RESEARCH LETTER

Characterization of a recombinant transferrin-binding protein A (TbpA) fragment from *Haemophilus parasuis* serovar 5

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Abstract

Haemophilus parasuis, the etiological agent of Glässer's disease in pigs, possesses iron acquisition pathways mediated by a surface receptor that specifically bind porcine transferrin. This receptor is composed of transferrin-binding protein A (TbpA) and TbpB. As it has been reported for other gram-negative organisms, *H. parasuis* TbpA could be useful as a candidate target for *H. parasuis* vaccination. In this study, a 600-bp *tbpA* fragment of the gene encoding TbpA from *H. parasuis* serovar 5, the Nagasaki strain, was amplified by PCR and cloned into a pBAD/Thio-TOPO expression vector, generating the pBAD-Thio-TbpA-V5-His (TbpA-His) construction. *Escherichia coli* LMG194-competent cells were transformed with this this construction, followed by the induction of protein expression with arabinose. A band (38.5 kDa) corresponding to a 200-amino acid recombinant TbpA (rTbpA) fragment was seen on the sodium dodecyl sulfate polyacrylamide gel electrophoresis and confirmed by immunoblotting. Polyclonal antibodies raised against this fragment were specific for *H. parasuis* and *Actinobacillus pleuropneumoniae*, reacted at the cell surface with *H. parasuis*, and a significant bactericidal activity was also detected. Therefore, this rTbpA fragment induces an immunological response and might be useful as an antigen for vaccination against Glässer's disease.

Introduction

Haemophilus parasuis is the causative agent of Glässer's disease in pigs, whose main symptoms are fibrinous polyserositis, polyarthritis and meningitis; furthermore, some strains can also be found as a commensal of the upper respiratory tract in healthy pigs. Glässer's disease has historically been considered a sporadic, stress-associated disease of young pigs; however, in recent years, in pigs of all ages, herds with high sanitary standards have suffered a significant increase in the morbidity and mortality rates due to this disease (Oliveira & Pijoan, 2004).

Outbreaks of Glässer's disease have been controlled by means of bacterins. These vaccines usually confer protection against challenge with the homologous serovar, but variable results have been reported in cross-protection surveys (Rapp-Gabrielson *et al.*, 1997). Antibodies against outer membrane proteins (Omps) of *H. Parasuis*, but not against lipoolygosaccharide or capsule, have been developed in pigs, suggesting that Omps are more immunogenic than other bacterial components (Miniats *et al.*, 1991). Recently, an Omp formulation has resulted in partial protection against challenge with *H. parasuis* (Martín de la Fuente *et al.*, 2009). In addition, 15 novel immunogenic Omps have been identified, and four of them (PalA, Omp2, D15 and HPS 06257) have been shown to have a strong potential to be vaccine candidates (Zhou *et al.*, 2009). In a similar way, Zhang *et al.* (2009) have purified a recombinant *H. parasuis* OmpA showing good antigenicity.

Among Omps, transferrin-binding proteins (Tbps) in other gram-negative organisms have been considered important targets for the development of attenuated live vaccines because an impairment of iron uptake mechanisms is likely to reduce virulence. Although TbpB have already

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Fig. 1. Nucleotide sequence (1.9 kb fragment) of the *tbpA* gene from the *Haemophilus parasuis* Nagasaki strain, amplified with the TbpAF and tbpA33 primers, nucleotide sequence selected and cloned (600 nucleotides), and protein yielded (200 amino acids).

been characterized in *H. parasuis* (del Río *et al.*, 2005), information regarding TbpA is scarce in this species, and *tbpA* gene has only been used for genotyping purposes by PCR-RFLP (de la Puente Redondo *et al.*, 2003; Li *et al.*, 2009). Here, we report the characterization of a recombinant TbpA (rTbpA) fragment from *H. parasuis* serovar 5 for further immunoprotective studies.

Materials and methods

Bacterial strains and growth conditions

Haemophilus parasuis Nagasaki strain (reference strain of serovar 5) and *Actinobacillus pleuropneumoniae* WF83 (reference strain of serotype 7) were cultured onto a chocolate agar and incubated for 24 h at 37 °C under 5% CO_2. *Escherichia coli* LMG194 and TOP10 cells were grown in LBA (Luria–Bertani medium + 100 μg mL^{-1} ampicillin). *Staphylococcus aureus* CIP 5710 was grown in TSA. The iron chelator 2.2 dipyridyl (100 μM) was added to 0.025% NAD-supplemented PPLO broth to ensure restricted iron availability.

DNA isolation and PCR amplification

Extraction of bacterial genomic DNA, RNA and protein removals, and DNA purification were carried out as reported previously (del Río *et al.*, 2005). Forward primer TbpAF (5′ TGG TGG CTT CTA TGG TCC AA 3′), designed in this study based on the nucleotide sequence from *H. parasuis* Nagasaki strain (GenBank accession nos. AY818058 and AY818059), and reverse primer tbpA33 (5′ AAG CTT GAA ACT AAG GTA CTC TAA 3′) (de la Puente Redondo *et al.*, 2000) were used for PCR amplification (Fig. 1). The PCR mixture was the same as that described by del Río *et al.* (2005), and the reaction was performed in a thermal cycler (Eppendorf Mastercycler Gradient, Germany) under the conditions reported previously (de la Puente Redondo *et al.*, 2000). The PCR fragments were purified using Qiagen PCR purification or Gel extraction kits (Qiagen Inc.).

DNA sequencing, and selection and cloning of the protein fragment

DNA sequencing of the *H. parasuis tpbA* gene was carried out using an Abi-Prism Apparatus (Perkin-Elmer, Spain) at Secugen S.L. (Madrid, Spain). The sequence obtained was analyzed using DNA Strider 1.4fl3 (CEA, France) and BLAST computer program at the National Center for Biotechnology Information. The DNAMAN program was used for predicting the secondary and tertiary structures of proteins, and for predicting transmembrane domains and hydrophobicity analyses. From 303 to 903 bp of the *tbpA* gene was the selected fragment (Fig. 1), and two primers were designed for amplification: GJM-F (5′ GGC TTG GCA TTG GAT GGG TTG 3′) and GJM-R (5′ AAC CAA CCA AGA ATC AGA TTT 3′).

The amplified PCR product was cut from the agarose gel, purified and cloned using a pBAD/TOPO Thiofusion Expression kit (Invitrogen), using the topoisomerase activity of the vector. The method described by del Río *et al.* (2005) was carried out. In order to confirm that clones contained the pBAD-Thio-TbpA-V5-His (TbpA-His) construction, a PCR with primers Trx Seq (5′ TTC CTC GAC GCT AAC CTG 3′) and GJM-R was used. Plasmidic DNA from positive clones was then extracted using the Plasmid Midi and QIAprep Spin Miniprep kits (Qiagen Inc.), and sequenced as described above.

Expression and purification of the fusion protein

Escherichia coli transformants containing the TbpA-His fusion protein were grown in LBA. Arabinose at concentrations from 0.02 to 0.2 $\mu g\,mL^{-1}$ was added to LBA so as to induce the recombinant fusion protein. Various time periods of incubation at 37 °C under agitation were tested to determine the optimal expression conditions of the TbpA-His fusion protein. Thereafter, the cultures were centrifuged, bacteria were resuspended in lysis buffer and sonicated (three cycles of 20 s, 40% duty cycle, Branson sonifier 450 Branson, VWR, Spain) before being centrifuged. The protein concentration was measured from the supernatants obtained using Bradford's method. These samples were then analyzed by sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE).

Immunoblots were carried out as described previously (Pyle & Schill, 1985) in order to confirm the TbpA-His fusion protein in these gels. The membranes were blocked with 5% skim milk in Tris-buffered saline (TBS) for 2 h at 37 °C, and incubated for 1 h at 37 °C with HRPO-labeled murine anti-V5 monoclonal antibodies (mAbs) (Invitrogen) diluted 1 : 5000 in TBS. These mAbs recognize the V5 epitope, which is located in the C-terminal domain of the protein fusion. Bound antibodies were detected adding an enhanced chemiluminescent substrate (GE Healthcare, Spain) (Bronstein *et al.*, 1992).

Nickel affinity chromatography (His-Select™ HC Nickel affinity gel, Invitrogen) was used for the purification of the TbpA-His fusion protein, which was eluted using a phosphate-buffered saline (PBS) buffer containing imidazole (from 75 to 250 mM). Crude extracts, unbound and eluted fractions were analyzed by SDS-PAGE to monitor the optimal conditions for expression and purification.

Production of polyclonal antibodies

Five groups of two 3-month New Zealand rabbits (Charles River, Spain) were immunized with different rTbpA antigens: (a) minced pieces of a Ponceau Red-stained nitrocellulose membrane containing a purified rTbpA fragment, (b) the same antigen as (a), but treating the nitrocellulose membrane with dimethyl sulfoxide, (c) small pieces of a minced Coomassie-blue-stained electrophoresis gel containing an rTbpA fragment band, (d) the purified protein extract, and (e) PBS. Fifty micrograms of each antigen was emulsified in Montanide IMS 2215 VG PR (Seppic Inc., France) at a 1 : 4 ratio and injected intramuscularly. Booster immunizations were administered 21, 42 and 63 days later in the same way, and rabbits were bled 7 days after the last injection. Sera were collected, inactivated at 56 °C for 30 min, adsorbed as reported earlier (del Río *et al.*, 2005) for reducing background staining and stored at −80 °C until use. The animals were handled and cared in accordance with European Animal Care guidelines.

Immunoblotting for testing the specificity of polyclonal antibodies

Bacterial extracts containing iron-binding proteins from *H. parasuis* (Nagasaki), *A. pleuropneumoniae* (WF83) and *S. aureus* were obtained under iron-starved conditions using 2.2 dipyridyl (100 μM). These samples were analyzed by SDS-PAGE. The nitrocellulose membranes were incubated with polyclonal antibodies diluted 1 : 100 for 1 h and then with HRPO-labeled goat anti-rabbit IgG (Sigma) diluted 1 : 5000 in TBS. Bound antibodies were revealed on adding an enhanced chemiluminescent substrate as described above. The assay was performed three times.

Indirect enzyme-linked immunosorbent assay (ELISA)

The plates (Poylsorp, Nunc, Denmark) were coated with 10 μg per well of a purified rTbpA fragment diluted in carbonate buffer and incubated overnight at 4 °C. After blocking with 3% bovine serum albumin (BSA) in PBS for 2 h at 37 °C, 50 μL of each serum diluted 1 : 100 in PBS+0.05% Tween-20 (PBST) was incubated for 1 h at 37 °C. After three rinses with PBST, 50 μL of HRPO-labeled goat anti-rabbit IgG (whole molecule) (1 : 5000 in PBST) (Sigma) was incubated for 1 h at 37 °C, followed by five other rinses. Plates were read at 450 nm after adding TMB+0.002% H_2O_2 for 10 min, and stopping with H_2SO_4 2 M. Samples were run in triplicate, and a serum was considered positive when its OD was at least two times higher than that of the mean before immunization+SD. ODs were analyzed using the GRAPHPAD PRISM statistical program 5.0. Tukey's multiple comparison test was used for comparing the ODs of the five types of sera. Significance was set at $P < 0.05$.

Bactericidal assays

The bactericidal activity of the sera was tested as described earlier (Danve *et al.*, 1993; Rokbi *et al.*, 1997). Sera (50 μL of

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serial twofold dilutions) were mixed in 96-well microplates with 25 µL of an iron-starved *H. parasuis* Nagasaki strain suspension (2×10^4 CFU mL^{-1}) and 25 µL of commercial baby-rabbit serum (Sigma), screened previously for the lack of antibodies to *H. parasuis* by ELISA, as the complement source. After incubation for 1 h at 37 °C, the mixture was plated onto a chocolate agar and incubated as described above. Sera were considered to be bactericidal when < 50% of *H. parasuis* were able to grow in comparison with the complement control. All bactericidal assays were performed four times and the results are shown as mean ± SD. ANOVA and Tukey's multiple comparison tests (GRAPHPAD PRISM statistical program 5.0) were used for comparing the five types of sera. Significance was set at $P < 0.05$.

Immunogold labeling

Immunogold labeling was performed using the method of Li *et al.* (1992). A single colony of *H. parasuis* Nagasaki strain was inoculated into PPLO broth+NAD (40 µg mL^{-1}), Iso-vitalex$^{®}$ (1.25 µL mL^{-1}; BD) and glucose (250 mg mL^{-1}) and incubated overnight at 37 °C under agitation. After centrifugation and washing, the cells were resuspended in 2 mL of PBS+1% BSA and sodium azide (PBSB) and 25 µL was placed on Formvar-coated grids and incubated for 30 min at room temperature. Then, unbound cells were removed and grids were blocked for 10 min with 25 µL of 2% BSA, before being incubated for 30 min with 25 µL of rabbit anti-rTbpA fragment serum diluted 1 : 100 in PBSB. After rinsing five times, the grids were incubated for 30 min with goat anti-rabbit antibody gold particles (25 nm; Aurion, the Netherlands), and observed using JEOL 1010 transmission electron microscopy. The study was carried out using *H. parasuis* grown in both iron-sufficient and deficient media.

Results

Amplification of *tbp*A gene, cloning of the selected fragment, expression and purification of rTbpA

The two primers selected resulted in the synthesis of a 1.9-kb DNA fragment from chromosomal DNA, representing the partial *tbpA* gene sequence of the reference strain of *H. parasuis* serovar (Fig. 1). This gene was then cloned into the pBAD/Thio-TOPO expression vector, and a second PCR was carried out for identifying the colonies containing the pBAD-Thio-TbpA-V5-His construction and its correct insertion. The positive clones yielded a 600-bp amplified band (Fig. 2b), and one of them was selected. DNA plasmidic was extracted and no mutations in the sequence of the inserted fragment were shown by sequentiation.
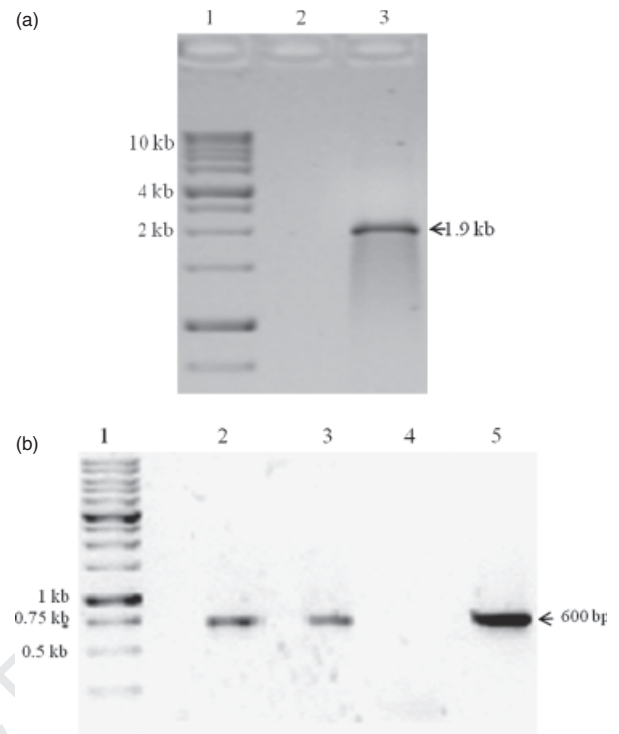

Fig. 2. (a) Results of the *tbpA* amplification from *Haemophilus parasuis*. Lane 1, molecular weight marker; lane 2, negative control (without DNA); lane 3, Nagasaki strain. (b) Positive clones showing the selected fragment from the *tbpA* amplification. Lane 1, molecular weight marker similar to Fig. 1a; lanes 2, 3 and 5, positive clones exhibiting a 600-bp band; lane 4, negative clone.

A difference in 18 nucleotides was detected between this sequence and that of the *tbpA* gene from *H. parasuis*, serovar 5, strain SH0165 (Yue *et al.*, 2009), resulting in two different amino acids (99% homology): Arg to Ser in position 127 and Leu to Asn in position 154 (Fig. 3). Similar results were obtained on analyzing the protein sequence of the *tbpA* gene from *A. pleuropneumoniae* serotype 7, strain AP76 (Gen-Bank accession no. ACE62281.1).

The TbpA-His fusion protein was expressed in *E. coli* LMG194 cells, and the optimal condition of arabinose as an inductor of the protein expression was 0.075% arabinose for 2 h, when 2400 µg mL^{-1} of the fusion protein was obtained. This rTbpA fragment had an estimated molecular mass of 38.5 kDa (Fig. 4a) and contained thioredoxin, the V5 epitope and six histidine tags. An immunoblotting using HRPO-labeled murine anti-V5mAb was carried out for confirming this, and the expected band of 38.5 kDa was observed for the rTbpA fragment under the optimal induction conditions (Fig. 4b, lane 4) and also with 2% arabinose (Fig. 4b, lane 5), but no band was detected in the absence of arabinose (Fig. 4b, lane 3). Different concentrations of imidazole were tested for the purification of the fusion

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HpsNga  GLALDGLPQIQSYVSQYSRSSSGAINEIEYENLRSIQISKGASSSEFGSGSLGGSVQFRT  60
SH0165  GLALDGLPQIQSYVSQYSRSSSGAINEIEYENLRSIQISKGASSSEFGSGSLGGSVQFRT
AP76    GLALDGLPQIQSYVSQYSRSSSGAINEIEYENLRSIQISKGASSSEFGSGSLGGSVQFRT

HpsNga  KEVSDIIKPGQSWGLDTKSAYSSKNQQWLNSLAFAGTHNGFDALVIYTHRDGKETKAHKD  120
SH0165  KEVSDIIKPGQSWGLDTKSAYSSKNQQWLNSLAFAGTHNGFDALVIYTHRDGKETKAHKD
AP76    KEVSDIIKPGQSWGLDTKSAYSSKNQQWLNSLAFAGTHNGFDALVIYTHRDGKETKAHKD

HpsNga  AESRSKRIQRVDLADNNPQGSNWFKVKNDCPSLKCEPRQQVGVSYDGQKYITEQLSAKEY  180
SH0165  AESRSKSIQRVDLADNNPQGSNWFKVKNDCPSLNCEPRQQVGVSYDGQKYITEQLSAKEY
AP76    AESRSKSIQRVDLADNNPQGSNWFKVKNDCPSLNCEPRQQVGVSYDGQKYITEQLSAKEY

HpsNga  TGEERALPDPVKYKSDSWLV  200
SH0165  TGEERALPDPVKYKSDSWLV
AP76    TGEERALPDPVKYKSDSWLV
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Fig. 3. Representation of the alignment of the amino acid sequences of the TbpAs from *Haemophilus parasuis* strains Nagasaki (HpsNga) and SH0165, and *Actinobacillus pleuropneumoniae* strain AP76.
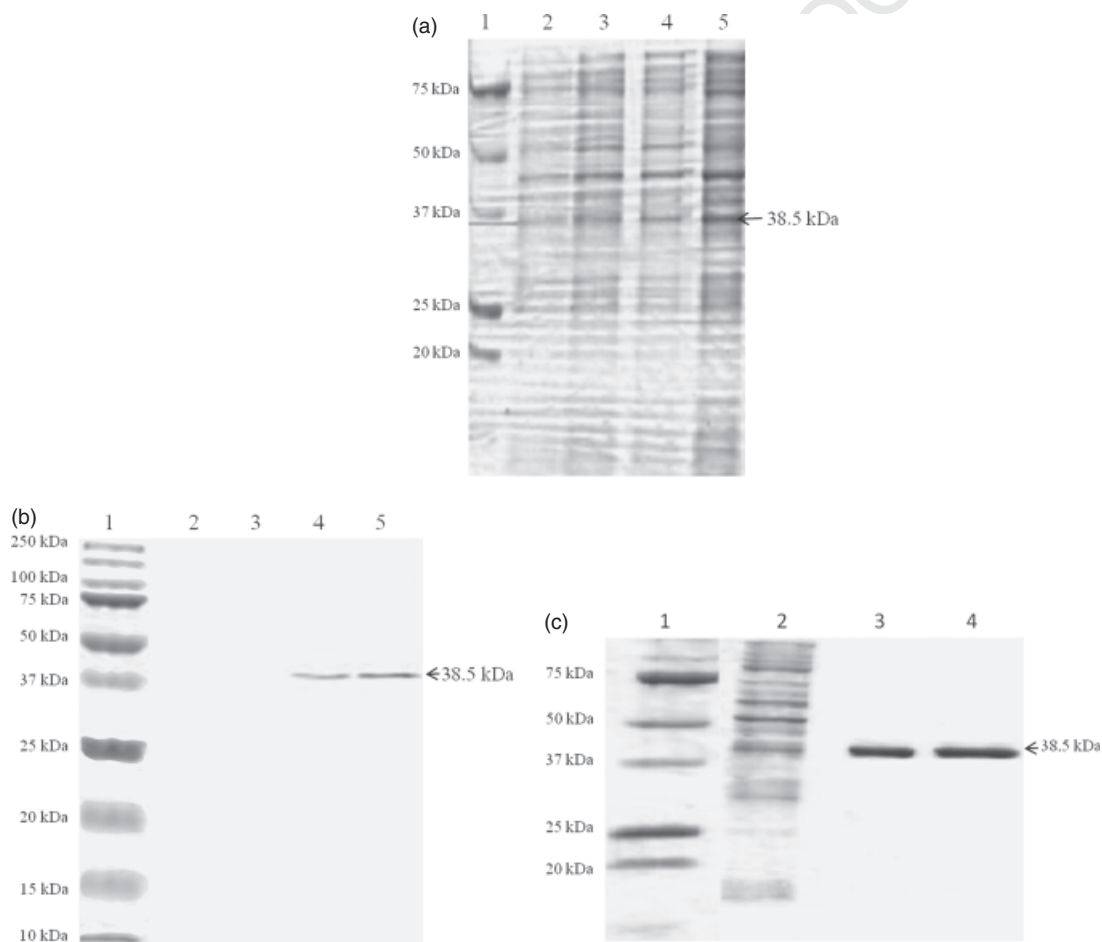

Fig. 4. (a) SDS-PAGE depicts the expression of the TbpA-His fusion protein from *Haemophilus parasuis* Nagasaki strain after 120 min of incubation at 37 °C with different arabinose concentrations. Lane 1, molecular weight marker (Precision Plus Protein™ standards, Biorad); lane 2, *Escherichia coli* LMG194 cells transformed with pBAD-Thio-TbpA-V5-His induced without arabinose; lane 3, *E. coli* LMG194 cells transformed with pBAD-Thio-TbpA-V5-His induced with 0.02% arabinose; lane 4, *E. coli* LMG194 cells transformed with pBAD-Thio-TbpA-V5-His induced with 0.05% arabinose; lane 5, *E. coli* LMG194 cells transformed with pBAD-Thio-TbpA-V5-His induced with 0.075% arabinose. (b) Immunoblotting using an HRPO-labeled murine anti-V5 monoclonal antibody shows the expression of the TbpA-His fusion protein. Lane 1, molecular weight marker similar to Fig. 3a; lane 2, nontransformed *E. coli* LMG194 cells (negative control); lane 3, *E. coli* LMG194 cells transformed with pBAD-Thio-TbpA-V5-His induced without arabinose; lane 4, *E. coli* LMG194 cells transformed with pBAD-Thio-TbpA-V5-His induced with 0.075% arabinose (optimal induction conditions); and lane 5, *E. coli* LMG194 cells transformed with pBAD-Thio-TbpA-V5-His induced with 2% arabinose. (c) SDS-PAGE depicts the correct purification of the TbpA-His fusion protein after nickel affinity chromatography. Lane 1, molecular weight marker similar to Fig. 3a; lane 2, TbpA fusion protein before being purified; lanes 3 and 4, eluted fractions of TbpA.
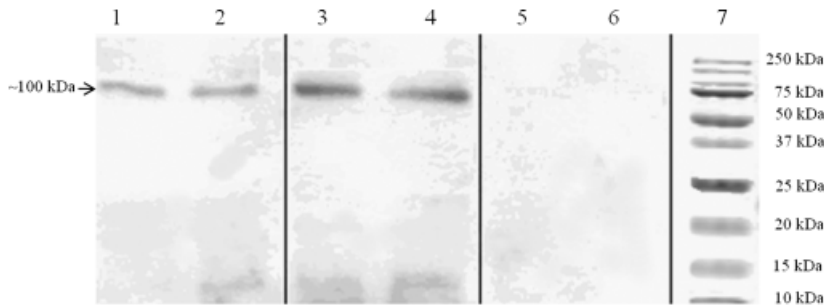
Fig. 5. Immunoblotting using rabbit polyclonal antibodies against the rTbpA fragment in some gram-negative and gram-positive strains. Lanes 1 and 2, iron-binding protein extract from *Haemophilus parasuis* Nagasaki strain (serovar 5); lanes 3 and 4, iron-binding protein extract from *Actinobacillus pleuropneumoniae* WF83 (serotype 7); lane 5, iron-binding protein extract from *Staphylococcus aureus* CIP 5710; and lane 6, iron-binding protein extract from *H. parasuis* without the first antibody.
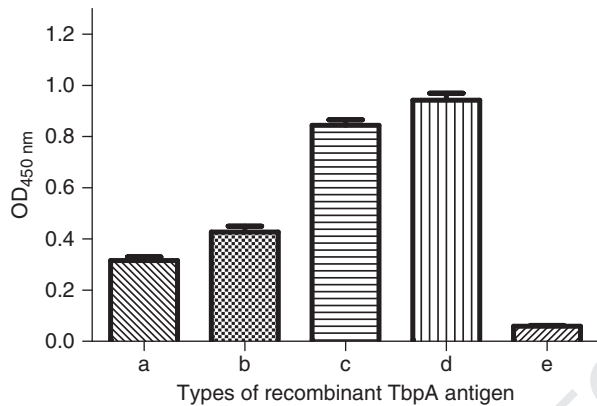

Fig. 6. Indirect ELISA results: mean levels ± SD of rabbit sera after immunization with different preparations of rTbpA. (a) Minced pieces of nitrocellulose membrane containing purified rTbpA, (b) the same antigen as (a), but treating a nitrocellulose membrane with DMSO, (c) small pieces of an electrophoresis gel containing the band corresponding to rTbpA, (d) the purified protein extract and (e) only PBS instead of rTbpA.
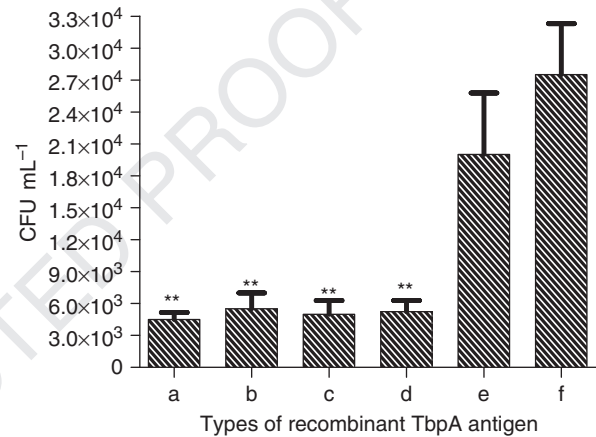

Fig. 7. Bactericidal activity of rabbit polyclonal antibodies specific for different preparations of rTbpA against the *Haemophilus parasuis* Nagasaki strain. (a) Minced pieces of a nitrocellulose membrane containing purified rTbpA, (b) the same antigen as (a), but treating a nitrocellulose membrane with DMSO, (c) small pieces of an electrophoresis gel containing the band corresponding to rTbpA, (d) the purified protein extract, (e) only PBS instead of rTbpA and (f) complement control (*H. parasuis* + commercial baby-rabbit serum as the source of the complement). **Significant differences ($P < 0.01$) compared with groups (e) and (f).

protein, and 250 mM in PBS showed the highest rate of separation from sepharose. The eluted fraction was subjected to a new SDS-PAGE in order to confirm purity (Fig. 4c).

Immunoblotting for testing the specificity of polyclonal antibodies

In order to demonstrate the specificity of the rabbit antibodies against the rTbpA fragment, immunoblots using other *Pasteurellaceae* were performed. Positive results (a 100 kDa band corresponding to a bacterial extract containing iron-binding proteins) were obtained for the *H. parasuis* Nagasaki strain and *A. pleuropneumoniae* WF83. In addition, *S. aureus* CIP 5710 was included in the study, and no bands were revealed for this gram-positive organism (Fig. 5).

ELISA results

The highest antibody levels were reached for antigens *c* and *d*, the ODs being about 15 and 17 times higher, respectively, than that obtained when immunizing with only PBS (Fig. 6). Antigen *b* resulted in antibody levels about one-half those measured for antigen *c*, while those of antigen

a were approximately one-third those of antigen *d*. This latter antigen resulted in significantly higher titers than the remaining preparations ($P < 0.01$ vs. antigen *c*, and $P < 0.001$ vs. antigens *a* and *b*). Similarly, significant differences ($P < 0.001$) were found between antigen *c* vs. antigens *a* and *b*.

Bactericidal assays

Haemophilus parasuis counts were significantly lower for all sera developed against any of the rTbpA fragment preparations, ranging from $(4.5 \pm 1.3) \times 10^3$ CFU mL^{-1} for group (a) to $(5.5 \pm 3.0) \times 10^3$ CFU mL^{-1} for group (b), compared either with group (e) (PBS) or (f) (without serum) ($P < 0.01$ in both cases). No significant differences were found when comparing any of the groups (a) to (d) with each other (Fig. 7).
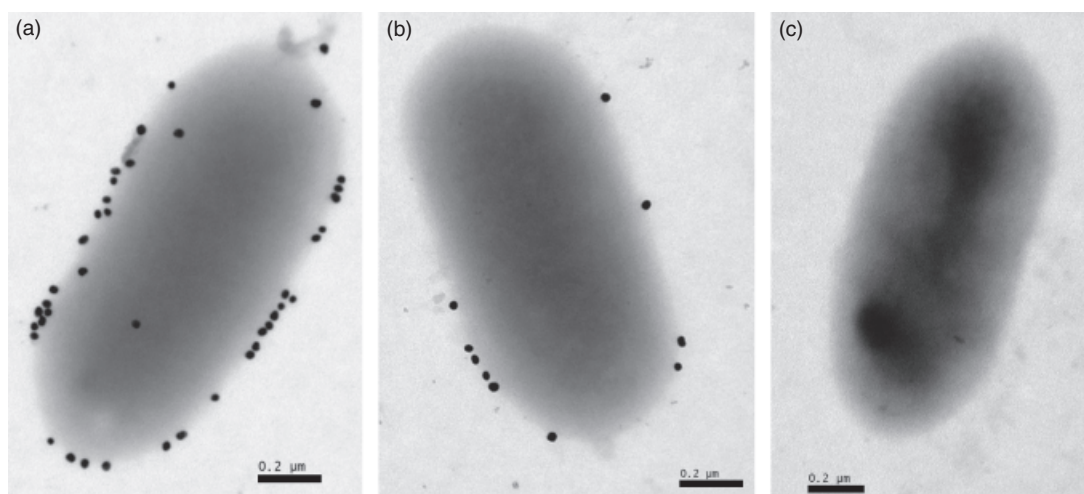
Fig. 8. Immunogold labeling of *Haemophilus parasuis* Nagasaki strains (a) grown in an iron-sufficient medium using rabbit antisera developed against minced pieces of a nitrocellulose membrane containing purified rTbpA, (b) the same, but using *H. parasuis* grown in an iron-deficient medium, (c) negative control (without the first antibody). Scale bar = 0.2 μm.

Immunogold labeling

Haemophilus parasuis Nagasaki strain cells (0.2–2.0 × 1.0–7.0 μm), grown in an iron-deficient medium and exposed to any of the sera developed, were covered with an irregular and discontinuous layer of gold particles (Fig. 8a). A minor amount of gold particles was seen when this *H. parasuis* strain was grown in an iron-sufficient medium (Fig. 8b). Finally, these particles were absent on cells in which the first antibody was excluded (Fig. 8c).

Discussion

For access to these limited resources of iron, pathogenic bacteria from the family *Pasteurellaceae* can either synthesize siderophores (del Río *et al.*, 2006) or utilize high-affinity iron uptake systems, such as Tbps (Litwin & Calderwood, 1993). The organization of the TonB region, involved in transferrin iron uptake and composed of *tonB*, *exbB*, *exbD*, *tbpB* and *tbpA* genes, has already been described in *H. parasuis* (del Río *et al.*, 2005), but the expression of the *tbpA* gene has not been reported previously. The TbpA forward primer designed in this study, along with the reverse primer tbpA33 reported previously (de la Puente Redondo *et al.*, 2000), successfully allowed the amplification of the complete *tbpA* gene, unlike the forward primer designed by de la Puente Redondo *et al.* (2000), which was unable to amplify the first 21 nucleotides of the *tbpA* gene. As the amplification product of *tbpA* gene obtained in *H. parasuis* is different in size from the 2.8-kb fragment revealed in *A. pleuropneumoniae* and *A. suis* (de la Puente Redondo *et al.*, 2000), the amplification of this gene could be a good candidate for an effective diagnostic tool for porcine respiratory infections caused for *Pasteurellaceae*.

On the other hand, the molecular mass of the predicted, mature TbpA of *A. suis* was 104.3 kDa (Bahrami *et al.*, 2003), while that of a complete rTbpA of *A. pleuropneumoniae* was 110 kDa (Kim & Lee, 2006). After selection of a 600-bp *tbpA* fragment from *H. parasuis*, purification and elution of rTbpA, there was clear evidence of the production of a 38.5 kDa protein on the SDS-PAGE gel, which represents about one-third of the estimated size for the complete TbpA of other *Pasteurellaceae*. In a previous study, an rTbpB from *H. parasuis* was generated (del Río *et al.*, 2005) using a similar methodology; however, in that case, rTbpB contained the first 102 amino acids of its N-terminal domain and it was shown that this partial rTbpB behaved as an immunodominant antigen, in agreement with that described for the N-terminal domain of other gram-negative organisms (del Río *et al.*, 2005). This N-terminal domain was not selected for the rTbpA fragment tested here because an earlier report about gonococcal TbpA (Yost-Daljev & Cornelissen, 2004) showed that the most exposed fragments are located in intermediate domains, which therefore are more readily accessible to antibodies.

According to the data gathered in our study, the intermediate domain of *H. parasuis* rTbpA might also represent an immunodominant region, as the rabbit antibodies raised against it developed high titers by ELISA and also reacted against TbpA from other *Pasteurellaceae*, such as *A. pleuropneumoniae*, revealing the high conservation of this protein, as reported in other species (González *et al.*, 1995; Myers *et al.*, 1998). In this respect, other porcine rTbps generated from *A. pleuropneumoniae* have developed a strong humoral immune response in experimental studies in pigs, being comparable to that induced by natural infection (Rossi-Campos *et al.*, 1992). On the other hand,

the bactericidal activity revealed by any of the four sera developed clearly shows that our rTbpA fragment, about one-third of the full length of native TbpA, was sufficient for the induction of bactericidal antibodies against the homologous serovar of *H. parasuis*. In this sense, a hypothetical protection induced by this rTbpA fragment against *H. parasuis* infection might be due to complement-mediated lysis, and serum bactericidal activity might be an appropriate predictor of efficacy for a potential vaccine based on this recombinant protein fragment. Finally, electron microscopy confirmed that the native TbpA appears to be accessible to antibodies at the cell surface, because the rabbit antibodies raised against this rTbpA fragment were able to bind specifically to *H. parasuis*.

Protective responses against TbpA from other gram-negative organisms, such as *Neisseria meningitidis* (Ferreiros & Criado, 1994; West *et al.*, 2001) and *A. pleuropneumoniae* (Kim & Lee, 2006), have demonstrated the potential efficacy of this protein as a vaccine candidate. The production of a soluble and purified form of *H. parasuis* rTbpA fragment, which is likely to be surface accessible to antibodies, provides an opportunity to directly assess whether this antigen can serve as a good candidate to protect not only against serovar-specific *H. parasuis* but also against other serovars. In conclusion, this work reports for the first time the characterization of a rTbpA fragment from *H. parasuis* serovar 5, a highly virulent and one of the most prevalent serovars (Oliveira & Pijoan, 2004). Further studies are needed to demonstrate whether this 200-amino acid fragment could be used as an effective vaccine to prevent Glässer's disease.

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Authors' contribution

S.M. and R.F. contributed equally to this work.

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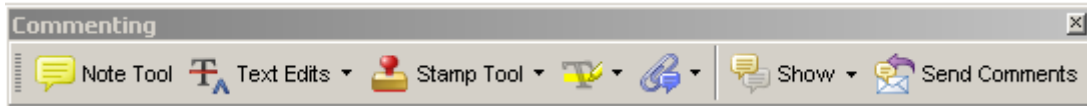
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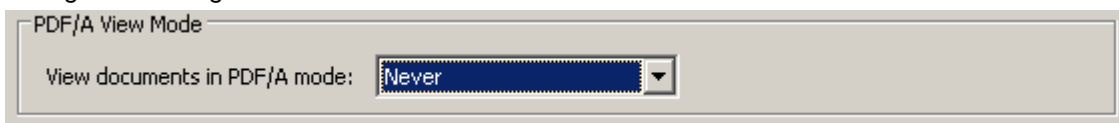
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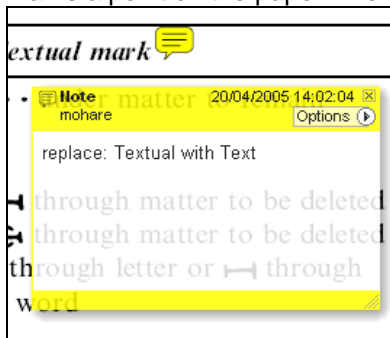
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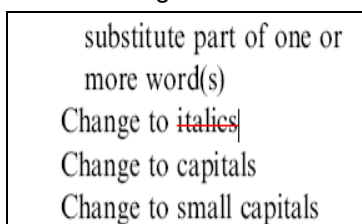Strikes red line through text and opens up a replacement text box.
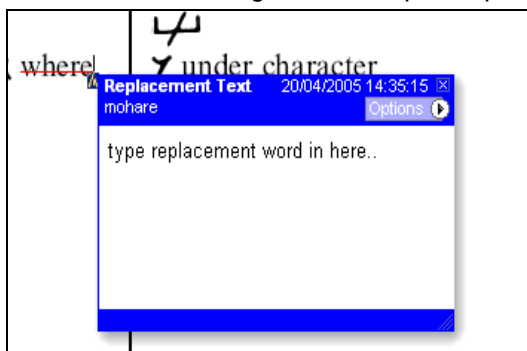
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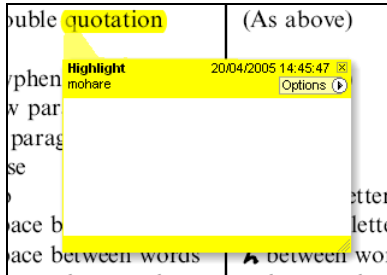

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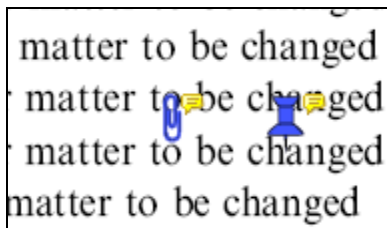
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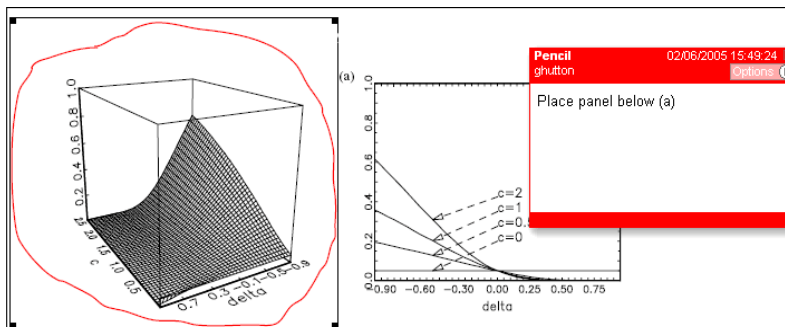
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