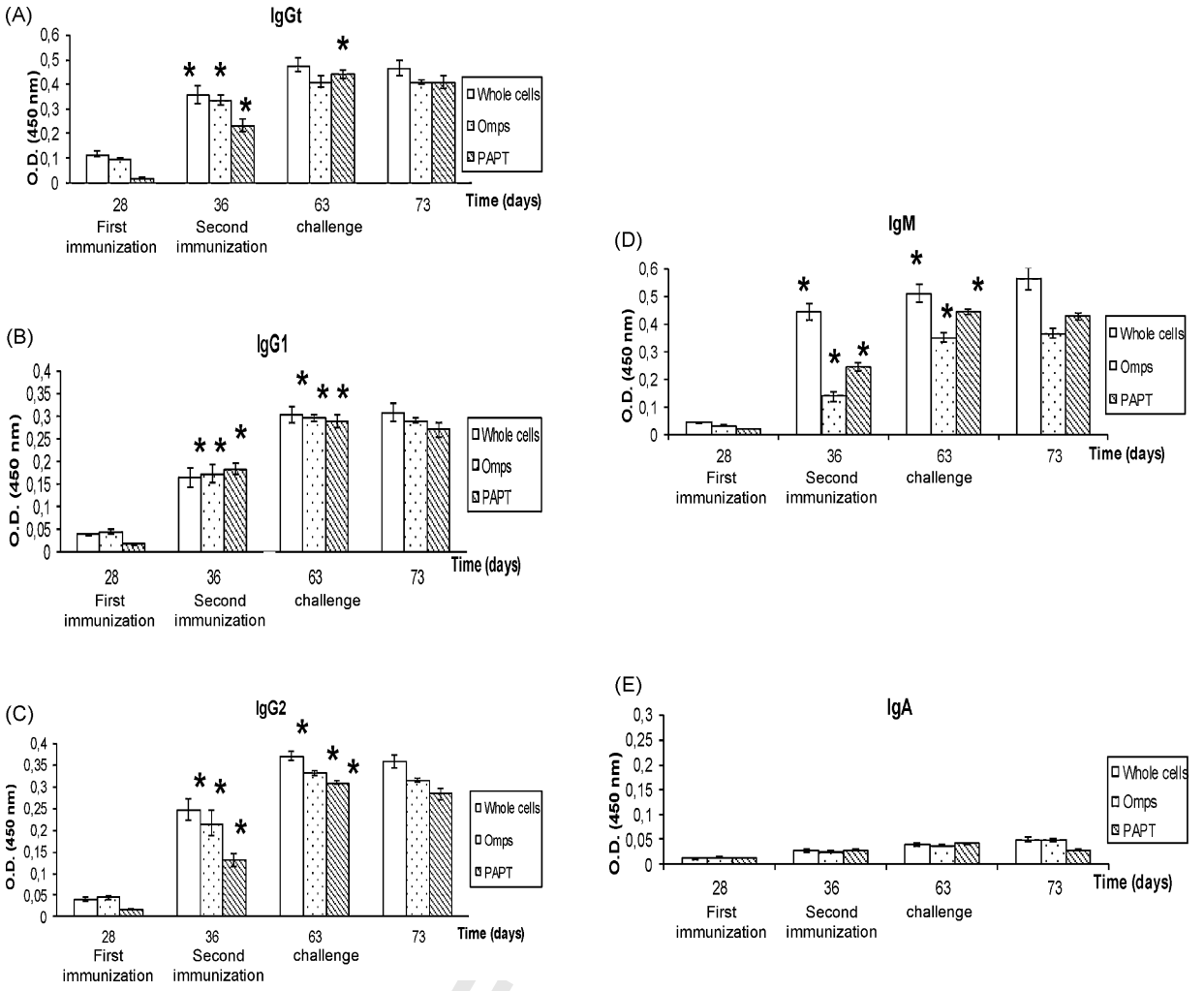

Fig. 8. Levels of immunoglobulins in the animals immunized with proteins with the commercial vaccine administered intramuscularly (PG group): Mean optical densities at 450 nm ± standard deviation of serum total IgG (IgGt) (A), IgG1 (B), IgG2 (C), IgM (D) and IgA (E) measured at different times for whole-cell, Omp and PAPT antigens. (*) Significant differences ($P < 0.005$) compared to the time before.

(Tables 1 and 2). This latter protein has been previously identified as immunogenic in *Neisseria meningitidis* (Williams et al., 2009) and *Streptococcus suis* (Zhang et al., 2008). Although the mucosal IgA response was not tested in our study, only significant increases of serum IgA antibodies were detected working with $PAPT_{Cp}$ and intratracheal route, but not working with intramuscular inoculation. This finding could be explained by the different site of inoculation of PAPT extract but also by the different adjuvant chosen (type IV neuraminidase *versus* Montanide 2215 VG PR respectively) or by both parameters. In a previous study using recombinant virulence-associated trimeric autransporters of *H. parasuis* as immunogens and an intramuscular route, serological IgA response could be measured in one of the six animals used (Olvera et al., 2011).

5. Conclusion

Six proteins (FKBP-type peptidyl-prolyl cis-trans isomerase, neuraminidase exo-α-sialidase, xanthine-guanine phosphoribosyl transferase, CMP-N-acetylneuramic acid synthetase, phenylalanyl-tRNA synthetase and glyceraldehyde 3-phosphate dehydrogenase) were identified as novel immunogenic proteins in *H. parasuis*. These molecules could have a high potential as candidates in future subunit vaccines to control Glässer's disease. Further investigation is required to prove if these molecules might be also effective in cross-protection with other serovars. The immunogenicity was also underlined by the antibody response developed, regardless of the inoculation route, especially when PAPT were used as antigen in ELISA. Finally, a clear relationship between the humoral response developed after immunization and protection against *H. parasuis* challenge could be established in the three vaccinated groups.

Uncited reference

Lichtensteiger and Vimr (1997).

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