Development and Characterization of Protective *Haemophilus parasuis* Subunit Vaccines Based on Native Proteins with Affinity to Porcine Transferrin and Comparison with Other Subunit and Commercial Vaccines

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*Haemophilus parasuis* is the agent responsible for causing Glässer’s disease, which is characterized by fibrinous polyserositis, polyarthritis, and meningitis in pigs. In this study, we have characterized native outer membrane proteins with affinity to porcine transferrin (NPAPT) from *H. parasuis* serovar 5, Nagasaki strain. This pool of proteins was used as antigen to developed two vaccine formulations: one was adjuvanted with a mineral oil (Montanide IMS 2215 VG PR), while the other was potentiated with a bacterial neuraminidase from *Clostridium perfringens*. The potential protective effect conferred by these two vaccines was compared to that afforded by two other vaccines, consisting of recombinant transferrin-binding protein (rTbp) A or B fragments from *H. parasuis*, Nagasaki strain, and by a commercially available inactivated vaccine. Five groups of colostrum-deprived piglets immunized with the vaccines described above, one group per each vaccine, and a group of nonvaccinated control animals were challenged intratracheally with a lethal dose (3 × 10⁶ CFU) of *H. parasuis*, Nagasaki strain. The two vaccines containing rTbps yielded similar results with minimal protection against death, clinical signs, gross and microscopic lesions, and *H. parasuis* invasion. In contrast, the two vaccines composed of NPAPT antigen and commercial bacterin resulted in a strong protection against challenge (without deaths and clinical signs), mild histopathological changes, and no recovery of *H. parasuis*, thus suggesting their effectiveness in preventing Glässer’s disease outbreaks caused by serovar 5.

Respiratory disorders induced by bacterial pathogens are one of the major problems in intensive production systems. Among them, *Haemophilus parasuis* has emerged in the last few years as one of the main causes of nursery mortality in modern swine herds, causing significant financial losses worldwide (12). This organism, a Gram-negative bacillus classified in the Pasteurellaceae family, is commonly found in the upper respiratory tract of healthy conventional pigs, preferentially colonizing the nasal mucosa and/or the tonsillar area (2). Some strains can migrate into the lungs, causing pneumonia, and disseminate to produce a severe systemic disease, characterized by fibrinous polyserositis, polyarthritis, meningitis, and more rarely myositis of the masseter muscles, known as Glässer’s disease (3, 29, 30). Fifteen serovars of *H. parasuis* have been recognized thus far by means of an immunodiffusion test (15); however, there are often a large number of nontypeable strains reported depending on geographic region and typing method (8, 32). Although there is not a strong correlation between serovars and degree of pathogenicity, serovars 1, 5, 10, and 12 to 14 are classified as highly virulent; serovars 2, 4, and 15 showed moderate virulence; and serovars 3, 6 to 9, and 11 are considered nonvirulent (27). It is believed that stress factors, such as transport, unfavorable environment (41), and some practices, such as early weaning, may have influenced the epidemiology of *H. parasuis* within herds, especially regarding the early colonization of pigs by virulent strains and the spread of them throughout a swine population (27).

Because most swine herds are colonized by *H. parasuis* and therefore have a degree of protective immunity, reproduction of systemic infection in conventional pigs is often difficult. Nevertheless, both caesarian-derived, colostrum-deprived pigs and naturally farrowed, colostrum-deprived pigs have been used successfully to study this disease experimentally (7, 26, 42). Control of Glässer’s disease outbreaks has traditionally been achieved by use of commercial or autogenous bacterins. These vaccines usually give strong protection against challenge with the homologous serovar (5, 13, 40), but more inconsistent results have been described when testing the development of cross-protection, depending on strains and serovars of *H. parasuis* (5, 16, 28, 33, 38). With regard to modern vaccines based on molecular techniques, outer membrane proteins (Omps) have rendered a high immunogenicity. In this respect, an Omp formulation has resulted in partial protection against the homologous serovar (21), while the purified recombinant OmpA has showed a good antigenicity (44), and four other Omps (PalA, Omp2, D15, and HPS 06257) have also yielded a strong potential to be vaccine candidates (45).

We examined here the immunoprotective effect of two vaccines based on Omps with affinity to porcine transferrin from *H. parasuis* serovar 5 (a highly virulent serovar of worldwide
prevalence [27]) and compared them to other subunit vaccines, also designed in our laboratory, and one commercially available inactivated vaccine. As an animal model, colostrum-deprived piglets (6) challenged with a lethal dose of *H. parasuis* serovar 5, were used.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** *H. parasuis* Nagasaki strain (reference strain of serovar 5) was used in the present study. It was cultured on chocolate blood agar for 24 h at 37°C under 5% CO₂. Overexpression of proteins with affinity to porcine transferrin was carried out by culturing *H. parasuis* in iron restrictive conditions, as described previously (10), but adding the iron chelator 2,2-dipyridyl at a final concentration of 200 μM to 0.025% NAD-supplemented pleuropneumonia-like organisms (PPLO) broth, instead of the chelator Na₃CaDTPA.

**Escherichia coli** TOP10 and LMG194 cells were grown in Luria-Bertani medium supplemented with 100 μg of ampicillin/ml.

**Antigen preparation.** rTbpA antigen consisted of an *H. parasuis* TbpA fragment, cloned in *E. coli* TOP10 and expressed in *E. coli* LMG194 (22). TbpB antigen consisted of a N-terminal fragment from *H. parasuis* TbpB, cloned and expressed in *E. coli* TOP10 (9).

**NPAPt antigen consisted of native outer membrane proteins (Omps) from *H. parasuis* exhibiting affinity to porcine transferrin, which were purified by gel filtration on a fast-protein liquid chromatography (FPLC)-CNBr-activated Sepharose 4B (GE Healthcare) column, according to the manufacturer’s instructions. Briefly, 20 mg of iron-loaded porcine transferrin (First Link, Ltd.) was dissolved in coupling buffer (0.1 M NaHCO₃ containing 0.5 M NaCl), the mixture were gently rotated for 2 h at room temperature, and excess ligand was washed with 5 volumes of coupling buffer. Then, any remaining active group was blocked with 0.1 M Tris-HCl buffer (pH 8.0) for 2 h, and the medium was washed with three cycles of alternating pH (0.1 M acetic acid-sodium acetate [pH 4.0] containing 0.5 M NaCl, followed by 0.1 M Tris-HCl [pH 8] containing 0.5 M NaCl). Omps were obtained from liquid cultures of *H. parasuis* grown in iron restrictive conditions as described previously (10). The cell-free supernatants were dialyzed against coupling buffer (10 mM Tris-HCl, 50 mM NaCl, 0.5% Sarkosyl) and an Amicon Ultra 30K centrifugal filter. The dialyzed sample was then adjusted to 60 mg of protein/ml of Sepharose in a 20 ml-volumed and the packed column was connected to the FPLC apparatus (Aktta Prime; GE Healthcare) with a flow rate of 0.2 ml/min. After the sample was passed through the column, it was washed with 50 ml of coupling buffer (flow rate, 0.5 ml/min), and the proteins were eluted with 2 M guanidine-HCl, with monitoring of the elution peak at 280 nm. The eluted proteins were collected in tubes containing 1 mM Tris (pH 9.0), immediately dialyzed against 0.1% Sarkosyl-PBS (pH 7.5), divided into aliquots, and frozen at −20°C until use.

**Identification of Tbps in NPAPt antigen.** Tbp identification was carried out on the Proteomic Service Facility from the University Complutense of Madrid (Spain), a member of ProteoRed Network. The protein spots of interest were internally calibrated by using peptides from the autodigestion of trypsin.

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**Statistical analysis.** The one-way analysis of variance was used for comparison of rectal temperatures at various times after challenge in each group and between groups at each time up to 48 h, while the Tukey’s multiple comparison test was used for comparison between groups at 72 and 144 h. The GraphPad Prism statistical software, version 5.0, was used for comparison between groups in survival studies. *P* values of <0.05 were considered significant.

**RESULTS**

**Characterization of rTbpA, rTbpB, and NPAPt antigens.** rTbpA antigen was composed of 200 amino acids located in an intermediate region of complete *H. parasuis* TbpA. The specific location of this rTbpA fragment has been recently pub-
lished by us (22). The rTbpB antigen contained the first 102 amino acids of N-terminal domain of TbpB. Two bands of 38.5 and 27.0 kDa (Fig. 2B and C, respectively) were revealed by SDS-PAGE for these recombinant proteins, respectively. The purified NPAPT antigen was further analyzed by SDS-PAGE, and seven other proteins able to bind to iron-loaded porcine transferrin could be observed, together with the well-characterized TbpA (100-kDa band, lane 1, in Fig. 2D) and TbpB (75-kDa band, lane 2, in Fig. 2D). The molecular masses of these seven transferrin-binding proteins were lower than those of TbpA and TbpB and comprised between 75 and 25 kDa (Fig. 2D). These native proteins were then characterized by using a MALDI-TOF/TOF mass spectrometer and compared to the sequences previously included in the NCBI protein database. In addition to TbpA and TbpB, transferrin sus scrofa, ABC transporter, periplasmic binding protein, catalase, elongation factor Tu, Omp2, periplasmic iron-binding protein, and chelated ABC transporter, periplasmic binding protein could be identified. Their molecular masses, theoretical isoelectric points, number of matched peptides, protein coverage, and scores are shown in Table 1.

Clinical signs. All of the animals belonging to the control group died between 24 and 48 h postchallenge (hpc), while eight of the piglets receiving rTbpA or rTbpB vaccines (four from each group) died between 24 and 72 hpc, with survival rates of 33.3 and 20%, respectively, in these two latter groups. In contrast, all of the animals immunized with the NPAPTM, NPAPT_CDP, or PG vaccines survived challenge with $3 \times 10^8$ CFU of H. parasuis Nagasaki strain (Fig. 3). These three later vaccines showed significantly higher survival rates ($P < 0.0001$) compared to the rTbpA, rTbpB, and nonimmunized groups.

The nonvaccinated control animals showed high temperatures until death (3°C above those at the time of challenge), and significant differences were obtained at 24 and 48 hpc compared to challenge ($P < 0.0005$) (Table 2). A similar tendency was observed for the groups receiving rTbpA or rTpbB formulations: significantly higher temperatures were also recorded at 24 hpc for rTbpA ($P < 0.005$) and until 48 hpc for rTbpB ($P < 0.005$ for both times), but these increases reached only about 1 to 1.5°C compared to challenge. However, no significant differences in temperature were observed at differ-

![FIG. 1. Immunization, infection, and necropsy schedule for colostrum-deprived piglets before and after challenge with H. parasuis.](http://cvi.asm.org/)

![FIG. 2. SDS-PAGE (Coomassie blue colloidal stain) analysis. (A) Molecular mass marker (Precision Plus Protein standards; Bio-Rad). (B and C) rTbpA (B) and rTbpB (C) eluted from nickel affinity columns. (D) Native proteins (1 to 9) with affinity to porcine transferrin eluted from a CNBr-transferrin affinity column. These nine proteins were further identified by MALDI-TOF/TOF mass spectrometry (see Table 1).](http://cvi.asm.org/)
ent times after challenge for the NPAPT<sub>M</sub>, NPAPT<sub>Cp</sub>, and PG groups. When we compared test groups to one another, control piglets had significantly higher temperatures than the rTbpA group ($P < 0.05$ at 24 hpc and $P < 0.005$ at 48 hpc), the rTbpB group ($P < 0.005$ at 24 hpc and $P < 0.0005$ at 48 hpc), and the NPAPT<sub>M</sub>, NPAPT<sub>Cp</sub>, and PG groups ($P < 0.0005$ at 24 and 48 hpc).

Clinical signs suspicious of Glässer’s disease, such as limb uncoordination, swollen joints, severe dyspnea, and coughing, together with other more nonspecific signs (apathy, weakness, and anorexia), were seen in all of the nonimmunized control piglets. Similar but considerably milder clinical signs were observed in piglets vaccinated with rTbpA or rTbpB; however, two animals belonging to rTbpA group and one belonging to rTbpB group showed as only signs weakness, loss of appetite, and a mild transient rise in temperature until 3 dpc, resulting in a complete recovery at 7 dpc. No appreciable clinical signs were seen in piglets immunized with the NPAPT<sub>M</sub>, NPAPT<sub>Cp</sub>, or PG vaccines. On the other hand, no adverse reactions to any of the four formulations developed in the present study (i.e., the rTbpA, rTbpB, NPAPT<sub>M</sub>, and NPAPT<sub>Cp</sub> vaccines) or to the commercial bacterin were detected.

**Gross and histopathological findings.** All nonimmunized piglets and most of those receiving rTbpA or rTbpB vaccines showed the characteristic inflammatory changes caused by a *H. parasuis* infection (Table 3). Severe fibrinous polyserositis was observed in the pericardial, pleural, and peritoneal cavities, characterized by the presence of fibrin strands or layers on serosal surfaces (Fig. 4a and 4c). Moreover, some piglets presented a moderate fibrinous polyarthritis, located mainly in carpal and hock joints (Fig. 4d), and a fibrinous exudate was also identified in the occipito-cervical joint in a piglet immunized with rTbpB. Another frequent inflammatory lesion was a moderate to severe fibrinous-suppurative meningitis identified by histopathological examination (Fig. 4e). Likewise, most organs showed vascular alterations of varying severity such as edema, congestion, hemorrhages, disseminated intravascular coagulation (consumptive coagulopathy), and vascular thrombosis (Table 3 and Fig. 4f). The spleens of these animals seemed normal in gross appearance, but microscopic examination revealed that the spleens in some of the piglets presented a transudation of plasma proteins (fibrin deposits) in the marginal zone of white pulp and in red pulp, as well as a lymphocyte reduction in periarteriolar lymphoid sheaths and follicles due to a lympholysis, with the production of nuclear debris (Table 3 and Fig. 4g). These splenic lesions and the vascular alterations described above were compatible with an acute septicemia, and death by septic shock was suspected.

In contrast, all piglets immunized with the NPAPT<sub>M</sub>, NPAPT<sub>Cp</sub>, or PG vaccine survived the challenge with the lethal dose of *H. parasuis* Nagasaka strain, and a considerable reduction of pathological changes was shown (Table 3). The only lesions observed grossly were a mild (PG vaccine) to moderate (NPAPT<sub>M</sub> and NPAPT<sub>Cp</sub> vaccines) fibrinous peritonitis in some animals (Fig. 4b), as well as a mild fibrinous polyarthritis in some piglets immunized with the PG commercial vaccine. In addition, microscopically, generalized congestion was observed, and mild edema and hemorrhages were found occasionally in piglets immunized with the NPAPT<sub>M</sub> or NPAPT<sub>Cp</sub> vaccines. Vascular thrombosis was only seen in a piglet immunized with PG vaccine. A lymphoid hyperplasia of spleen, characterized by multiple prominent nodules of white pulp in its cut surface, was a constant finding in these three test groups, these nodules being made up of expanded periarteriolar lymphoid tissue and follicles due to an increase in lymphocytes. No necrotic lymphocytes in the white pulp were seen (Fig. 4h).

**Bacteriological findings.** *H. parasuis* was isolated from the heart blood, kidneys, and pleural cavities from any of the six groups compared. However, pure cultures were recovered from the brains, spleens, and peritonea of all of the control piglets and from the livers, lungs, and pericardial cavities of most of them. *H. parasuis* was isolated from the eight sites

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**Table 1. Proteins identified in the extract containing NPAPT antigen**

| Band | Access no. | Protein                           | Mol wt | Theoretical isoelectric point | No. of matched/searched peptides | Protein coverage (%) | Score |
|------|------------|-----------------------------------|--------|-------------------------------|----------------------------------|----------------------|-------|
| 1    | gi/33305754| Transferrin binding protein A     | 106,614| 9.11                          | 18/32                            | 21                   | 152   |
| 2    | sp/P09571 | Transferrin sus scrofa            | 78,971 | 6.93                          | 8/20                             | 14                   | 77    |
| 3    | gi/161408008| Transferrin binding protein B    | 59,835 | 6.40                          | 16/37                            | 31                   | 157   |
| 4    | gi/167855391| ABC transporter, periplasmic binding protein | 57,908 | 8.23                          | 15/30                            | 30                   | 156   |
| 5    | gi/219692447| Catalase                         | 54,954 | 6.50                          | 14/22                            | 33                   | 180   |
| 6    | gi/219692224| Elongation factor Tu             | 43,501 | 5.23                          | 11/17                            | 32                   | 140   |
| 7    | gi/209968888| Outer membrane protein 2           | 38,552 | 9.20                          | 5/13                             | 17                   | 60    |
| 8    | gi/219691519| Periplasmic iron-binding protein | 37,755 | 8.80                          | 15/39                            | 50                   | 174   |
| 9    | gi/219691491| Chelated ABC transporter, periplasmic binding protein | 32,632 | 7.74                          | 7/22                             | 32                   | 84    |

* As listed in the NCBI protein database.
shown in Table 4 in two control piglets. Similar decreased isolation rates were found for rTbpA group, but *H. parasuis* was recovered from all of the samples taken in at least one of the dead animals. In rTbpB group, *H. parasuis* was isolated from the liver parenchyma in three piglets, and from the brains, lungs, pericardial and peritoneal cavities, and carpal and hock joints in two piglets. In the rTbpA and rTbpB treatment groups, *H. parasuis* was only recovered from the piglets that died (Table 4). All of these positive cultures were confirmed by PCR. In contrast, no *H. parasuis* recovery was obtained from any of the swabs taken from piglets vaccinated with the NPAPT<sub>M</sub>, NPAPT<sub>Cp</sub> or PG formulations.

**DISCUSSION**

It has become evident that many bacterial virulence determinants are synthesized in response to specific environmental signals, and those that are expressed in host are especially attractive for studies of pathogenesis and rational vaccine development (31). In this way, the transferrin-binding proteins produced by some Gram-negative organisms play a pivotal role in virulence (11). Indeed, their potential utility as vaccine immuno gens has been supported by studies carried out in some members of the Porcine Pasteurellaceae family, such as *A. pleuropneumoniae* (36) or *H. influenzae* (20), in which TbpB behaved as a protective antigen. Therefore, we have developed in the present study a subunit vaccine from *H. parasuis* composed of several native Omp proteins with affinity to porcine transferrin, which were adjuvanted with two types of compounds (the commercial mineral oil Montanide IMS 2215 VG PR and a neuraminidase from *Clostridium perfringens* (100 mU/ml); PG, Porcilis Glaesser (Intervet)).

The numbers of surviving piglets at each time point (n) are indicated in parentheses. Superscript capital letters indicate significant differences as follows: A, P < 0.005 from result at 0 h (challenge); B, P < 0.005 from result at 0 h (challenge); C, P < 0.05 compared to control group; D, P < 0.005 compared to control group; and E, P < 0.0005 compared to control group.

| Pathology                  | Control (4) | rTbpA (6) | rTbpB (5) | NPAPT<sub>M</sub> (6) | NPAPT<sub>Cp</sub> (6) | PG (6) |
|---------------------------|-------------|-----------|-----------|----------------------|----------------------|--------|
| Vascular lesions          |             |           |           |                      |                      |        |
| Congestion                | +           | +         | +         | +                    | +                    | +      |
| Pulmonary edema           | ++          | +         | ±         | ±                    | ±                    | ±      |
| Gallbladder edema         | ++          | +         | +         | –                    | –                    | –      |
| Brain edema               | +           | ±         | ±         | –                    | –                    | –      |
| Hemorrhages               | +           | +         | +         | ±                    | ±                    | ±      |
| Disseminated intravascular coagulation | + | + | + | – | – | – |
| Vascular thrombosis       | +           | +         | +         | –                    | –                    | –      |
| Inflammatory lesions      |             |           |           |                      |                      |        |
| Meningitis                | ++          | +         | +         | –                    | –                    | –      |
| Fibrous pleuritis         | ++          | +         | +         | –                    | –                    | –      |
| Fibrous pericarditis      | ++          | +         | +         | –                    | –                    | –      |
| Fibrous peritonitis       | ++          | +         | +         | +                    | +                    | ±      |
| Fibrous polyarthritis     | +           | +         | +         | –                    | –                    | –      |
| Splenic changes           |             |           |           |                      |                      |        |
| Lymphoid hyperplasia      | –           | ±         | ±         | –                    | +                    | +      |
| Fibrin deposits           | ±           | ±         | ±         | –                    | –                    | –      |
| Lympholysis               | ±           | ±         | ±         | –                    | –                    | –      |

<sup>a</sup> Experimental groups: control, no vaccine; rTbpA, vaccine containing rTbpA plus Montanide IMS 2215 VG PR in a 1:4 ratio; rTbpB, vaccine containing rTbpA plus Montanide IMS 2215 VG PR in a 1:4 ratio; NPAPT<sub>M</sub>, native proteins with affinity for porcine-transferrin plus Montanide IMS 2215 VG PR in a 1:4 ratio; NPAPT<sub>Cp</sub>, native proteins with affinity for porcine-transferrin plus neuraminidase from *Clostridium perfringens* (100 mU/ml); PG, Porcilis Glaesser vaccine (Intervet). Severity: –, no changes; ±, minimal to mild changes; +, moderate changes; and ++, severe changes. n, Number of animals per group.
cially available bacterin against Glässer’s disease. We chose an intratracheal inoculation for challenge with a lethal dose of *H. parasuis* because this is the most commonly used route for inducing a successful infection under experimental conditions (3, 6, 7, 21, 40); in addition, this administration route circumvented the innate immune system located in the upper respiratory tract of hosts.

The groups immunized with recombinant TbpA or TbpB fragments showed a similar behavior against challenge, with low survival rates and clinical signs resembling those of control piglets, although of a milder intensity in some cases. Moreover, as in the nonvaccinated group, rTbpA- and rTbpB-immunized animals had significantly higher temperatures at 24 hpc compared to the challenge group, and for rTbpB-immunized animals this was also the case at 48 hpc. In addition, this administration route circumvented the innate immune system located in the upper respiratory tract of hosts.

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Myers et al. (25), after generating antibodies against rTbpA from *Moraxella catarrhalis*, showed that anti-rTbpA antiserum was capable of recognizing epitopes located on the bacterial surfaces of entire cells but it was not bactericidal. Loosmore et al. (20) confirmed that infant rats passively immunized with rabbit anti-*H. influenzae* rTbpA serum were not protected from challenge with *H. influenzae*. The observations seen in these two studies in other closely related Gram-negative organisms seem to corroborate the scarce level of protection obtained for *H. parasuis* rTbpA antigen in the present study. Nevertheless, Potter et al. (31) demonstrated that the combination of the native TbpA and the recombinant TbpB from *Mannheimia haemolytica* (an organism also belonging to the Pasteurellaceae family) enhanced considerably the protection conferred by these two proteins when administered separately to calves to prevent shipping fever and that TbpA solely might contribute to protection through a cell-mediated immune response. The scarce protection conferred by rTbpA formulation in our study might be explained by the reduced length of the protein fragment that was cloned (only 200 amino acids) and/or by the fact that the selected sequence might not have included the most exposed epitopes in the bacterial surface and, consequently,

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**TABLE 4. Recovery of *H. parasuis* from piglets after challenge**

| No. of piglets positive for *H. parasuis* | Brain parenchyma | Liver parenchyma | Lung parenchyma | Spleen parenchyma | Pericardial swab | Peritoneal swab | Carpal joint | Hock joint |
|----------------------------------------|-----------------|-----------------|-----------------|------------------|-----------------|----------------|--------------|------------|
| Control | 4 | 3 | 3 | 3 | 4 | 3 | 4 | 2 | 2 |
| rTbpA | 4 | 3 | 3 | 1 | 3 | 3 | 2 | 1 | 1 | 1 |
| rTbpB | 2 | 2 | 3 | 2 | 2 | 2 | 0 | 0 | 0 |
| NPAPTM | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| NPAPTP | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| PG | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

* a Experimental groups: control, no vaccine; rTbpA, vaccine containing rTbpA plus Montanide IMS 2215 VG PR in a 1:4 ratio; rTbpB, vaccine containing rTbpA plus Montanide IMS 2215 VG PR in a 1:4 ratio; NPAPTM, native proteins with affinity for porcine-transferrin plus Montanide IMS 2215 VG PR in a 1:4 ratio; NPAPTP, native proteins with affinity for porcine-transferrin plus neuraminidase from *Clostridium perfringens* (100 mU/ml); PG, Porcilis Glässer vaccine (Intervet). n, Number of animals per group.
this antigen could not have been accessible to the immune response.

Retzer et al. (34) cloned and expressed a larger N-terminal portion of TbpB from different pathogens of the Pasteurellaceae and Neisseriaceae families and proved that this portion possesses a high binding affinity for iron-loaded transferrin. Several reports have demonstrated the immunoprotective capacity of the A. pleuropneumoniae (36), H. influenzae (20), and Neisseria meningitidis (35) full-length recombinant TbpBs in pigs, rats, and in vitro tests, respectively. According to these studies in other Gram-negative organisms, the successful protection of this recombinant protein against challenge with H. parasuis might be achieved with the inoculation of either a larger N-terminal portion of H. parasuis rTbpB or the entire rTbpB, thus resulting in a greater antigenic exposure to the porcine immune system than that obtained by us with the first 102 amino acids of N-terminal domain of TbpB.

On the other hand, of the nine native proteins showing affinity to porcine transferrin described here, the function of three of them (catalase, elongation factor Tu, and Omp2) have never been associated, to our knowledge, with the acquisition, binding, or transport of iron in H. parasuis. In a recent immunoeproteomic analysis of N. meningitidis (43), several immunoreactive proteins were found, including an ABC transporter, periplasmic protein, which was also identified in our NPAPT antigen. Similarly, previous studies have reported as immunogenic proteins elongation factor Tu in Bordetella pertussis (1) or elongation factor Tu and Omp2 in Helicobacter pylori (19). In addition, Zhou et al. (45) demonstrated a strong potential for Omp2 of H. parasuis serovar 5 as a vaccine candidate, among other Omps. Catalase has been used in other organisms, such as corroboration by the absence of deaths, hyperthermia (or any other relevant clinical sign), and H. parasuis recovery from any of the locations sampled, as well as by the scarce gross and histopathological changes recorded. That the results obtained with the NPAPT_M and NPAPT_Cp groups were practically identical suggests that the efficacy of these vaccine formulations was not related to the adjuvant chosen and/or the administration route but rather to the quality of the antigens themselves.

Montaniad adjuvant could not be used for the formulation of the vaccine being inoculated intratra cheally because the administration of mineral oils is not recommended by this route. Neuraminidase from C. perfringens (type VI) was used as a replacement. This glycoprotein possesses enzymatic action degrading the sialic acid, and desialylation of cell surface glycoproteins is frequently observed during inflammation and infection episodes (39). In this sense, Kuroiwa et al. (17) reported that neuraminidase increased interleukin-8 (IL-8) production in human lung epithelial cell cultures, while Stamatos et al. (39) showed that the desialylation of glycoconjugates on the surfaces of monocytes by neuraminidase activated increased production of specific cytokines such as IL-6, macrophage inflammatory protein 1α (MIP-1α), and MIP-1β. Based on these findings, we decided to use this glycoprotein as a potentiator in the vaccine administered intratra cheally in order to enhance the local immune response, because the secretion of proinflammatory cytokines would attract phagocytes and other antigen-presenting cells, thus improving the presentation of NPAPT antigens.

The protection results exhibited by NPAPT_M and NPAPT_Cp vaccines were similar to those obtained by the commercial PG vaccine, also formulated with an H. parasuis strain belonging to serovar 5. Other commercial inactivated vaccines containing serovar 5 strains demonstrated equally strong protection against experimental infection with the homologous serovar (5, 13).

Some gross and microscopic lesions have already been reported in similar experiments (7, 21, 24, 26, 42). The vascular lesions seen in piglets receiving rTbps, which are more severe in nonvaccinated animals, were previously described by Amano et al. (3) in pigs with acute septicemia after intratracheal inoculation with lower concentrations (10^9 to 10^7 CFU) of the H. parasuis Nagasaki strain. These authors associated septicemia in these animals with endotoxin shock caused by the endotoxins released by bacterial lysis, resulting in disseminated intravascular coagulation and death within a short time. Interestingly, the piglets that survived challenge (the NPAPT_M, NPAPT_Cp, and PG groups) showed a reactive hyperplasia in spleen, which was considerably milder in the minimally protected animals (rTbpA and rTbpB groups) and absent in nonvaccinated piglets. This finding could be explained by the invasiveness of this pathogen and by the activation and clonal expansion of memory cells as a response to bacterial antigens.

In a previous trial with conventional piglets (data not shown), the animals were inoculated with the same concentrations of rTbpA, rTbpB, and NPAPT antigens by the same administration routes. No clinical signs, fibrinous peritonitis or polyarthritis, or other macroscopic lesions were seen; consequently, the lesions reported here must be attributable to H. parasuis invasion. On the other hand, no H. parasuis was isolated from piglets that survived until they were euthanized 15 days after challenge (not even from those surviving in rTbpA and rTbpB groups), and this finding seems to indicate that H. parasuis could have been largely cleared by this time.

In conclusion, we developed a strategy to obtain Omps from H. parasuis grown under iron-restricted conditions, and the pool of nine native proteins with affinity for porcine transferrin reported in the present study provides effective subunit vaccines to control Glässer’s disease caused by H. parasuis serovar 5, Nagasaki strain. Further studies are required to demonstrate whether the two vaccine formulations described here might be also effective in cross-protection experiments with other serovars.

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