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A vaccine based on a mutant transferrin binding protein B of *Haemophilus parasuis* induces a strong T-helper 2 response and bacterial clearance after experimental infection

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ABSTRACT

This study aimed to characterize the type of immune response induced by an experimental vaccine based on a mutant *Haemophilus parasuis* transferrin binding protein (Tbp) B (Y167A) defective in its ability to bind porcine transferrin. Clinical and pathological signs, bacterial clearance, antibody response and the cytokine profile in alveolar macrophages and spleen after the vaccination and challenge of twenty-two colostrum-deprived pigs with 10^8 CFU of *H. parasuis* were analysed. Pigs vaccinated with Y167A were compared to those vaccinated with native TbpB (nTbpB), those treated with a commercial bacterin (CB) against Glässer's disease, those unvaccinated challenged (CH) and those unvaccinated unchallenged (UNCH) pigs. The rectal temperatures of Y167A pigs resembled those of UNCH pigs and were significantly lower than those of the nTbpB, CB and CH animals. A major reduction in pathological changes of the challenged pigs was observed in the Y167A group. *H. parasuis* was cleared from 88.9% of the samples from Y167A pigs *versus* 60.0% and 55.6% from those of the CB and nTbpB groups, respectively. The antibody response elicited by Y167A by ELISA was notably higher than that observed for nTbpB and CB pigs and was capable of preventing the expression and secretion of IL-8. The expression of IL-4 and IL-5, which were associated with the specific antibody levels, suggests that the main mechanism of protection conferred by Y167A vaccine is based on a strong T-helper 2 response.

1. Introduction

Haemophilus parasuis, a γ-proteobacteria that belongs to the *Pasteurellaceae* family, is a gram-negative, non-haemolytic, nicotinamide adenine dinucleotide-dependent organism. Although it is a commensal bacterium of the upper respiratory tract of healthy pigs, in combination with other pathogens of the porcine respiratory disease complex, *H. parasuis* can transform into a pathogen responsible for the development of Glässer's disease (GD), which is typically characterized by serofibrinous to fibrinopurulent polyserositis, arthritis, meningitis and sometimes acute pneumonia. This disease causes high morbidity and mortality in piglets, which results in one of the main causes of economic loss in swine industries worldwide (Aragón et al., 2010). Of the 15 serovars identified to date, serovar 5 is more frequently isolated from respiratory and systemic infection; it is considered a high-virulence serovar and is one of the major agents of neonatal mortality over the world (Oliveira and Pijoan, 2004).

A revolution in the design of vaccines has emerged from the use of postgenomic technologies, and much information about immunogenic components can be derived from immunoproteomic studies (Serruto and Rappuoli, 2006). In fact, the development of subunit vaccines seems to have become an effective alternative to conventional bacterins and, in this respect, several *H. parasuis* outer membrane proteins have resulted in good immunogenicity with diverse protection values (Martín de la Fuente et al., 2009; Zhang et al., 2009; Zhou et al., 2009; Olvera et al., 2011; Fu et al., 2013). Among them, transferrin-binding proteins (Tbps) play a crucial role in H. parasuis pathogenesis because they specifically bind porcine transferrin. Thus, the protective effect of a vaccine based on native proteins with affinity to porcine transferrin has been confirmed (Frandoloso et al., 2011).

A mutant TbpB that is defective in binding porcine transferrin has been recently developed from the *H. parasuis* Nagasaki strain; the Y167A mutant had more than a 280-fold reduction in binding affinity compared to the recombinant native TbpB (nTbpB); however, the mutant and the wild-type proteins were virtually identical except for

Abbreviations: Y167A, mutant transferrin-binding protein B; nTbpB, native transferrin-binding protein B; CB, commercial bacterin; CH, unvaccinated challenged pigs; UNCH, unvaccinated unchallenged pigs; GD, Glässer's disease; PBST, PBS + 0,5% tween 20; OD, optical density; SD, standard deviation; qPCR, quantitative real-time PCR; BALF, bronchoalveolar lavage fluid

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the absence of an amino acid side chain located in loop 8, the first loop in the cap region of the barrel domain of the N-lobe (Frandoloso et al., 2015). As a continuation of these studies, we describe here some clinical, pathological and immunological parameters of the porcine host response after immunization with Y167A, nTbpB or a commercial bacterin (CB) developed against Glässer's disease (GD) and further challenge with the *H. parasuis* Nagasaki strain.

2. Materials and methods

2.1. Immunization, challenge experiments and ethical guidelines

Twenty-five colostrum-deprived Large White × Pietrain piglets were 4 week old when they were randomly assigned to five experimental groups, taking into account body weight (the five heaviest pigs were assigned one each of the five groups and so successively). One group (Y167A group, $n = 6$) was vaccinated with the Y167A mutant TbpB from *H. parasuis* serovar 5, Nagasaki strain; the second received the recombinant wild-type TbpB from the same strain (nTbpB group, $n = 6$); the third was inoculated with a commercial bacterin (Porcilis Glässer, Intervet, Salamanca, Spain) composed of *H. parasuis* cells belonging to serovar 5, strain 4800 (CB group, $n = 5$); and the fourth remained as a challenge control group (CH group, $n = 5$) and received PBS alone. Finally, the fifth group (UNCH group, $n = 3$) was neither immunized nor challenged. The Y167A and nTbpB groups received 200 μg of protein antigen in 2 ml of a mixture of PBS and Montanide IMS 2215 VG PR adjuvant (Seppic, Inc., Paris, France). The first four experimental groups were inoculated twice (at 28 and 49 days of age for Y167A and nTbpB vaccines, and at 28 at 42 days for commercial bacterin, as recommended by the manufacturer) intramuscularly and then challenged by intratracheal injection of 10^8 CFU of *H. parasuis* Nagasaki strain. The pigs' rectal temperatures and other clinical signs were monitored every 12 h for the first seven days post-challenge (pc) and then monitored once a day until the end of the study (day 14 pc). A one-way analysis of variance was used for the comparison of temperatures at various times after challenge in each group and between groups until 60 h pc.

Pigs with severe signs of distress were humanely euthanized for necropsy, and those that survived challenge were euthanized 14 days pc. The experiment was approved by the Executive Commission of the Ethical Committee of the University of León (protocol 1-2011) and adhered to the guidelines of the Spanish Government and the European Community.

2.2. Pathological studies

All pigs were subjected to necropsy, and gross lesions were recorded, with special attention paid to the pleural, pericardial and peritoneal cavities; joints; lungs; spleen; heart; and the central nervous system. The severity of pathological changes was recorded blindly and indicated by - (no changes), + (mild changes, <25% in extent), ++ (moderate changes, 25- < 50% in extent) or +++ (severe changes, ≥50% in extent).

2.3. Bacterial isolation and Haemophilus parasuis confirmation by polymerase chain reaction (PCR)

Swabs were collected aseptically and cultured aseptically from the lung, spleen, brain, abdomen, hock joints and heart blood on chocolate blood agar for 24 h at 37 °C in 5% CO_2. Suspicious colonies were confirmed by PCR (Angen et al., 2007).

2.4. Enzyme-linked immunosorbent assays (ELISAs)

All pigs were bled from the jugular vein before the first and second immunizations and before challenge, and those surviving were also bled at one, three, eight and 14 days after challenge. Five micrograms of biotinylated recombinant nTbpB or Y167A diluted in 100 μl of PBS + Tween 20 (PBST) were added to each well of a streptavidin-coated ELISA plates (ELISA Duo-Set®, Vitro, Madrid, Spain) and incubated at room temperature for 1 h. Non-binding proteins were washed, and then the plates were blocked with 5% skim milk in PBST (PBS + 0,5% Tween 20). Sera from CH, nTbpB, Y167A and CB pigs were added at a 1:100 dilution and incubated for 60 min at room temperature. After washing with PBST, goat anti-swine IgG (Sigma, Madrid, Spain) horseradish peroxidase-conjugated antibody at a 1:10,000 dilution was added, and the plates were developed at 450 nm (Curran et al., 2015).

In addition, IL-8 cytokine quantification was assessed by indirect ELISA using a commercial Porcine IL-8 ELISA immunoassay kit (Thermo Fisher Scientific, Madrid, Spain). Microplates were incubated with the porcine sera from CH pigs, UNCH pigs and the three vaccinated groups taken before immunization, after the second immunization and two days after challenge.

The samples were run in triplicate for both ELISAs, and a serum sample was considered positive when its optical density (OD) was at least twice as high as that of the mean before the first immunization + standard deviation (SD). The ODs were analysed by Prism 6 (Graph Pad Software, La Jolla, CA, USA). The normality of the data (ODs) was confirmed, and multiple comparisons were performed using a two-way ANOVA. Significance was set at $p < 0.05$.

2.5. Quantitative RT-PCR (qPCR)

qPCR was used to verify cytokine gene expression after challenge using *ex-vivo* tissues. After necropsy, small fragments (about 0,5 cm) of the spleen tissue were collected and maintained as described by Frandoloso et al. (2013). In parallel, the bronchoalveolar lavage fluid (BALF) was sampled, according to Kowalczyk et al. (2014). Briefly, each lung was washed twice with PBS, the cell suspension was filtered with cell strainers (70 μm) (Fisher Scientific, Madrid, Spain) and centrifuged at 300 × *g* for 5 min, and the resulting pellet was counted. Total cellular RNA was purified from 30 mg (tissue) or from 3×10^6 cells (BALF) using the RNeasy Mini Kit (Qiagen, Hilden, Germany). The genomic DNA was digested with RNase-Free DNase Set (Qiagen). cDNA synthesis was performed by reverse transcription using a First-Strand cDNA Synthesis Kit (Invitrogen, Madrid, Spain). The set of primers and hydrolysis probes used to determine the expression of all molecules is described in Table 1.

qPCR was conducted in 96-well plates (LightCycler 480 Multiwell Plates 96, white, Roche, Madrid, Spain) in a total volume of 20 μl containing 500 nM of each primer, 100 nM of each specific probe, 1× Light Cycler Probes Master (Roche) and 5 μl of cDNA (diluted 1:10). The PCR reactions were run on a Mastercycler® RealPlex (Eppendorf, Endfield, USA) with the following conditions: an initial denaturation step (95 °C, 10 min) followed by 40 cycles of 95 °C for 20 s (denaturation), 60 °C for 30 s (annealing) and 72 °C for 20 s (extension). qPCR reactions were run in duplicate, and no-template controls for reverse transcription and qPCR were included. Calibration curves for each cytokine and reference gene were obtained using tenfold serial dilutions of a plasmid DNA with the specific nucleotide sequence of each gene (Frandoloso et al., 2013). The relative quantification of each gene was calculated using the

Table 1
Oligonucleotides used in qPCR analysis.

Genes	Sequences (5′ to 3′)	Efficiency (%)	Reference
IL1-β	CCAAAGGCCGCCAAGATATAA GGACCTCTGGGTATGGCTTTC	100 ± 0.2	Arce et al. (2010)
	FAM-CTGACTTCACCATGGAAGTCCTCTCTCCCTAAG-BHQ2		
IL-2	GAGCCATTGTCGCTGGATT CCTCCAGAGCTTTGAGTTCTTCTACTA	98.1 ± 0.7	This study
	FAM-TCAATGCCCAAGCAGGCTACAGAATTG-BHQ2		
IL-4	CTGCCCCAGAGAACACGA ACAGAACAGGTCATGTTTGCC	96.2 ± 1.1	This study
	FAM-CCGCTCAGGAGGCTCTTCATGCAC-BHQ2		
IL-5	CGTTAGTGCCATTGCTGTAGAAA CAAGTTCCCATCGCCTATCAG	98.7 ± 0.6	This study
	FAM-AGACCTTGACACTGCTCTCCATTCATCGA-BHQ1		
IL-6	CTGGCAGAAAACAACCTGAACC TGATTCTCATCAAGCAGGTCTCC	98.6 ± 0.2	Duvigneau et al. (2005)
	FAM-TTGAACCCAGATTGGAAGCATCCGTCTTTT-BHQ1		
IL-8	TTCGATGCCAGTGCATAAATA TGACAAGCTTAACAATGATTTCTGAA	99.8 ± 0.8	Arce et al. (2010)
	FAM-CATTCCACACCTTTCCACCCCAAATTTATC-BHQ2		
IL-10	CGGCGCTGTCATCAATTTCTG CCCCTCTCTTGGAGCTTGCTA	99.8 ± 0.3	Duvigneau et al. (2005)
	FAM-AGGCACTCTTCACCTCCTCCACGGC-BHQ1		
IL-13	CCAGAACCAGAAGACACCCCTA CTTGCCAGGAACTTGCTCG	97.8 ± 0.9	This study
	FAM-CGCCCTGGAATCCCTCATCAACATCTC-BHQ2		
IL-17A	CTGTCACTGCTGCTTCTGCT CATGCTGAGGGAAGTTCTTG	98.1 ± 0.5	This study
	FAM-TCATGATCCCACAAAGTCCAGGATG -BHQ1		
CCL-2	ACCAGCAGCAAGTGTCCTAAAG TCCTGGACCCACTTCTGCTT	99.4 ± 0.7	Arce et al. (2010)
	FAM-AGCAGTGATCTTCAAGACCATCGCGG-BHQ2		
TLR-4	GCCATCGCTGCTAACATCATC CTCATACTCAAAGATACACCATCGG	99.7 ± 0.2	Frandoloso et al. (2013)
	FAM- CAAAAGTCGGAAGGTTATTGTCGTGGTGTC-BHQ1		
TNF-α	GCCCTGGTACGAACCCATCTA CAGATAGTCGGGCAGGTTGATCTC	99.1 ± 0.3	Arce et al. (2010)
	FAM-CCAGCTGGAGAAGGATGATCGACTCAGT-BHQ2		

Table 1 (*Continued*)

Genes	Sequences (5′ to 3′)	Efficiency (%)	Reference
IFN-γ	CGATCCTAAAGGACTATTTTAATGCAA TTTTGTCACTCTCCTCTTTCCAAT	98.4 ± 0.3	Duvigneau et al. (2005)
	FAM-ACCTCAGATGTACCTAATGGTGGACCTCTT-BHQ1		
cyclophilin	TGCTTTCACAGAATAATTCCAGGATTTA GACTTGCCACCAGTGCCATTA	100.0 ± 0.1	Duvigneau et al. (2005)
	FAM-TGCCAGGGTGGTGACTTCACACGCC-BHQ1		

ΔΔCT method (Livak and Schmittgen, 2001). The calculated PCR efficiencies ranged from 97% to 100%.

Data were compared using Student's *t*-test or a Mann-Whitney test, depending on the data normality (assessed by the Bartlett's test) for significant effects. Significance was set at $p < 0.05$. Because the number of animals was not sufficient to compare all groups in the same moment (14 days after the challenge), we could compare only data from the nTbpB ($n = 3$), Y167A ($n = 6$) and UNCH ($n = 3$) groups. The expression data from the nTbpB and Y167A groups were first compared with those observed for the UNCH group. The corresponding mathematical results were used to compare the gene expression between the nTbpB and Y167A groups.

3. Results

3.1. Clinical and pathological results

All CH pigs died between 14 and 72 h after infection and showed temperatures above 41 °C from 12 h pc until death (for instance, 41.5 ± 0.3 at 48 h), which were significantly higher than those recorded before infection ($p < 0.05$, Table 2). Other clinical signs suggesting GD (limb incoordination, swollen joints, claudication, coughing and severe dyspnea) were observed in all animals in this group. In contrast, two of the five CB animals died from 18 h to 20 h pc, and two others were euthanized at 108 h and six days after infection because of the severity of clinical signs. Three of the nTbpB pigs died at 48, 72 and 108 h pc. Their clinical signs were similar to those of CH pigs, and their temperatures are shown in Table 2.

All Y167A pigs survived the challenge. Their rectal temperatures were significantly lower (approximately 2 °C below) than those recorded for CH ($p < 0.05$ from 12 h to 60 h pc), nTbpB ($p < 0.05$ at 12 h and from 48 to 60 h pc) and CB pigs ($p < 0.05$ from 36 h to 48 h pc) and resembled those for UNCH pigs. Even so, the temperatures recorded at 12, 24 and 60 h were significantly higher ($p < 0.05$) than those measured before the challenge (Table 2). In regards to other clinical signs, only mild lower limb claudication or weakness was observed in three of the six pigs in this group.

A summary of the main pathological changes found in the four experimental groups is shown in Table 3. In the three vaccinated groups, a notable reduction of pathological changes was shown compared to CH pigs. In fact, the Y167A pigs had the smallest lesions, followed by the nTbpB and CB groups, and the pigs exhibited quite similar lesion severity in the different locations sampled.

3.2. Recovery and confirmation of Haemophilus parasuis

H. parasuis was recovered from only 11.1% (one from each of the lung or abdominal cavity samples and two isolates from the spleen) of all the samples tested in the Y167A group, a rate notably lower than that recorded from CB and nTbpB pigs (40.0 and 44.4%, respectively, Table 4). Specifically, *H. parasuis* was isolated from at least one-third of the tissues sampled (spleen, brain and hock joints) in the nTbpB group, and from at least 40% of the tissues sampled (these three same locations + blood taken from heart) in the CB group. In contrast, *H. parasuis* was recovered from 92.0% of the total samples in CH pigs, with 100% from the abdomen and 80% from the remaining tissues except for the brain, with only two isolations from this organ.

Table 2
Rectal temperatures in control pigs or surviving pigs at different intervals after challenge with *Haemophilus parasuis.*

Pig group[b]	Temperature (°C) at intervals after challenge (*n*)[a]					
	before	12 h	24 h	36 h	48 h	60 h
CH (5)	39.3 ± 0.3 (5)	41.1 ± 0[c d] (5)	41.2 ± 0.7[c d] (4)	41.2 ± 0.4[c d] (4)	41.5 ± 0.3[c d] (3)	41.6[c d] (1)
Y167A (6)	38.3 ± 0.3 (6)	39.6 ± 0.1[c] (6)	39.9 ± 0.5[c] (6)	39.3 ± 0.7 (6)	39.3 ± 0.2 (6)	39.9 ± 0.1[c] (6)
nTbpB (6)	39.1 ± 0.3 (6)	40.8 ± 0.6[c d] (6)	40.3 ± 0.7[c] (6)	40.5 ± 0.8[c] (6)	41.3 ± 0.3[c d] (6)	41.1 ± 0.7[c d] (5)
CB (5)	38.9 ± 0.4 (5)	40.2 ± 0.6[c] (5)	40.1 ± 0.5[c] (3)	40.7 ± 1.2[c d] (3)	40.9 ± 0.4[c d] (3)	40.7 ± 0.9[c] (3)
UN (3)	39.5 ± 0.5 (3)	39.6 ± 0.2 (3)	39.6 ± 0.1 (3)	39.0 ± 0.2 (3)	39.3 ± 0.4 (3)	39.3 ± 0.5 (3)

[a] The number of surviving pigs at each time point is given in brackets.

[b] CH: unvaccinated, challenged pigs; Y167A: pigs vaccinated twice with Y167A mutant TbpB and challenged; nTbpB: pigs vaccinated twice with native recombinant TbpB and challenged; CB: pigs vaccinated twice with a commercial bacterin (Porcilis Glässer) and challenged; UNCH: unvaccinated, unchallenged pigs.

[c] Significant differences ($p < 0.05$) compared to the time before infection in each group.

[d] Significant differences ($p < 0.05$) compared to the Y167A group.

Table 3
Pathological changes found in each of the four groups infected with the *Haemophilus parasuis* Nagasaki strain.

Pathology	Group[a]			
	CH	Y167A	nTbpB	CB
Fibrinous polyarthitis	+++[b] (1)	+ (3)	++ (2)	++ (2)
	++ (1)	− (3)	+ (2)	− (3)
	+ (1)		− (2)	
	− (2)			
Fibrinous peritonitis	+++ (4)	++ (1)	++ (2)	++ (2)
	++ (1)	+ (4)	+ (4)	+ (3)
		− (1)		
Fibrinous pleuritis	+++ (2)	+ (3)	++ (4)	+++ (2)
	++ (1)	− (3)	+ (2)	++ (1)
	− (2)			− (2)
Fibrinous pericarditis	+++ (3)	+ (5)	+++ (1)	++ (2)
	++ (1)	− (1)	++ (2)	+ (1)
	− (1)		+ (1)	− (2)
			− (2)	
Meningitis	+++ (3)	− (6)	+ (2)	+ (3)
	++ (1)		− (4)	− (3)
	− (1)			
Fibrin deposits in spleen	+++ (4)	+ (2)	++ (2)	++ (1)
	+ (1)	− (4)	+ (2)	+ (2)
			− (2)	− (2)
Lymphoid hyperplasia in spleen	++ (3)	+ (2)	++ (2)	++ (1)
	− (2)	− (4)	− (4)	− (4)
Lung edema	++ (1)	− (6)	− (6)	+ (2)
	− (4)			− (3)
Lung haemorrhages	+ (1)	− (6)	++ (1)	++ (1)
	− (4)		− (5)	+ (1)
				− (3)
Heart haemorrhages	− (5)	− (6)	+ (2)	++ (1)
			− (4)	+ (1)
				− (3)
Congestion	++ (3)	+ (3)	++ (1)	++ (2)
	+ (1)	− (3)	+ (4)	− (3)
	− (1)		− (1)	

[a] CH: unvaccinated, challenged pigs; Y167A: pigs vaccinated twice with Y167A mutant TbpB and challenged; nTbpB: pigs vaccinated twice with native recombinant TbpB and challenged; CB: pigs vaccinated twice with a commercial bacterin (Porcilis Glässer) and challenged.

[b] Lesion severity: -, no changes; +, mild changes; ++, moderate changes; +++, severe changes. The number in brackets indicates the number of pigs experiencing this change in each experimental group.

Table 4
Bacterial recovery followed by PCR confirmation in each of the four groups infected with the *Haemophilus parasuis* Nagasaki strain.

	Number of pigs positive for *H. parasuis* in[a]			
Sample	CH (5)	Y167A (6)	nTbpB (6)	CB (5)
Lung	4	1	3	3
Spleen	4	2	2	2
Brain	2	0	2	2
Abdominal cavity	5	1	4	3
Hock joints	4	0	2	2
Heart blood	4	0	3	2

[a] CH: unvaccinated, challenged pigs; Y167A: pigs vaccinated twice with Y167A mutant TbpB and challenged; nTbpB: pigs vaccinated twice with native recombinant TbpB and challenged; CB: pigs vaccinated twice with a commercial bacterin (Porcilis Glässer) and challenged. The number in brackets indicates the number of pigs in each group.

3.3. Antibody results

The ODs for the UNCH group were always approximately 0.1 (data not shown), similar to the remaining groups before the first immunization and the CH group until after challenge (Fig. 1A and B). When nTbpB was used as an antigen, the Y167A and CB pigs became seropositive for IgG before the second immunization ($p < 0.001$), as did the nTbpB pigs to a lesser extent ($p < 0.05$) (Fig. 1A). These values increased significantly ($p < 0.001$) on the challenge day and at 24 h pc compared to the CH group for the three vaccinated groups, and from then on, the ODs remained similar until day 14 pc or varied slightly in each group. The IgG response elicited by Y167A was higher than that found for nTbpB throughout the study and was especially higher than that recorded for the CB group, with the latter group showing OD values significantly lower ($p < 0.001$) than those of the Y167A pigs on the challenge day and thereafter (Fig. 1A).

When Y167A was used to coat the plates, a similar tendency was observed (Fig. 1B). In this case, the highest value was measured for the Y167A and nTbpB groups at 24 h pc. The greatest ODs were always those recorded for the Y167A pigs, with values ranging from 0.25 on day 3 pc ($p < 0.001$) to below 0.05 on the challenge day, in comparison to the nTbpB pigs. Major differences were detected relative to CB pigs, whose antibody levels decreased slightly and continually after challenge (Fig. 1B).

3.4. Cytokine expression by ELISA or qPCR

Concerning the proinflammatory molecules, we have analysed the expression of IL-1β, IL-6, IL-8, CCL-2 and TNF-α by ELISA or qPCR. To verify the molecular impact produced by all vaccines tested systemically, we performed an ELISA to quantify the soluble amount of IL-8 present in the serum from all animals. Of the five groups compared, the group exhibiting the lowest IL-8 concentration in the serum was UNCH, followed by the Y167A, CB, nTbpB and CH groups (Fig. 2). The IL-8 in the serum was significantly higher in the CH, nTbpB and CB pigs ($p < 0.001$) at 48 h pc than the levels present before infection. In addition, the IL-8 concentration in the Y167A group after challenge was significantly lower than those recorded in the nTbpB and CB groups and especially lower than that measured in CH pigs ($p < 0.001$) (Fig. 2).

The BALF cells from the two groups with the best clinical protection showed differences in the IL-8 transcription. The animals from the nTbpB group transcribed approximately 200 times more IL-8 than the physiological level (UNCH) 14 days after the challenge; this value was significantly greater ($p < 0.05$) than that detected in the Y167A group (Fig. 3).

Although we were interested in verifying the impact of vaccine-mediated protection on alveolar macrophages from all experimental groups, the comparison of the mRNA expression could be performed only for three groups (nTbpB, Y167A and UNCH) at 14 days after the infection due to the deaths that occurred at different times after the challenge. Following the trends of the clinical signs observed, the expression of IL-1β, IL-6, and MCP-1 were significantly higher ($p < 0.05$) in the nTbpB than in the Y167A group (Fig. 3). Although we did not observe significant differences between the expression of IL-17A and TNF-α in both vaccinated groups, we observed that the expression of IL-17A was approximately 50 and 30 times higher in the nTbpB and Y167A groups than in the UNCH group, respectively (Fig. 3).

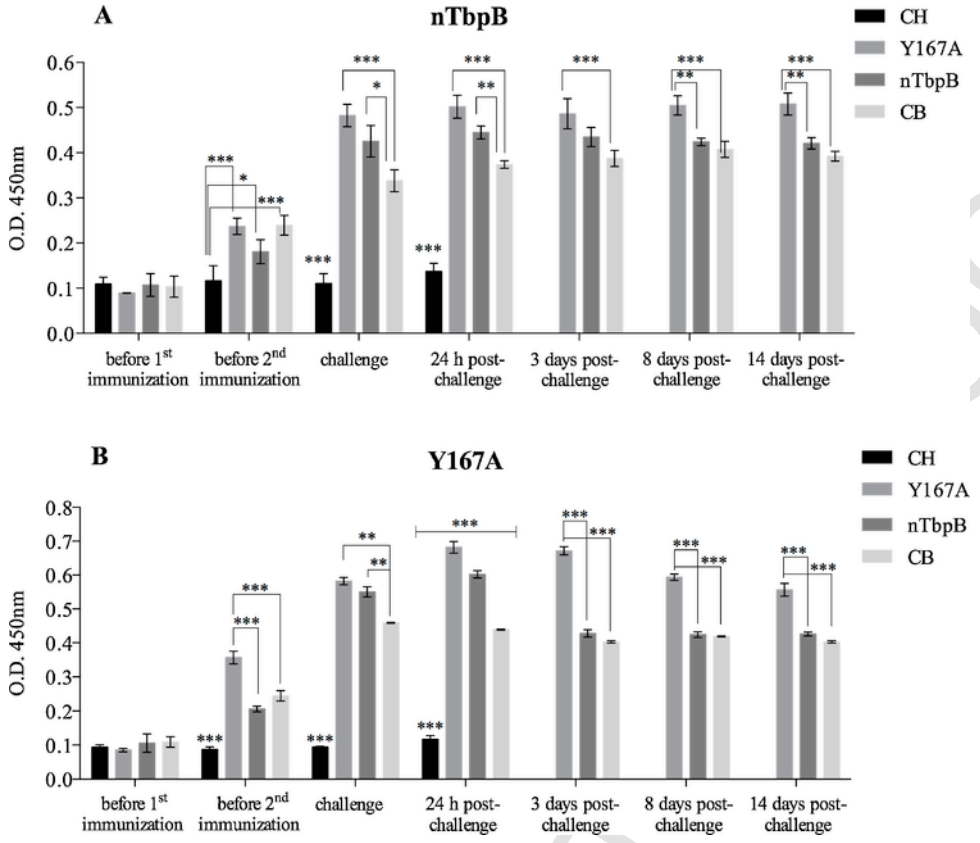

Fig. 1. IgG antibody levels in different experimental groups measured at different times. Plates were coated with wild-type native TbpB (a) or with Y167A mutant TbpB (b). nTbpB: pigs vaccinated twice intramuscularly with native recombinant TbpB and challenged; Y167A: pigs vaccinated twice intramuscularly with Y167A mutant TbpB and challenged; CH: unvaccinated, challenged pigs; CB: pigs vaccinated twice intramuscularly with a commercial bacterin (Porcilis Glässer) and challenged. The data were compared between groups. */**/*** Significant differences ($p < 0.05$, $p < 0.01$ or $p < 0.001$, respectively).

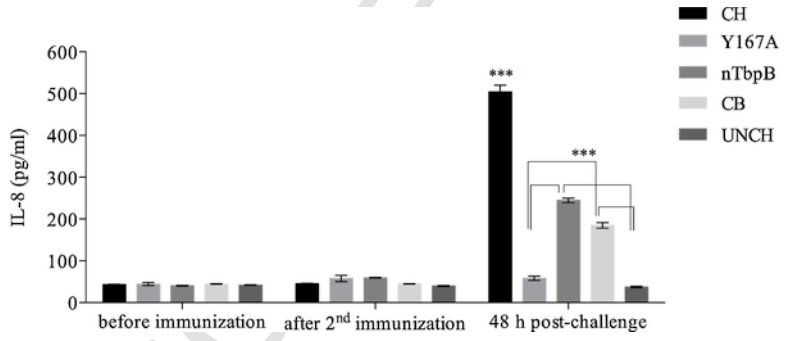

Fig. 2. Concentration of IL-8 in the serum (pg/ml) from the different experimental groups measured at different times. CH: unvaccinated, challenged pigs; Y167A: pigs vaccinated twice intramuscularly with Y167A mutant TbpB and challenged; nTbpB: pigs vaccinated twice intramuscularly with native recombinant TbpB and challenged; CB: pigs vaccinated twice intramuscularly with a commercial bacterin (Porcilis Glässer) and challenged; UNCH: unvaccinated, unchallenged pigs. *** Significant differences ($p < 0.001$) compared to the time before and between all groups at 48 h post-challenge.

To understand the molecular modulation of the specific immune response developed after the infection, we analysed in spleen a panel of cytokines involved in Th1 (IL-2 and INF-γ) and Th2 (IL-4, IL-5, IL-10 and IL-13) immune responses. Both vaccines, nTbpB and Y167A, showed a similar mRNA expression profile of IL-5, IL-13, IFN-γ and IL-10; however, IL-2 and IL-4 were significantly more expressed in the nTbpB group than in the Y167A group ($p < 0.05$ and $p < 0.01$, respectively) (Fig. 4).

4. Discussion

A subunit vaccine based on a site-directed mutant of TbpB from the *H. parasuis* Nagasaki strain (serovar 5) being defective in its ability to bind porcine transferrin was compared in colostrum-deprived pigs with the native form of TbpB and with a commercial serovar 5 vaccine. Clinical and pathological signs, bacterial clearance, antibody response to mutant and native TbpB antigens and cytokine profile in bronchoalveolar lavage fluids and spleen were evaluated after immunization and challenge with a lethal dose of Nagasaki strain.

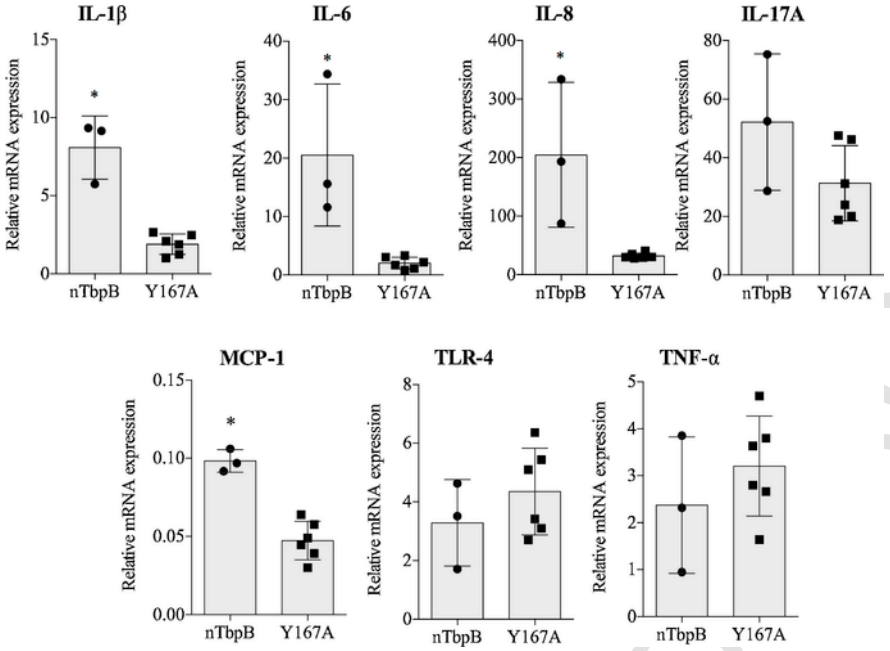

Fig. 3. Relative mRNA expression rates of cytokines from alveolar macrophages from the nTbpB (pigs vaccinated twice intramuscularly with native recombinant TbpB and challenged) and Y167A (pigs vaccinated twice intramuscularly with Y167A mutant TbpB and challenged) groups. The data shown here are those obtained after comparison to those measured in the UNCH (unvaccinated unchallenged pigs) group. * Significant differences ($p < 0.05$) compared to the remaining groups.

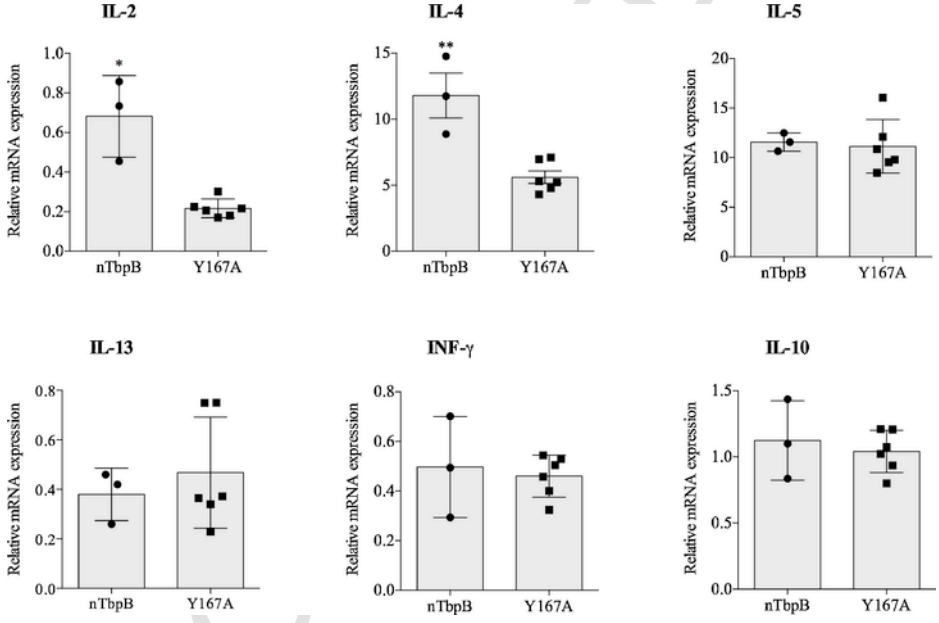

Fig. 4. Relative mRNA expression rates of cytokines from the spleens of the nTbpB (pigs vaccinated twice intramuscularly with native recombinant TbpB and challenged) and Y167A (pigs vaccinated twice intramuscularly with Y167A mutant TbpB and challenged) groups. The data shown here are those obtained after comparison to those measured in the UNCH (unvaccinated unchallenged pigs) group. */** Significant differences ($p < 0.05$/$p < 0.01$) compared to the remaining groups.

The rectal temperatures recorded for Y167A group showed a similar behaviour as those found for the unvaccinated and unchallenged pigs. This absence of hyperthermia, deaths and the scarcity of other relevant clinical signs in the pigs immunized with this mutant TbpB clearly state the stronger protection provided against challenge compared with the lower efficiency showed by the native TbpB and especially by the commercial bacterin. Similarly, the gross pathological results clearly demonstrate the better efficiency of the Y167A vaccine relative to the vaccine formulated with the wild-type TbpB molecule and the vaccine composed of inactivated whole cells from *H. parasuis* strain 4800 (CB). The gross lesions described in Table 3 closely resembled those previously reported in similar experiments, in which colostrum-deprived pigs were also infected intratracheally with a *H. parasuis* Nagasaki strain (Martín de la Fuente et al., 2009; Frandoloso et al., 2011).

In addition, the results listed in Table 4 reveal that *H. parasuis* was markedly cleared on day 14 pc in the pigs inoculated with the Y167A vaccine, with full clearance in the brain, heart blood and hock joints and almost total clearance in the lung and abdominal cavity. These findings show again that the Y167A mutant has the best protective effect of the three vaccines compared. As in our study for the Y167A group, no *H. parasuis* was recovered from the brain parenchyma and hock joints in a previous report in which colostrum-deprived pigs were immunized with a pool of nine proteins with affinity to porcine transferrin and then challenged with the Nagasaki strain (Frandoloso et al., 2011, 2012).

Concerning humoral response, the fact that the antibodies developed by the pigs immunized with Y167A were able to specifically bind to the nTbpB antigen to a higher extent than those developed by the pigs immunized with the native nTbpB seems to suggest that the antibodies against the mutant TbpB protein (Y167A) are capable of recognizing more diverse epitopes from the N-lobe than the wild-type protein, a finding already demonstrated in the report in which Y167A is described (Frandoloso et al., 2015). Furthermore, these data also highlight the superior humoral protective response elicited by the Y167A immunogen.

IL-8 has been described as the main neutrophil-activating chemoattractant chemokine, and the high expression of this proinflammatory molecule has been considered one of the main inflammatory markers of *H. parasuis* infection by the Nagasaki strain (Frandoloso et al., 2013). The results obtained both by ELISA and qPCR from the BALF cells show that the Y167A vaccine is capable to maintain the level of soluble IL-8 in serum as well as the mRNA transcription from immune cells at levels similar to the physiological values recorded for the UNCH group. Consequently, this experimental vaccine is capable of preventing the local and systemic inflammatory reaction mediated by this chemokine. This provides new evidence of the enhanced protective capability of the Y167A vaccine to control GD caused by the Nagasaki strain.

Several different types of cells can produce IL-17A, such as Th-17 cells (mainly), $CD8^+$ (Happel et al., 2003), γδT cells (Stark et al., 2005) and neutrophils (Ferretti et al., 2003); in the lung tissue, IL-17A can also play an important protective role against infection produced by intracellular (Ye et al., 2001) and extracellular bacterial pathogens (Curtis and Way, 2009). Taking into account this information, the BALF samples collected in our study were analysed by FACS analysis (FSC *vs* SSC dot plot) and the results showed the presence of a small neutrophil population in both groups, being the lymphoid cell infiltration more prevalent (data not shown).

Notably, proinflammatory cytokines such as IL-1β and IL-6 can trigger Th17 cell differentiation (Tsai et al., 2013), and these molecules were overexpressed in the nTbpB group compared to the Y167A group. We observed that the elevated expression of these molecules was accompanied by higher expression of IL-17A in the BALF. Although it is difficult to explain why IL-17A is expressed at similar levels in these two groups with different protection levels, we believe that the amount of residual bacteria in this tissue after challenge could be different. Additionally, it should be noted that γδT cells represent a large subset of lymphocytes present in the peripheral blood of swine, and their migration to the lung tissue could be a source of this molecule in the BALF. Nevertheless, IL-17A expression appears to be useful as a sensitive marker of lung inflammation in swine. Further studies should be performed to determine the cell profiles present in the BALF and the molecules produced by these cells in animals vaccinated and challenged with *H. parasuis*.

Although the nTbpB vaccine has been shown to cause higher mRNA expression of IL-2 and IL-4 at 14 days after the challenge in spleen, we find significantly more IgG in the sera of the animals from the Y167A group than in those from the nTbpB group (Fig. 1). Our data do not allow us to construct a clear justification for this unexpected finding; however, the difference in the systemic protection capacity of these two vaccines could be involved.

In conclusion, the protective mechanism induced by the vaccine based on the Y167A antigen is centred on a strong specific humoral immune response. The single mutation in the N-lobe of the TbpB protein (Y167A) made this antigen more immunogenic than nTbpB, allowing it to induce specific IgG antibodies with high antigenicity and clinical protection against *H. parasuis* infection. Thus, the Y167A vaccine seems to be a suitable candidate to control GD in pigs.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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