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Molecular analysis of lungs from pigs immunized with a mutant transferrin binding protein B-based vaccine and challenged with *Haemophilus parasuis*

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Abbreviations:

GD: Glässer's disease; **Tbp**: Transferrin binding protein; **UNCH**: Unvaccinated challenged pigs; **UNUN**: Unvaccinated unchallenged pigs; **DIGE**: Difference gel electrophoresis; **IPA**: Ingenuity pathway analysis; **APP:** Acute-phase protein.

Highlights

- Inflammatory response genes were seen in Glässer's disease by microarray and qPCR
- Lower expression of them was seen in pigs vaccinated with an *H. parasuis* mutant TbpB
- Cell damage genes were seen in Glässer's disease by microarray and qPCR
- Lower expression of them was seen in pigs vaccinated with an *H. parasuis* mutant TbpB
- Gene and protein expression was similar in mutant TbpB vaccinated and healthy pigs

Abstract

The molecular analysis of pigs vaccinated with a mutant transferrin-binding protein B (Y167A) from *Haemophilus parasuis* was compared with that performed for unvaccinated challenged (UNCH) and unvaccinated unchallenged (UNUN) pigs. Microarray analysis revealed that UNCH group showed the most distinct expression profile for immune response genes, mainly for those genes involved in inflammation or immune cell trafficking. This fact was confirmed by real-time PCR, in which the greatest level of differential expression from this group were *CD14*, *CD163*, *IL-8* and *IL-12*. In Y167A group, overexpressed genes included *MAP3K8, CD14, IL-12* and *CD163*. Proteomics revealed that collagen α-1 and peroxiredoxins 2 and 6 were overexpressed in Y167A pigs. Our study reveals new data on genes and proteins involved in *H. parasuis* infection and several candidates of resistance to infection that are induced by Y167A vaccine. The expression of proinflammatory molecules from Y176A pigs is similar to their expression in UNUN pigs.

Keywords: *Haemophilus parasuis,* Glässer's disease, subunit vaccine, vaccination, mutant transferrin binding protein B, proteomics, microarray, quantitative real-time PCR.

1. Introduction

Haemophilus parasuis causes one of the most important diseases reported in modern swine production, known as Glässer's disease (GD). Piglets are infected by direct contact with sows soon after birth, and the colonization of the nasal mucosa and/or the tonsillar area reaches the highest level at about two months [1,2]. Virulence factors and pathogenic mechanisms have not yet been fully elucidated; however, it is known that virulent strains of *H. parasuis* can overcome the innate immune response of the lung [3,4] extending to the bloodstream. There, this organism is resistant to complement system attack [5] and triggers TCRγδ lymphocyte depletion [6], causing systemic disease. Meningitis, fibrinous polyserositis and polyarthritis are the most common lesions caused by *H. parasuis*; however, this organism can also cause rhinitis, otitis media and pneumonia [7].

Currently, classical available typing tests can identify 15 serovars of *H. parasuis*, but there are many non-typeable isolates [8]; however a multiplex PCR has been recently developed for a rapid serotyping, with much lower rates of non-typeability [9]. Several serovars may be present within a herd, but some of them, such as serovars 4 and 5, are more frequently associated with outbreaks of GD, which causes significant financial loss to the modern swine industry worldwide [10]. GD primarily appears in nursery to early finishing swine, and this appearance is often associated with stress factors. These circumstances strongly influence the epidemiology of *H. parasuis* within a swine population of a given barn, resulting in the spread of virulent isolates from the upper respiratory tract to systemic sites [11].

Vaccination is considered to be the most effective practice used to control GD [11], and some bacterins [12,13] and recombinant antigens have been tested experimentally as vaccines [14-16]. Among *H. parasuis* virulence factors with a

known function are transferrin-binding proteins (Tbps), which play a crucial role in survival by mediating the main pathway for iron uptake [17]. Consequently, they are essential in the pathogenesis of this infection.

The use of Tbps as vaccine antigens has been evidenced in relevant pathogens of the *Pasteurellaceae* family [18,19], to which *H. parasuis* belongs. The effect of a subunit vaccine composed of a TbpA recombinant fragment [20] and the protection conferred by a mutant TbpB (Y167A) [21] have been confirmed in previous studies. In this study, as a continuation of the latter report, we have investigated the impact of *H. parasuis* infection on genomic and proteomic profiles of porcine lungs from animals vaccinated with antigen Y167A or from those left unvaccinated.

2. Materials and methods

2.1. Expression and purification of recombinant TbpB fragments

Recombinant proteins were expressed and purified as previously described [22]. The gene encoding mutant TbpB from the *H. parasuis* Nagasaki strain was cloned into a T7 expression vector encoding a histidine tag and TEV cleavage site preceding the region encoding the mature TbpB protein. After expression and cell disruption, the recombinant fusion protein was isolated from the crude lysate supernatant by Ni-NTA chromatography [21]. The purified fusion protein was treated with TEV protease, and the mixture was subjected to Ni-NTA chromatography and Q-Sepharose chromatography to achieve purity [21].

2.2. Immunization and challenge experiments

Sixteen colostrum-deprived Large White × Pietrain piglets were 4 weeks old when were randomly assigned to three groups. One group, Y167, was vaccinated with the Y167A mutant TbpB (n=6), another (the unvaccinated challenged group, UNCH) only received buffer (n=5), and the third group remained unvaccinated and

unchallenged (UNUN, *n*=5). The Y167A group 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) administered intramuscularly at 28 and 49 days of age, and the other two groups received 2 ml of PBS (pH 7.4) administered intramuscularly on the same days. At 74 days of age, the first two groups, Y167A and UNCH, were intratracheally challenged with 10^8 CFU of *H. parasuis* Nagasaki strain in 2 ml of RPMI 1640. The details of the immunization and challenge protocols have been extensively described [21]. Pigs with severe signs of distress were immediately euthanized. Animals that survived challenge were humanely euthanized at 88 days of age. This 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.

2.3. Nucleic acids and purification

Two-cubic-centimetre samples from each lung, taken from a damaged area or, if not, from the same undamaged area, were frozen at -80º C and treated with RNAlater® solution on ice. Thirty mg of sample were obtained by scraping the surface of the lung samples with a razor blade and homogenizing the resulting samples in 0.6 ml RLT buffer (Qiagen, USA) using a rotor-stator homogenizer. Further RNA extraction was performed using the Qiagen RNeasy Mini Kit (Qiagen). Eluted RNA was treated with DNase using a TURBO DNA-free kit (Ambion). RNA was resuspended in RNase free water and quantified using a Nano-Drop TM1000 Spectrophotometer (Thermo Scientific, USA). RNA quality was checked by agarose gel electrophoresis, and the RNA integrity was evaluated using Experion RNA chips (Bio-Rad, Spain).

2.4. Microarray analysis

Gene expression analysis was carried out using the Gene-Chip Porcine Genome Array by Affymetrix (Affymetrix Inc., Santa Clara, USA) at the Genomics Unit of CABIMER (Andalusian Center of Molecular Biology and Regenerative Medicine, Seville, Spain). This chip contained 23,937 probe sets to interrogate 23,256 transcripts in pig, which represented 20,201 genes. The One-Cycle Eukaryotic Target Labelling Array was used to obtain biotinylated cDNA targets, and they were then cleaned up, fragmented and hybridized to the array following the manufacturer's protocol. Arrays were stained with a Gene-Chip Fluidics Station 450 (Affymetrix) and scanned with an Affymetrix Gene-Chip Scanner 3000. Probe signal intensities were captured and processed with the Gene-Chip Operating Software 1.4.0.036 (Affymetrix), and the results were normalized using multi-array average normalization. Bioinformatics analysis was carried out by the Bioinformatics Platform of Andalucía (University of Málaga, Spain).

Differential expression analysis of genes in the three experimental groups was conducted using Bayesian analysis [23]. A Bayes factor value of 0.05 was used as the cut-off for significantly regulated transcripts. Genes showing significant changes in expression were further investigated to understand the biological pathways involved. The Ingenuity Pathway Analysis tool was used (IPA, Ingenuity Systems, www.ingenuity.com). Gene interaction networks were automatically generated, ranked by score and depicted on IPA. Each node in the network diagram represented a gene, and its relationship with the other molecules was represented by a line. Solid and dotted lines represented direct and indirect associations, respectively. Nodes with a red background were genes detected in this study while grey nodes were molecules

inserted by IPA based upon the Ingenuity Knowledge Base to produce a highly connected network.

For statistical analysis of enriched functions or pathways, IPA Knowledge Base was used as a reference set, and Fisher's exact test was used to estimate the significance of association. *P*-values < 0.05 were considered significant. In a graphical representation of the canonical pathway, "ratio" indicated the percentage of genes taking part in a pathway that could be found in the uploaded data set, and "log" (*P*-value) indicated the level of association. The threshold line represented a *P*-value of 0.05.

2.5. Quantitative real-time PCR (qPCR)

qPCR was used to determine the relative expression of 27 immune response-related genes (Table 1) at the time of necropsy and furthermore to validate microarray results. Lung total RNA (1.5 µg) from four pigs from each experimental group (the minimum number for which the data can be treated statistically) was reverse transcribed to cDNA using the iScript cDNA Synthesis kit (Bio-Rad) in a total volume of 20 µl. cDNA solutions were diluted by adding 60 µl of water and stored at -20ºC. The qPCR assays were performed with an iQ5 Thermo Cycler (Bio-Rad) using 96 well PCR plates. All samples were amplified in duplicate in the same PCR plate, and plates were repeated at least twice. Thirty microliters of qPCR reactions were prepared using 2.5 µl of cDNA template and iQ SYBR Green Supermix (Bio-Rad). The final concentration of primers in the PCR reactions was 0.4 mM. The sets of primers used are shown in Table 1. The qPCR conditions were: 95 ºC for 3 min, and 35 cycles of 95 ºC for 30 s, 57 ºC for 30 s and 72 ºC for 30 s. After amplification, a melting curve was run to ensure correct amplification of the expected amplicons. Expression of β-actin and cyclophilin-A genes was used as an internal control for each

plate. All amplification primers were designed using Beacon Designer (Biosoft International) [23].

The relative gene expression was assessed using the $2^{-\Delta\Delta Cq}$ method [24]. Afterwards, fold change values ($2^{-\Delta\Delta Cq}$) were standardized by a series of sequential corrections [25], after which a log change of 1 denoted no change in gene expression. However, values lower than 1 or higher than 1 denoted downregulation or upregulation, respectively. Standardized data were analysed using the software SPSS 15.0 for Windows. Data were tested for normality and equality of variances by Shapiro-Wilk and Levene's tests, respectively. These data showed a normal distribution, and those which had variances that were constant across groups were analysed using the one-way ANOVA and Duncan´s post hoc tests. The remaining data were analysed using the Kruskal-Wallis and Mann-Whitney tests. A *P*-value < 0.05 was considered significant.

2.6. Protein extraction and labelling

Lung samples from experimental groups were homogenized on ice in 1.5 ml of lysis buffer (7 M urea, 2 M thiourea, 4% w/v cholamidopropyl-dimethylammonio-propanesulfonate (CHAPS), 0.5 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail, Roche, Spain) per 100 mg of tissue by means of a glass tissue lyser. After sonication (4×15 s pulses on ice) and centrifugation (8,000 ×*g* for 10 min at 4 ºC), supernatants (200 µg of protein) were washed and concentrated with 2-D Clean-Up Kit (GE Healthcare, Spain) and resuspended in a buffer for difference gel electrophoresis (DIGE) (8 M urea, 30 mM Tris and 4% v/w CHAPS). Protein concentrations were determined using the Bradford protein assay (Bio-Rad).

DIGE protein analysis was carried out [26], and the three experimental groups, containing four biological replicates each, generated 12 individual samples distributed

across six DIGE gels. A pooled internal standard sample (Cy2 labelled) was also included in each gel. Cy dye labelling was performed (GE Healthcare) using a ratio of 400 pmol/μL of dye per 50 μg of protein (30 min on ice in the dark). Dye swap was carried out, and 10 mM lysine was used as the stop buffer and incubated for 10 min. The samples were combined with up to 350 μL of rehydration buffer (8 M urea, 2% w/v CHAPS, 60 mM dithiothreitol -DTT- and 1% pharmalytes pH 3.0–11.0, GE Healthcare). Isoelectrofocusing was carried out on 18 cm immobilized pH gradient strips with a pH range of 4.0-7.0 (GE Healthcare) at 20 °C according to the following program: 1 h at 0 V and 12 h at 30 V (rehydration); 2 h at 60 V; 1 h at 500 V; 1 h at 1,000 V; a 30 min gradient up to 8,000 V; and up to 7 h at 8,000 V until 50 kV h are reached. Equilibration steps were conducted twice for 15 min in a buffer containing 50 mM Tris–HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 0.002% bromophenol blue and 1% (w/v) DTT (first step) or 4.0% (w/v) iodoacetamide (second step). The second dimension was run on a 12.5% SDS-PAGE gel in an Ettan DALT six apparatus (GE Healthcare) for 45 min at 3 W/gel and, subsequently, 3.5 h at 18 W/gel. Precision Plus Protein Standards (Bio-Rad) were run on each gel as molecular weight markers.

An Ettan DIGE Imager (GE Healthcare) with Cy Dye filters and a 100 μm pixel size was used for protein visualization. The DeCyder 2-D differential analysis software 7.0 (GE Healthcare) was used for image analysis. First, the DIA module assigned spot boundaries and calculated parameters (e.g., normalized spot volumes). Second, the BVA module corrected intergel variability by matching and normalizing to the internal standard spot maps. The average ratio, unpaired Student's t-test and ANOVA were also calculated for each the group. Third, the EDA module was used to develop an unsupervised multivariate analysis. Thus, protein spots were considered to

be differentially expressed with statistical significance when: i) spots were present in at least 70% of gel images, ii) the average ratio of spots between groups met the 1.5-fold threshold and iii) *P*-values of differentially expressed spots were less than 0.05.

2.7. Mass spectrometry and protein identification

DIGE gels were stained with colloidal Coomassie [27] to detect total proteins. These spots of interest were manually excised by biopsy punches. Protein digestion was carried out [28], and differentially expressed spots were excised from gels for subsequent in-gel reduction, alkylation and digestion with trypsin. Spots were washed twice with water, shrunk for 5 min with acetonitrile and dried in a Speed-Vac concentrator (Thermo Scientific) for 15 min. Samples were then reduced with 10 mM DTT in 25 mM ammonium bicarbonate (56 ºC, 30 min) and alkylated with 55 mM iodoacetamide in 25 mM ammonium bicarbonate for 15 min in the dark. Finally, samples were digested overnight with 5 ng/µl sequencing grade trypsin (Promega, Spain) in 25 mM ammonium bicarbonate (pH 8.5) at 37 ºC. After digestion, the supernatant was collected, and 1 µl was spotted onto a MALDI target plate and allowed to air-dry at room temperature. Matrix solution of α-cyano-4-hydroxy-cinnamic acid (LaserBio Labs, Spain) in 50% acetonitrile/0.1% trifluoroacetic acid in water was added to the dried peptide digest spots and again allowed to air-dry at room temperature.

MALDI-TOF MS analysis was performed on a 4800 Proteomics Analyzer MALDI TOF/TOF Mass Spectrometer (Applied Biosystems). A 4700 Proteomics Analyzer Calibration Mixture (Cal Mix 5; AB Sciex, Spain) was used for external calibration. All mass spectra were calibrated internally using peptides from trypsin autodigestion. MALDI-TOF MS analysis produced peptide mass fingerprints, and the peptides observed were collected and represented as a list of monoisotopic molecular weights. Protein identification was conducted using a local license of Mascot 2.2.04 from Matrix Science through the Global Protein Server 3.6 (AB Sciex). The search parameters for the resulting peptide mass fingerprints and tandem MS spectra were set

as follows: i) NCBInr sequence databases were used; ii) all taxonomy entries were used; iii) modifications were considered (Cys as a S-carbamidomethyl derivative and Met as an oxidized methionine); iv) one missed cleavage was allowed; v) the precursor tolerance was 100 ppm and MS/MS tolerance was 0.3 Da; vi) the peptide charge was 1+; and vii) trypsin use was indicated [29].

3. Results

3.1. Clinical signs

All UNCH animals died between 14 and 72 h after the Nagasaki strain challenge, with clinical signs suggesting GD. On the contrary, all Y167A pigs survived the challenge, showing only mild clinical symptoms (mild lower limb claudication and weakness in some of them). On the other hand, it was a mild pleuritis and pneumonia in some of the vaccinated animals while the lesions observed were considerably greater in the UNCH group were considerably greater.

3.2. Microarray

There were 489 differentially expressed genes between the UNCH and UNUN pigs; the most important of which, on the basis of the degree of differential expression or by the function of the gene in question, are shown in Table 2. The metabolic functions associated with these genes included acute phase response signalling, liver X receptor/retinoic acid receptor activation, inflammatory response, cell movement or development, cell-to-cell signalling and interaction and immune cell trafficking. Additionally, significantly decreased expression of the genes related to albumin and potassium was shown.

In contrast, only 84 differentially expressed genes were recorded between Y167A and UNUN pigs; the most important of which are shown in Table 2. In this case, the most affected functions or metabolic pathways were antigen presentation,

cell-to-cell signalling, molecular transport, liver damage and acute-phase proteins (APPs). A total of 563 genes were differentially expressed between the UNCH and Y167A animals. The main functions or metabolic reactions involved were tissue development and morphology, APP response signalling, cell-to-cell signalling and interaction, connective tissue disorders, immune cell trafficking and liver inflammation. In UNCH pigs, only the expression of the genes involved in metabolic pathways about production, regulation or degradation of creatinine and haematocrit increased in comparison to Y167A pigs.

3.3. Quantitative real-time PCR (qPCR)

The expression of genes coding for 27 molecules related to immunity were quantified using qPCR (Table 3). Of them, only the most differentially expressed are described. *TLR-5* was significantly downregulated in both the Y167A and UNCH groups, while *SOD* was significantly downregulated only in Y167A pigs. *IL-8*, *MIP-1*α and *MIP-1*β encoding chemoattractant cytokines were significantly upregulated in the UNCH group, and this effect was also observed to a lesser extent in Y167A pigs for *IL-8*.

There was an overexpression of other genes encoding proinflammatory cytokines (*IL-1*β, *IL-1RN*, *IL-6* and *TNF-*α) in the UNCH pigs compared to the other two groups. The expressions recorded for *IL-4* and *IL-12* were significantly higher in both the UNCH and Y167A pigs.

CD14 and *CD163* genes were overexpressed in both the UNCH and Y167A pigs. On the contrary, the *CD83* gene suffered a decrease in expression in UNCH pigs. *CCL19* encodes a chemokine that promotes the migration of mature dendritic cells that express chemokine receptor 7 to lymph nodes, while *F13A1* codes for the coagulation factor XIII A subunit. mRNA expression of both genes was more than

three times higher in the UNCH group and almost two times higher in the Y167A group than in the UNUN group. Finally, the gene encoding vascular cell adhesion protein VCAM1 was again overexpressed in the UNCH pigs.

Five of the genes tested by qPCR (*CXCL2, IL-1β, IL-4, IL-8* and *IL-12*) served to validate the microarray analyses, as their expression was similar in both techniques for Y167A and UNCH groups (Fig. 1).

3.4. DIGE and protein identification

Albumin and mitochondrial cytochrome b-c1 complex subunit 1 were only repressed in the UNCH pigs, while lamin β1 was repressed in this group and in the Y167A pigs (Table 4). On the other hand, haptoglobin was only overexpressed in the UNCH group. Collagen α-1 (VI) chain and peroxiredoxins (Prdxs) 2 and 6 were overexpressed in the Y167A pigs. In addition to being overexpressed in the Y167A group, actin cytoplasmic 2-like protein and apolipoprotein AI were also produced at higher levels in the UNCH group. Fig. 2 shows protein spots identified as differentially expressed between the three experimental groups, and Fig. 3 shows the difference in protein expression as visualized with DIGE.

4. Discussion

A subunit vaccine based on a site-directed mutant of TbpB from the *H. parasuis* Nagasaki strain (serovar 5) was capable of inducing greater protection against challenge compared with native TbpB or commercial serovar 5 vaccines [21]. In this study, the proteomic and genomic analyses of the lungs from pigs vaccinated with a Y167A mutant were compared with those from the UNCH and UNUN pigs. Differentially expressed genes were identified, and those related to the inflammatory response, to the response to cell damage or to maintaining cellular homeostasis were

the most abundant, as it was demonstrated by microarray studies (Table 2). These results agree with the proteomic data, where differentially expressed proteins were also connected to the inflammatory response and the response to vascular injury or tissue damage, among other functions.

Several genes identified by microarray to be differentially expressed between groups are notable. For instance, among those genes whose expression was much higher in the UNCH *versus* the UNUN pigs were: *ACKR3*, *ALOX15*, *α-1* (*HBA1*), *α-2* (*HBA2*) and *TRIM55*. The *ACKR3* gene encodes a chemokine receptor that controls chemokine levels and localization via its high affinity for chemokines, resulting in their sequestration, degradation or transcytosis [31]. *ALOX15* encodes an arachidonate 15-lipoxygenase, which is essential for immune and inflammatory responses, and whose expression has been associated with atherosclerosis and osteoporosis [32]. *α-1* (*HBA1*) and *α-2* (*HBA2*) are involved in oxygen transport from the lung to various peripheral tissues. Diseases associated with these genes include vascular cancer and body anaemias [33]. Upregulation of *α-1* and *α-2* in the UNCH pigs could be related to the invasion of endothelial cells by *H. parasuis* [34] or to some of the vascular lesions described in GD, such as haemorrhages and disseminated intravascular coagulation [12]. The protein encoded by *TRIM55* contains a RING zinc finger, a motif involved in protein-protein interactions. Overexpression of this gene could be the result of the cellular damage caused by *H. parasuis* in several tissues [12], while repression in the Y167A pigs could serve as an indicator of protection by this vaccine against GD.

Another set of genes was overexpressed in the Y167A *versus* the UNUN pigs. Among them, *CES1* encodes liver carboxylesterase 1, which is involved in drug detoxification [35]. Its overexpression in the Y167A group could be related to *H.*

parasuis clearance in the liver and consequently to overcoming GD. *CR2* encodes CR2 or CD21, a membrane glycoprotein found on B-cells that is capable of binding fragments of the complement system. Its increased expression in the Y167A pigs could be due to an increase in lung B-cells following migration of mature B-cells (αIgM^+CD21^+) from the peripheral blood [21]. Alternatively, taking into account the distribution of B-cells in the lung, we can speculate that the parenteral immunization had a positive impact on the acquired immune response of the bronchus-associated lymphoid tissue. *PCDH15* encodes integral membrane proteins which mediate calcium-dependent cell-cell adhesion; specifically, PCDH15 plays a crucial role in the maintenance of normal cellular function [36]; therefore, its overexpression in the Y167A group seems to suggest and indicator of protection against *H. parasuis*.

Of the genes being overexpressed in the Y167A *versus* the UNCH group, three are noteworthy: *BAG3*, *CCL3L1*, and *CLCA1*. *BAG3* encodes a protein that cooperates with two molecular chaperones in the degradation of cytoskeleton components in the lysosome, which is essential for maintaining muscle activity [37]. *CCL3L1* encodes a chemokine that binds some chemokine binding proteins, and it is an important factor in arthritis [38]. *CLCA1* encodes a protein that regulates tissue inflammation during the innate response in airways [39].

HLA-DRB4 is one of the genes underexpressed in Y167A compared with the UNUN group and encodes the class II major histocompatibility complex, DR β4. This protein is involved in the presentation of exogenous peptides for recognition by $CD4^+$ cells [40], indicating a possible mechanism of suppression of these molecules. Similarly, a reduction in SLADR molecules on alveolar macrophages has been shown after an intranasal infection with the *H. parasuis* Nagasaki strain [4], and this effect

was also observed in peripheral blood monocytes after intratracheal challenge with the same strain [41].

The qPCR technique validated the microarray results and could help to better understand the mechanisms of the porcine immune response against *H. parasuis* infection. Concerning TLRs, TLR5 was the only clearly downregulated in both groups of challenged pigs; this TLR recognizes flagellin, the major protein of bacterial flagella. *H. parasuis* lacks flagella, which could explain this result in Y167A and UNCH groups. However, it is difficult to justify the downregulation of *SOD* gene, encoding one of the enzymes implicated in the respiratory burst during phagocytosis, especially when other proinflammatory molecules were clearly upregulated in the two infected groups. For instance, *CD14* gene, encoding a molecule being found on macrophages and granulocytes, is significantly more expressed in Y167A and UNCH groups, thus suggesting an active phagocytosis against *H. parasuis*.

The significantly higher expression of several interleukins in UNCH group shows that they play an important role in the inflammatory response. A high level of IL-1 expression in porcine lungs infected with *A. pleuropneumoniae* [42] or *H. parasuis* [43] has been previously found. IL-8 stimulation in porcine brain endothelial cells and tracheal epithelial cells by the Nagasaki strain has been demonstrated *in vitro* [44,45] and in the brain during *in vivo* infection [43]. This fact agrees with the high overexpression of IL-8 detected in our study in the UNCH group, notably among those pigs that died as a result of infection, a finding already reported in these same animals for IL-8 measured in sera at 48 h post-challenge (more than 500 pig/ml) [30]. The high IL-8 level in those pigs was accompanied by high CD163 values, which have been described as a good marker for sepsis, morbidity and mortality. In fact, a steep increase in both IL-8 and soluble CD163 has been shown in serum at 3 to 4 days

post-challenge after intranasal inoculation of *H. parasuis* Nagasaki in colostrum-deprived pigs [4].

IL-6 has been related to the synthesis of APPs in hepatocytes and to Th2 differentiation [46], and the increase in IL-6 detected in the Y167A and UNCH groups could be associated with a Th2 response. This interleukin has been shown to be related to the differentiation of monocytes into macrophages [46], and the higher number of lung macrophages seen by immunohistochemistry (data not shown) could be associated with the increase in *MIP-1α, MIP-1β* and *CD14* expressions observed in the UNCH group when compared to the UNUN animals. The presence of TNF-α was previously detected in the lungs of pigs infected with *H. parasuis* [47] and was similar to our results in the UNCH pigs. The high expression levels of TNF-α and IL-1 have been associated with some symptoms of GD (oedema, lung congestion and vascular thrombosis) [43], and the higher levels of VCAM1 expression in the UNCH pigs could be explained precisely by the increase in IL-1β and TNF-α cytokines.

On the other hand, Y167A animals showed a strong protective humoral response [30], and therefore, they were capable of modulating the chemoattraction by CCL19 of mature lung dendritic cells (as revealed by the higher CD83 expression in the Y167A group compared to UNUN group) to draining lymph nodes where they may stimulate an adaptive immune response [48]. However, the scarce expression of CD83 observed in the UNCH group seems to indicate the absence of mature dendritic cells.

The higher *F13A1* expression, encoding for the coagulation factor XIII A subunit, in the Y167A and especially in the UNCH groups could justify some of the lesions described for GD, such as disseminated intravascular coagulation [20]. This is agreement with the upregulation seen by microarray for *HBA1/HBA2* genes.

With regard to proteomic studies, collagen α-1 (the major structural component of microfibrils which maintains the integrity of various tissues) and actin, cytoplasmic 2-like (one of the proteins that is most rapidly synthesized after cell damage [49]) are overexpressed when cellular damage or cellular homeostasis problems occur. Therefore, their overexpression in the two groups that received an *H. parasuis* challenge may be one mechanism of repairing injuries caused by this infection. Prdxs are a family of antioxidant enzymes that control cytokine levels and mediate signal transduction. The mRNA encoding Prdx6 is present in all major mammalian organs, with the greatest mRNA content and protein expression levels found in the lung [50]. Prdx6 shows the highest expression levels in respiratory epithelial cells. This fact could explain its overexpression in Y176A pigs; although these animals survived challenge, injury caused by *H. parasuis* infection forced the lungs to trigger Prdx6 production in response.

Apolipoprotein AI is associated with high density lipoproteins during the APP [51]. Surprisingly enough, our lung results disagree with those previously reported in sera from pigs infected with *H. parasuis*, in which a reduction in apolipoprotein AI was measured [52,53]. However, an increase in the expression of apolipoprotein AI have been previously reported after *A. pleuropneumoniae* infection [42], as in our study.

Albumin, which is synthesized by the liver, is the major plasma protein. When its concentration decreases, fluid moves from blood vessels into the tissues, resulting in oedema that mainly accumulates in the abdomen, lungs and ankles [54]. This may be a logical explanation for the decreased expression of this protein in the lungs from UNCH pigs, a finding also revealed by microarray, because one of the symptoms detected in GD is pulmonary oedema [20].

Cytochrome b-c1 complex, subunit 1 is part of the mitochondrial respiratory chain. Intracellular bacteria, including *H. parasuis* [34], attempt to paralyze cell growth, including mitochondrial respiration, upon colonization. This tactic could explain the decreased expression of cytochrome b-c1 complex, subunit 1 in the UNCH group. Haptoglobin binds and removes free haemoglobin from circulation, and haemoglobin-haptoglobin complexes are catabolized by the liver. Haptoglobin overexpression in the UNCH animals could be related to the presence of haemorrhages caused by the *H. parasuis* infection. This is in agreement with microarray data that showed the differential expression of genes, such as *PCDH15* and *HBA1/HBA2*, which are also related to vascular lesions and bleeding. Haptoglobin is also an APP that may occasionally increase in inflammations, such as those caused by this porcine pathogen. In fact, it was elevated in our study, which is in accordance with the results previously reported when directly analysing APPs in convalescent pigs infected with the *H. parasuis* Nagasaki strain [52,53] or with *Pasteurella multocida* [55].

5. Conclusion

Gene and protein expression profiles involved in susceptibility to and TbpB mutant-raised protection against infection by *H. parasuis* Nagasaki strain in colostrum-deprived pigs have been identified. In the Y167A group –vaccinated with the TbpB mutant, which is defective in its ability to bind porcine transferrin–, the expression of proinflammatory molecules was notably lower than that observed in the UNCH pigs suffering from disease. In some cases, levels of proinflammatory molecule expression were similar to the physiological levels found in the UNUN group. Therefore, our results add useful information about the molecular basis of

resistance to GD and, consequently, may contribute to the control of this prevalent swine disease.

Competing interests

The authors declare that they have no competing interests.

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Fig. 1. Comparison of the expression of five selected genes (*CXCL2*, *IL-1β*, *IL-4*, *IL-8* and *IL-12*) from the unvaccinated challenged (UNCH) and the Y167A vaccinated pigs by quantitative real-time PCR and microarray analyses.

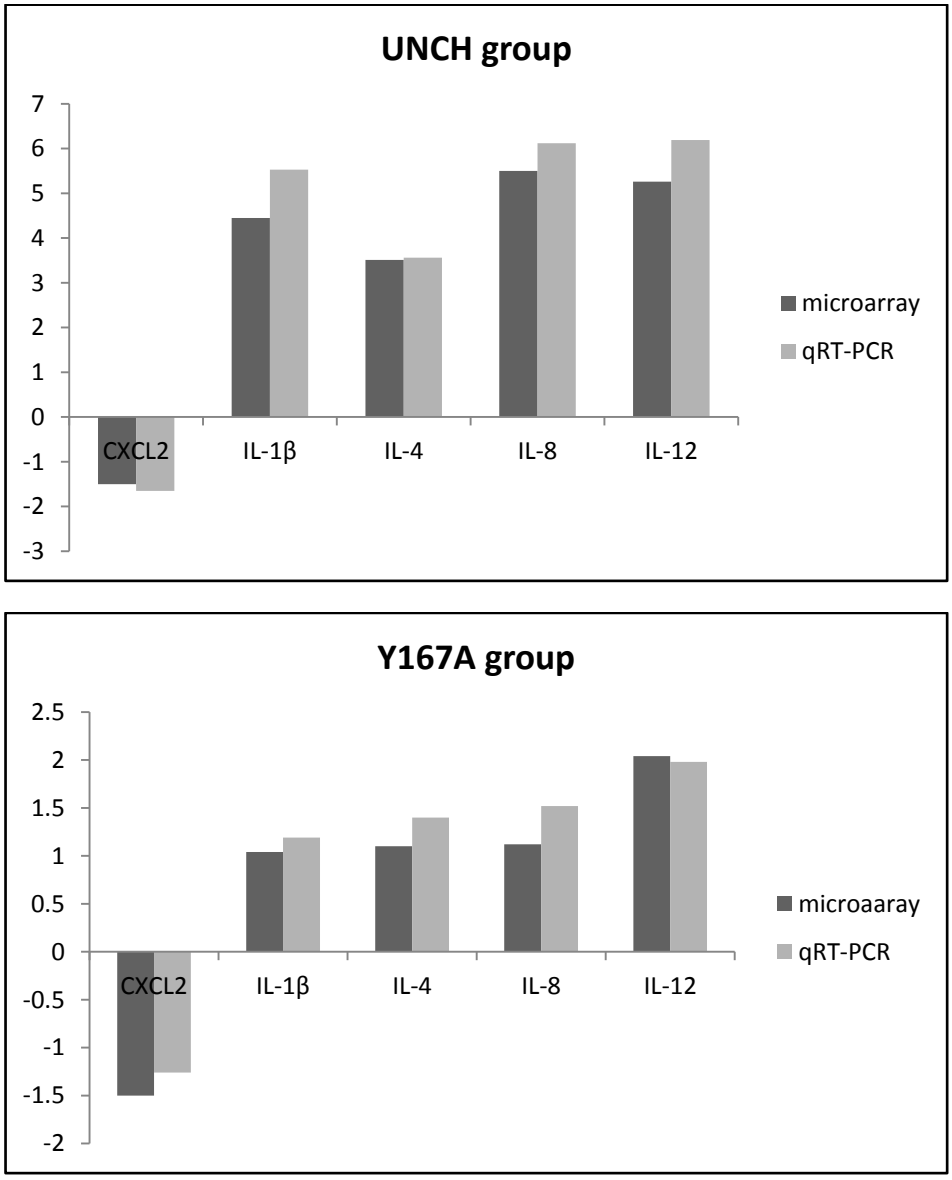


Fig. 2. Coomassie stained 2-DE gel showing proteins differentially expressed between experimental groups. The circled proteins represent spots identified using MS/MS analyses (see Table 4).

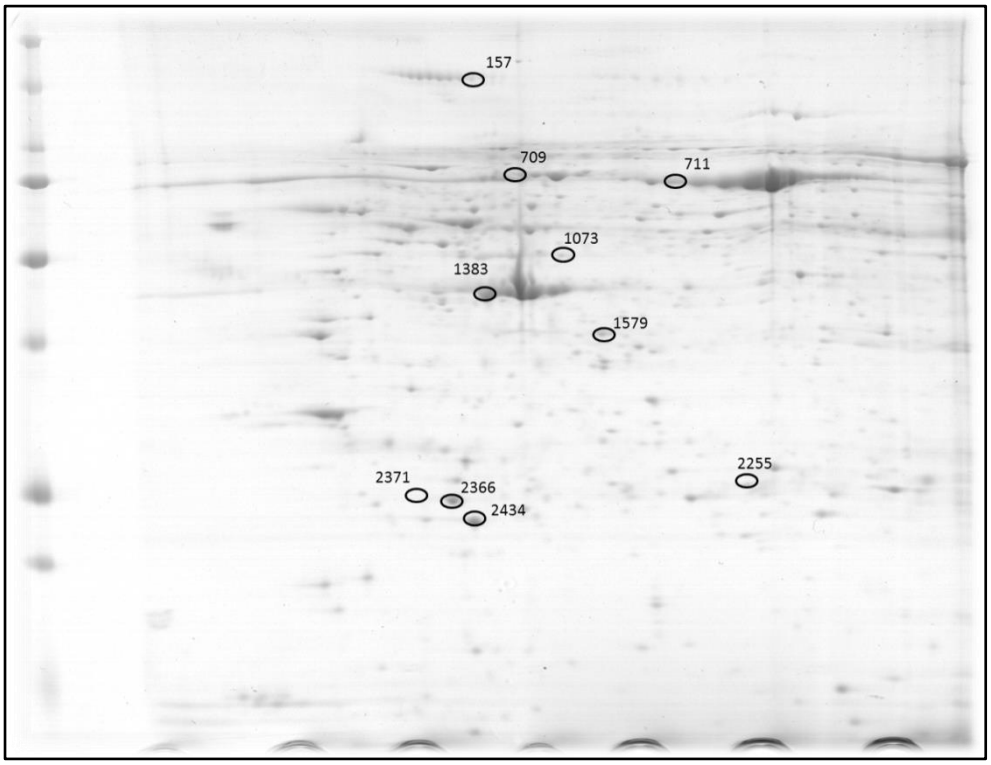
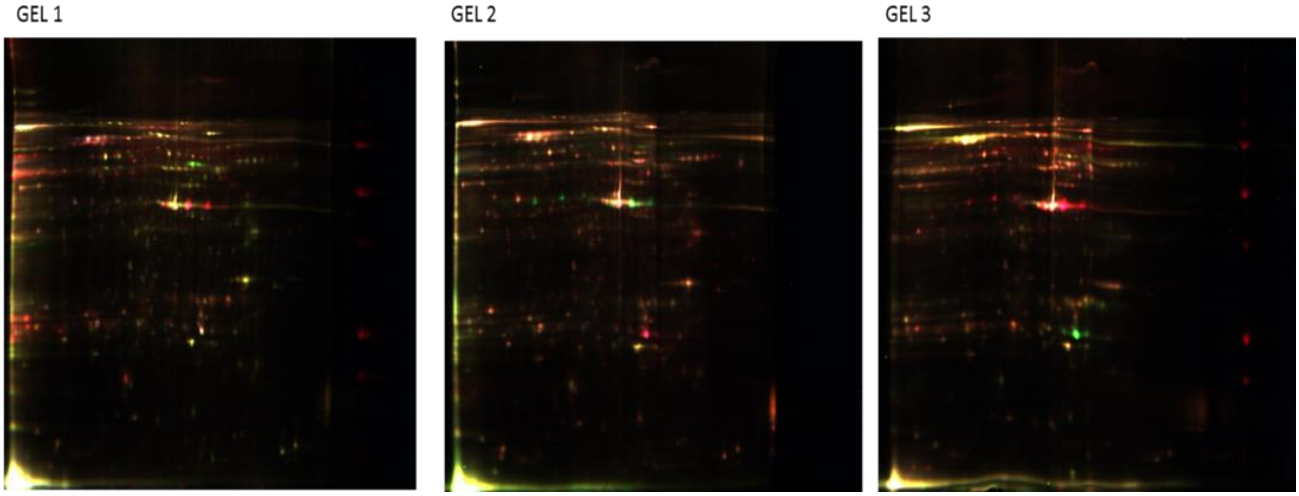


Fig. 3. Comparison of experimental groups by means of DIGE gels.



Gel 1: Unvaccinated unchallenged (UNUN) pigs are labelled with Cy3, and Y167A vaccinated pigs are labelled with Cy5

Gel 2: Unvaccinated challenged (UNCH) pigs are labelled with Cy3, and Y167A vaccinated pigs are labelled with Cy5

Gel 3: Unvaccinated unchallenged (UNUN) pigs are labelled with Cy3, and unvaccinated challenged (UNCH) pigs are labelled with Cy5

Table 1. Oligonucleotides used in quantitative real-time PCR analysis.

Target gene	Forward primer 5′→3′	Reverse primer 5′→3′	Accession number
CCL19	GAGATGCCAACGATGCTGAA	GAAAGGCTCGAACCAGATTCC	NM_001170516.1
CD14	ACCACCCTCAGACTCCGTAATG	TTGCGCCACTTTCAGTACCTT	EFO51626
CD83	CTGTTGGCTCTGGTCACTTTCT	CTCTGCTGTCGTGCAAACTTG	XM_001928655.2
CD163	GCCTGTCTCATCGCATTCCT	GGATTTAGCATATCCGTTTCATCTG	NM_213976.1
CXCL2	GGATAGGACGCTGTACCATC	ACTGTCTCAATAAATAACAACCGAC	AY578786
CYR61	TTCCAGCCCAACTGTAAACAC	GTCCTTGGCACCGTCCTC	EF990805
DEFB1	ACCGCCTCCTCCTTGTATTC	GGTGCCGATCTGTTTCATCT	NM_213838
DEFB2	CTGTCTGCCTCCTCTCTTCC	CAGGTCCCTTCAATCCTGTT	NM_214442
F13A1	TCTCAGCCCATGACAACAATG	TCACGTTCCCATCTTCTTCCA	XM_001927630.4
FOS	AAAAGGAGAATCCGAAGGGAAA	TGCAAAGCAGACTTCTCGTCTT	NM_001123113.1
IL-1RN	GAAAGAACAGCGAGCAGGAC	TGACCTTGACGGCTGCTTTG	NM_214262.1
IL-1β	GGCCGCCAAGATATAACTGA	GGACCTCTGGGTATGGCTTTC	NM_214055
IL-4	TTGCTGCCCCAGAGAAC	TGTCAAGTCCGCTCAGG	AY294020
IL-6	TGGCTAACTGCCTTCCCTACC	CAGAGATTTTGCCGAGGATG	NM_214399
IL-8	TTCGATGCCAGTGCATAAATA	CTGTACAACCTTCTGCACCCA	AB057440
IL-12	GGAGTATAAGAAGTACAGAGTGG	GATGTCCCTGATGAAGAAGC	U08317
MAP3K8	GCACAGGAAGCACTGAGGAAT	CCCCGGACAAGGTTGAAAT	XM_003359272.1
MIP-1α	TCTCGCCATCCTCCTCTG	TGGCTGCTGGTCTCAAAATA	
MIP-1β	CAGCACCAATGGGCTCAGA	TTCCGCACGGTGTATGTGA	EF107667
MYD88	TGGTGGTGGTTGTCTCTGATGA	TGGAGAGAGGCTGAGTGCAA	NM_002468
PTGS2	TCAACCAGCAATTCCAATACCA	TGTACTCGTGGCCATCAATCTG	NM_214321.1
SOD	GGGAGCATGCGTATTACCTTCA	CGGCGTATCGCTCAGTTACAT	NM_214127.2
TNF-α	CCTCTTCTCCTTCCTCCTG	CCTCGGCTTTGACATTGG	X57321
TLR-2	TCACTTGTCTAACTTATCATCCTCTTG	TCAGCGAAGGTGTCATTATTGC	AB085935
TLR-4	GCCATCGCTGCTAACATCATC	CTCATACTCAAAGATACACCATCGG	AB188301
TLR-5	CAGCGACCAAAACAGATTGA	TGCTCACCAGACAGACAACC	NM_001123202
VCAM1	CCGAGGAATATCGTCGTGATC	CACCAATCTGCGCAATCATT	NM_213891

Table 2. Microarray results comparing the expression of different genes from the three experimental groups: the unvaccinated challenged (UNCH), unvaccinated unchallenged (UNUN), and Y167 vaccinated pigs.

Gene	Function of the protein/s expressed for each gene	Difference in expression value between pairs of experimental groups		
		UNCH *versus* UNUN	**Y167A *versus* UNUN**	**UNCH *versus* Y167A**
ACKR3	Receptor for chemokines CXCL11 and CXCL12	+2.723		
ADAMTS17	Disintegrin and metalloproteinase. Cell growth and survival	+3.061		+3.597
AHSP	Chaperone binding α-globin. Involved in hemoglobin assembly			+3.071
ALOX15	Regulates macrophage function (inflammation). Response to IL-13	+2.943		
ASH2L	Part histone methyltransferase complex. Role in hematopoiesis		+1.742	
CES1	Carboxylesterase. Detoxification		+1.661	
CR2	Receptor for complement C3d. Activation of B-cells	-2.848	+1.804	
DDX3Y	ATP binding, hydrolysis, and intramolecular interactions	+3.666	+3.769	+7.366
CXCL2	Immunoregulatory and inflammatory processes	-1.500	-1.268	
EJF1AY	Molecular marker for damage lung	+3.066	+3.113	+6.103
EJF2S3	Molecular marker for damage lung	+3.477	+2.977	+2.827
HBA1/HBA2	Oxygen transport from the lung to the various tissues	+2.678		+2.862
IL-1β	Inflammatory response, differentiation cell and apoptosis	+4.453		
IL-4	B-cell activation. Class II MHC induction	+3.511		
IL-8	Attracts neutrophils, basophils and T-cells. Neutrophil activation	+5.564		
IL-12	Differentiation of Th1 and Th2 cells. Induction of interferon-γ	+5,266	+2,048	
KDM5C	Regulation of transcription and chromatin remodeling		+1.638	+3.478
PCDH15	Calcium-dependent cell-adhesion protein		+1.582	
PRSS35	Transport of hormones	+4.056		+3.085
TMSB10/TBSB4	Binds to and sequesters actin and inhibits its polymerization	+2.938	+3.050	+6.043
TRIM55	Protein-protein interactions	+3.334		+3.691
RGS5	Induction of endothelial apoptosis		+1.615	
BAG3	Cellular response to heat stress. Maintains muscle activity			-2.899
BRINP3	Vascular inflammation	-2.867		-6.255
CCL3L1	Chemotactic for lymphocytes and monocytes. CCR1, CCR3 and CCR5 ligands	-2.314		-2.688
CLCA1	Calcium-activated chloride conductance. Inflammation			-3.036
FAM65B	Myogenic cell differentiation and cytoskeletal formation		-1.547	
FAM78B	Intramolecular interactions	-2.584		-2.850
F13A1	Transglutaminase to catalyze the formation of fibrin	-2.353		
HIST1H2A	Interacts between nucleosomes in the compaction of chromatin	-2.594		-2.846
HLA-DRB4	Presents antigens on the cell surface for recognition by the Th-cells		-2.394	
HSPA6	Stabilizes preexistent proteins against aggregation		-1.447	
HSPB2	Maintenance of muscle structure and function		-1.388	
LPHN2	Regulation of exocytosis from neuroendocrine cells	-2.947	-3.208	-5.814
MT1E	Transport of heavy metals, drugs and glucocorticoids		-1.525	
NRXN1	Neurotransmission and synaptic contacts		-1.471	
RETN	Suppresses insulin ability to stimulate glucose	-4.251		-4.071
STEAP4	Reduces Fe(3+) to Fe(2+) using NAD(+) as acceptor	-3.032		-3.003
TMOD3	Contributes to the formation of the short actin protofilament		-2.146	
XIST	Essential for the initiation and spread of X-inactivation	-2.442	-3.433	-5.742
ZDHHC9	Member of the zinc finger DHHC domain-containing protein family		-1.773	

Table 3. Expression of 27 molecules related to immunity from the three experimental groups as determined by quantitative real-time PCR.

Molecules	Experimental group		
	UNUN	**Y167A**	**UNCH**
TLR-2	1.00	-1.13	1.21
TLR-4	1.00	1.18	1.70
TLR-5	1.00 (A)	-2.27 (B)	-1.31 (C)
SOD	1.00 (A)	-2.38 (B)	-1.35 (A)
MIP-1α	1.00 (A)	1.11 (A)	4.52 (B)
MIP-1β	1.00 (A)	1.43 (A)	3.02 (B)
IL-1β	1.00 (A)	1.19 (A)	5.53 (B)
IL-1RN	1.00 (A)	1.67 (B)	4.05 (C)
IL-4	1.00 (A)	1.40 (B)	3.56 (C)
IL-6	1.00 (A)	2.14 (B)	3.44 (C)
IL-8	1.00 (A)	1.52 (B)	6.12 (C)
IL-12	1.00 (A)	1.98 (B)	6.19 (C)
TNF-α	1.00 (A)	-1.49 (B)	3.75 (C)
MYD88	1.00 (A)	1.24 (A)	2.53 (B)
DEFEN I	1.00	1.45	1.08
DEFEN II	1.00	-1.81	1.81
CD14	1.00 (A)	2.41 (B)	7.33 (C)
CD163	1.00 (A)	1.81 (B)	4.79 (C)
CD83	1.00 (A)	1.40 (A)	-2.04 (B)
CXCL2	1.00 (A)	-1.26 (A)	-1.65 (B)
CYR61	1.00	1.04	1.05
PTGS2	1.00	1.59	1.75
CCL19	1.00 (A)	1.84 (B)	3.85 (C)
F13A1	1.00 (A)	1.66 (B)	3.44 (C)
MAP3K8	1.00 (A)	2.42 (B)	1.18 (A)
VCAM1	1.00 (A)	1.08 (A)	2.69 (B)
FOS	1.00 (A)	1.14 (A)	2.83 (B)

UNUN: unvaccinated unchallenged pigs; **Y167A**: pigs vaccinated with mutant TbpB; **UNCH**: unvaccinated challenged pigs

B Significant differences compared to A ($P< 0.05$)

C Significant differences compared to B and A ($P< 0.05$)

Table 4. Proteins identified using MALDI-TOF mass spectrometry for the three experimental groups.

Protein number	Spot number	Protein name	Accession number	Protein score	Total ion score	Protein molecular weight	Protein pI	Best ion score	Peptide count	Groups with	
										overexpression	underexpression
1	157	Collagen α-1 (VI) chain	gi/359323606	228	142	109650.5	5.36	60	12/65	Y167A	
2	709	Lamin-β1	gi/335283403	334	104	66673.7	5.08	49	28/65		UNCH and Y167A
3	711	Albumin	gi/833798	542	340	71361.5	5.92	73	23/65		UNCH
4	1073	Mitochondrial cytochrome b-c1 complex, subunit 1	gi/335299041	441	235	53349.4	5.76	94	22/65		UNCH
5	1383	Haptoglobin	gi/189409353	393	306	39006.6	6.51	95	11/65	UNCH	
6	1579	Actin cytoplasmic 2-like	gi/326935974	631	408	37314.6	5.37	140	19/65	UNCH and Y167A	
7	2255	Peroxiredoxin-6	gi/47523870	859	633	25078.1	5.73	194	20/65	Y167A	
8	2366	Apolipoprotein AI	gi/164359	370	181	30311.7	5.38	112	19/65	UNCH and Y167A	
9	2371	Peroxiredoxin-2	gi/347300176	381	328	22037.2	5.23	109	6/65	Y167A	
10	2434	Peroxiredoxin-2	gi/347300176	474	408	2237.2	5.23	133	6/65	Y167A	

UNCH: unvaccinated challenged pigs; **Y167A:** pigs vaccinated with mutant TbpB