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Original article

Glycoprotein analysis of porcine bronchoalveolar lavage fluid reveals potential biomarkers corresponding to resistance to *Actinobacillus pleuropneumoniae* infection

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Abstract – Biomarkers facilitating both pathogen-independent diagnosis of respiratory health and breeding selection of pigs with increased resistance to respiratory tract infections would be of considerable interest to the pig industry. Following this concept we performed a comparative glycoproteome analysis of bronchoalveolar lavage fluid (BALF) from healthy pigs and pigs 4 days (acute) and 20 days (chronic) after an experimental infection with *Actinobacillus pleuropneumoniae*. In order to identify possible differences in BALF glycoprotein patterns we investigated pigs of three different breeding lines (German Landrace, Piétrain, Hampshire). In total, 12 glycosylated proteins (alpha-1-acid glycoprotein, fetuin A, properdin, haptoglobin precursor, haptoglobin, hemoglobin, hyaluronidase, inter-alpha-trypsin inhibitor family heavy chain-related protein, alpha-1-antichymotrypsin 3, pulmonary surfactant-associated protein D (SP-D), transferrin, and alpha-1B-glycoprotein) were identified as being differentially expressed depending on the health status of the animal. Fetuin A levels were consistently low in chronically infected pigs thereby being a potential marker for chronic infection. Hyaluronidase levels were consistently high in all pigs after experimental infection independent on isolation of the pathogen thereby being a potential marker for previous pathogen contact and latent infection. High levels of fetuin A as well as low levels of haptoglobin and pulmonary SP-D correlated with the absence of lung lesions in pigs of the Hampshire breeding line, implying a potential application as selection markers for breeding programmes.

porcine respiratory disease / glycoprotein analysis / bronchoalveolar lavage fluid / biomarker

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1. INTRODUCTION

Respiratory diseases pose a severe problem to the pig industry worldwide as they cause production losses, increase veterinary costs and hamper marketing of finishing pigs [30, 36, 40]. Pathogen-specific diagnostics and vaccination programmes are limited to the most frequently occurring pathogens; furthermore, the detection of clinically healthy carrier animals is difficult due to the low number and poor diagnostic accessibility of colonizing pathogens [7, 23]. Therefore, these programmes only partly improve the situation by primarily protecting from clinical disease outbreaks. Lowering the infection pressure by improving husbandry conditions with respect to hygiene, climate, reduction of air pollution, and herd size also exhibits positive impacts on health [11] but does not prevent dissemination of airway pathogens from clinically healthy carrier animals [19]. Increasing the resistance to infection by breeding selection has also been approached [33] but is hampered by the lack of appropriate genetic or phenotypic markers facilitating an effective selection of adequate animals.

Innate immune mechanisms as well as the local immune response in the lung significantly participate in the control of respiratory tract infections [5]. Particularly glycosylated proteins have been shown to play a pivotal role in the defence system of the respiratory tract against bacteria and fungi [3, 42]. Thus, the protein alpha-1-acid glycoprotein has been reported to have anti-inflammatory and immunomodulatory functions that are exclusively mediated by its carbohydrate chains [14]. Furthermore, a number of respiratory pathogens are recognized and neutralized by members of the collectin protein family during the early stages of infection [16]. Expression of pulmonary surfactant protein D (SP-D) was shown as increased during acute and chronic *Actinobacillus pleuropneumoniae* infection. In that context it was assumed that SP-D could represent a link between the innate and adaptive immune system, facilitating bacterial antigen presentation by dendritic cells in bronchus-associated lymphoid tissue [44].

The identification of appropriate biomarkers would improve the situation as biomarkers can be used to diagnose infection [15, 28, 38, 50] and, potentially, are also applicable as markers for breeding selection. In case of respiratory tract infections local disease markers such as proteins present in bronchoalveolar lavage fluid (BALF) are likely to be most meaningful [13, 35, 53]. In order to identify potential markers, proteome analysis of BALF performed with two dimensional gel electrophoresis (2-D PAGE) followed by mass spectrometry has become the method of choice [37, 52]. Following this approach, initial studies of Hennig-Pauka et al. [20, 21] identified the antibacterial peptide PR-39 as a first marker for chronic respiratory tract infection in pigs.

Based on these considerations we investigated the distribution of BALF-derived glycoproteins using an experimental aerosol infection model with *A. pleuropneumoniae*. The protein patterns prior to infection were compared with the pattern in acute and in chronic infection in order to identify new disease markers as the availability of a number of markers would greatly improve the informative value of marker-based diagnostics. In addition, pigs from three different breeding lines with different resistance to respiratory tract infections [22] were investigated in order to identify proteins which might be usable as markers for breeding selection.

2. MATERIALS AND METHODS

2.1. Bacterial strains, media and aerosol infection, selection of BALF samples

A total number of 76 weaning-pigs, 42–49 days old, of three different breeding lines (German Landrace, Piétrain, Hampshire) from an *A. pleuropneumoniae*-free herd had been aerosol-infected with *A. pleuropneumoniae* serotype 7 clinical isolate AP76 [1] in a previous experiment [22]. Necropsy was performed either on day 4 (acute stage) or on day 20 (chronic stage) post infection. The chronic stage of infection on day 20 has already been confirmed during a previous study by immunohistological and macroscopic investigations [24].

In order to investigate glycoprotein expression during the course of infection a total of nine pigs of each breeding line with severe lung lesions (average scores on days 4 and 20 post infection of 9.4 and 10.9 in the German Landrace breeding line and, 9.9 and 7.3 in the Piétrain breeding line) were selected. Since no Hampshire pigs with lung lesions could be obtained pigs of this breeding line had an average lung lesion score of 0. In order to determine differences in BALF glycoprotein expression between the different breeding lines, BALF samples from three pigs each sacrificed on day 4 and on day 20 post infection, respectively, were pooled.

2.2. Glycoprotein purification

Pigs were anaesthetized by intramuscular application of 2 mg/kg azaperone (Stresnil®, Fa. Janssen-Cilag GmbH, Baar, Switzerland) and 15 mg/kg ketamine (Ursotamin®, Fa. Serum-Werk-Bernburg AG, Bernburg, Germany). BALF was obtained by lavaging the bronchus trachealis with 100 mL of 154 mM NaCl solution using a bronchoscope [4]. The yield of recovery normally ranged from 60 to 80 mL BALF. However, from severely diseased animals considerably lesser but more concentrated lavage fluid was obtained. The BALF was kept on ice until subsequent examinations. Cellular components of BALF were removed by centrifugation (5 000 × *g*) for 15 min at 4 °C. To the cell-free BALF, a protease inhibitor solution (25 µM PMSF, 375 µM trypsin-chymotrypsin inhibitor, 25 nM pepstatin, 250 nM antipain, 250 nM leupeptin, 375 µM aprotinin; final concentrations) was added and the salt concentration was increased to 500 mM NaCl. A Concanavalin A sepharose 4B column (Amersham Biosciences AB, Uppsala, Sweden) was equilibrated with 10 volumes of binding buffer (20 mM Tris-HCl, 500 mM NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, pH 7.4), and the BALF-supernatant was affinity-purified at a constant flow rate of 15 cm/h. The column was washed with 10 volumes of binding buffer; then the glycoprotein fraction was eluted with 10 volumes of elution buffer (20 mM Tris-HCl, 500 mM NaCl, 500 mM methyl-alpha-D-mannopyranoside, pH 7.4). To remove interfering salts and sugars, overnight dialysis was performed against buffer (20 mM Tris-HCl, pH 7.4, 1:2 500) in dialysis tubing with a cut-off of 7 000 Da (Spectra/Por® 7, Spectrum Laboratories, Inc., Breda, The Netherlands) at 4 °C in the presence of protease inhibitors. Total protein extract and glycoprotein fraction were visualized by 2-D PAGE (Supplementary Fig. S1 available online at www.vetres.org), thus confirming the purification step by Concanavalin A lectin chromatography.

2.3. 2-D gel electrophoresis

Protein concentrations were determined (Uptima Kit Micro BCA®, Interchim, Monluçon, France), and equal amounts of protein (150 µg) were precipitated with trichloro acetic acid (TCA: 10% w/v final concentration) overnight at 4 °C. After centrifugation (5 000 × *g*, 15 min, 4 °C) protein pellets were washed twice with ice-cold acetone and centrifuged again. The pellets were air-dried, resuspended in rehydration solution (2 M thiourea, 7 M urea, 4% (w/v) CHAPS), and supplemented with DTT (18 mM final concentration), IPG-buffer (2% f.c.; Amersham Biosciences AB), and bromphenol blue (0.001% f.c.). Each sample was anodal cup-loaded on a 24 cm Immobiline Dry Strip® pH 3-11 NL (Amersham Biosciences) which had been rehydrated with supplemented rehydration solution the night before. The proteins were focused in an Ettan IPGphor™ System (Amersham Biosciences) for 21 h in the following series of time blocks with increasing voltage: 3 h at 150 V, 3 h at 300 V, 6 h at 1 000 V gradient, 3 h at 8 000 V gradient and 6 h at 8 000 V. After isoelectric focussing, the strips were stored at −70 °C or directly used for second dimension as previously described [20]. Analytical gels were stained with silver nitrate [39] and preparative gels with colloidal Coomassie G-250 [34].

2.4. 2-D difference gel electrophoresis (DIGE)

Each protein pool was TCA precipitated and the pellets were washed as described in Section 2.3. The proteins were resuspended in 100 µL of lysis buffer (30 mM Tris-NaOH, 7 M Urea, 2 M Thiourea, 4% (w/v) CHAPS, pH 8.5) and left on ice for 10 min. The protein concentration varied from 1.8 to 3 mg/mL. A total amount of 150 µg of each protein pool was then labelled with 1 µL of the CyDye working solution (CyDye in dimethylformamide (250 pmol/µL)) for 30 min in the dark at 4 °C. The reaction was stopped by addition of 1 µL lysine solution (10 mM) with a subsequent incubation in the dark for 10 min. Afterwards, samples designated to run on the same gel were pooled and supplemented with DTT and IPG-buffer as described in Section 2.3. 2-D gels were scanned on a Typhoon Trio™ Scanner (Amersham Biosciences) at a resolution of 100 dots/cm using filters with specific excitation and emission

wavelengths for Cy2 (filter BP 40; 488 nm/520 nm), for Cy3 (filter BP 30; 532 nm/580 nm), and for Cy5 (filter BP 30; 633 nm/670 nm).

2.5. Protein quantification and statistical analysis

Silver-stained protein spots were quantified using ImageJ software (Image Processing and Analysis in Java), and expression ratios comparing day −7 with day 4 or day 20, respectively, were ascertained for each protein. As it is generally known that silver staining is limited by a low linear range only proteins with an average expression change of ≥ 1.5 in BALF from at least two animals were selected. Individually regulated proteins as well as not identified proteins were excluded from evaluation. Expression ratios higher than 25 were set to the threshold value of −25/+25. Determination of significance was carried out using SAS (Statistical Analysis Software) by performing a *t*-test for paired comparison under the term of a Gaussian distribution of the residual values. State-of-the-art DIGE analysis was applied as soon as the technology became accessible; fluorescence-labelled spots were analyzed by DeCyder™ (Differential Analysis Software, Amersham Biosciences), and only proteins with a minimum expression change of 1.5 were evaluated.

2.6. Identification of protein spots by mass spectrometry

After evaluation of the glycoproteome expression patterns, prominent protein spots were excised from preparative gels, trypsinized according to the method of Wilm and Mann [43] and identified by mass spectrometric methods as previously described [6]. Briefly, matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) MS was carried out by using a VoyagerDE Pro (Applied Biosystems, Foster City, CA, USA); 1 μL of the peptide solution was mixed with 1 μL of matrix (alpha-cyano-4-hydroxycinnamic acid (Bruker Daltonics, Billerica, MA, USA), 5 mg/mL, in 50% acetonitrile with 0.1% trifluoroacetic acid) and then spotted on the target plate. Peptide spectra were acquired in positive reflection mode, averaging about 1 000 laser shots per MALDI-TOF spectrum. Mass spectra were calibrated using calibration mixtures CalMix1 and CalMix2 (Applied Biosystems). The peptide mass spectra were analyzed with the peptide mass fingerprint algorithm on the Mascot web site[1] by searching against the NCBI non-redundant database (NCBInr 20080410 (6 417 748 sequences, 2 190 362 656 residues)). The search algorithm was set to allow carbamidomethylation on cysteine residues, oxidation on methionine residues, and a maximum of one missed trypsin cleavage. The peptide mass tolerance was 0.3 Da. Proteins were considered to be positively identified when the probability-based score was above the significance threshold ($p \leq 0.05$).

For electrospray injection-based quadrupole time-of-flight tandem mass spectrometry (ESI Q-TOF MS/MS), 4 μL of the peptide solution were loaded into a sample tip (Nanoflow Probe Tip, long; Waters, Milford, MA, USA). Peptide sequences were determined from MS/MS fragmentation data recorded with an ESI Q-TOF MS (Q-TOF Ultima, Waters) in positive reflection mode. Proteins were identified using the program ProteinLynx Global Server (version 2.1; Waters) by searching against the NCBI non-redundant database[2] as downloaded on 16th August 2006.

3. RESULTS

3.1. Breeding line-specific BALF glycoprotein regulation comparing day −7 and day 4 post infection

Glycosylated BALF proteins obtained from clinically healthy pigs (day −7 pre-infection) and pigs with acute disease (day 4 after infection) were separated by 2-D PAGE (Fig. 1). Expression levels of seven glycosylated proteins, namely alpha-1-acid glycoprotein, fetuin A, haptoglobin precursor, hemoglobin, hyaluronidase, alpha-1-antichymotrypsin 3, and pulmonary SP-D were compared for the three breeding lines between day −7 and day 4 post infection (Fig. 2, Tab. I). In pigs of the German Landrace breeding line three proteins were regulated significantly. Thus, the expression of SP-D was significantly decreased 17.6-fold and protein levels of alpha-1-acid glycoprotein and hyaluronidase were significantly increased 2-fold and 1.9-fold. In pigs of the Piétrain breeding line expression of two proteins, namely alpha-1-acid glycoprotein and alpha-1-antichymotrypsin 3 were significantly decreased 2.8-fold and 1.5-fold, respectively.

[1] http://www.matrixscience.com.

[2] ftp://ftp.ncbi.nlm.nih.gov/blast/db/FASTA/nr.gz.

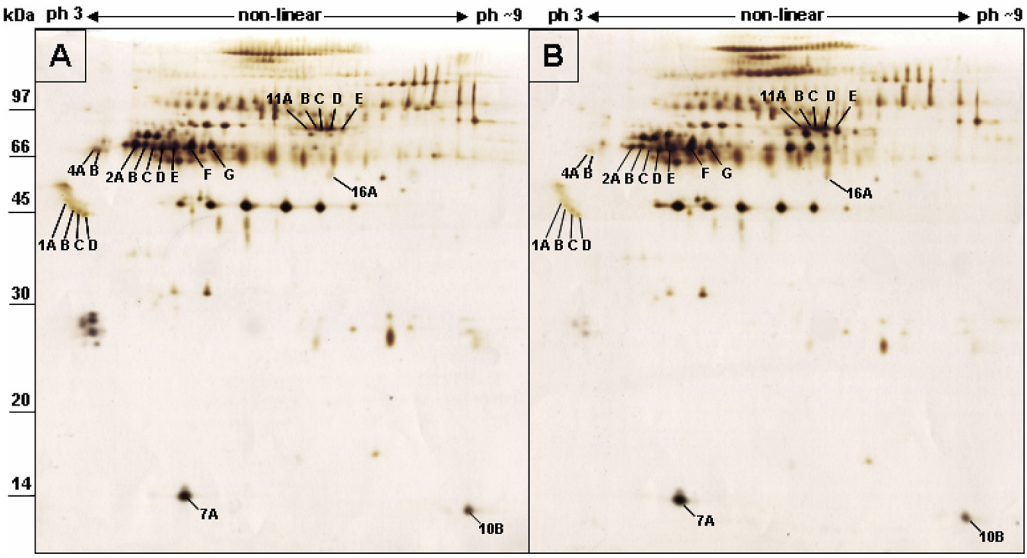

Figure 1. Silver-stained 2-D-PAGE from a representative Piétrain pig prior to infection (A) and on day 4 post infection (B) with *A. pleuropneumoniae*. Purification of glycoproteins was carried out by concanavalin A lectin chromatography. Identities of differential expressed proteins were detected by mass spectrometry (MALDI-TOF MS, ESI Q-TOF MS/MS) and data base analysis (MASCOT, BLAST).

The pigs of the Hampshire breeding line did not show any lung lesions on day 4 post infection, and no unanimous regulation of any of the 7 proteins was observed.

3.2. Breeding line-specific BALF glycoprotein regulation comparing day −7 and day 20 post infection

For the comparison of BALF obtained from clinically healthy pigs (day −7 pre-infection) and pigs with chronic disease (day 20 after infection), expression levels of the same seven proteins (as described in Section 3.1.) were monitored (Fig. 2). In pigs of the German Landrace breeding line three of these were regulated significantly. Thus, fetuin A and haptoglobin were downregulated 1.2- and 2-fold, respectively, and hyaluronidase was upregulated 1.8-fold. In pigs of the Piétrain breeding line two proteins were regulated significantly with fetuin A being downregulated 2-fold and hyaluronidase being upregulated 1.6-fold. In pigs of the Hampshire breeding line (none of which showed lung lesions) five proteins were regulated significantly. Thus, fetuin A, hyaluronidase, and alpha-1-antichymotrypsin 3 were significantly upregulated 3.6-fold, 3-fold, and 1.7-fold, respectively. In addition, haptoglobin and SP-D were downregulated 3-fold and 3.8-fold.

3.3. Differences in BALF glycoprotein expression between breeding lines on days −7, 4, and 20 post infection

In order to identify expression differences between the different breeding lines in uninfected pigs as well as on days 4 and 20 post infection expression levels of proteins pools of three animals per breeding line were compared (Fig. 3). Performing 2-D DIGE technology, we identified five additional differentially regulated proteins so that a total number of 12 glycoproteins were monitored (alpha-1-acid glycoprotein, fetuin A, properdin, haptoglobin precursor, haptoglobin, hemoglobin, hyaluronidase, inter-alpha-trypsin inhibitor family heavy chain-related protein (IHRP), alpha-1-antichy-

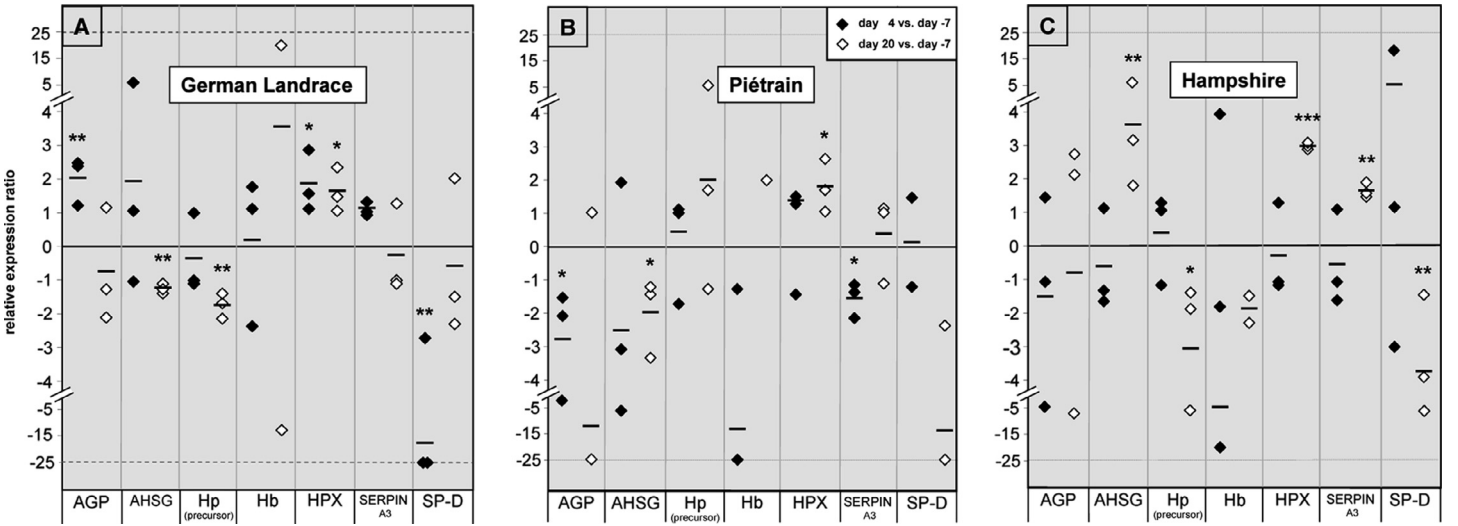

Figure 2. Breeding line-specific BALF glycoprotein regulation comparing day −7 and day 4/20 post infection. For three pigs of each breeding line, glycoproteins obtained from BALF on day −7, 4 and 20 were enriched by lectin affinity chromatography and separated by 2-D PAGE. Gels were performed for three biological replicates, and silver-stained protein spots were quantified by ImageJ software. Protein expression on day 4 or on day 20 was compared to day −7. If the quotient was below 1, the negative inverse value is given. Diamond symbols indicate relative expression differences of individual proteins on day 4 or 20 in comparison to day −7. Mean values are depicted by black bars. Significant differences are indicated by asterisks (* $\leq$ 0.05, ** $\leq$ 0.01, *** $\leq$ 0.001). AGP: alpha-1-acid glycoprotein, AHSG: fetuin A, Hp: haptoglobin, Hb: hemoglobin, HPX: hyaluronidase/ hemopexin, SERPINA3: alpha-1-antichymotrypsin 3, SP-D: pulmonary surfactant-associated protein D. For German Landrace (Hb, day 20), Piétrain, (AGP day 20, Hb and SP-D days 4 and 20), and Hampshire (Hb day 20) the respective proteins were below detection level in individual pigs and, therefore, no expression ratios could be obtained in these cases.

Table I. BALF-glycoproteins that have been identified by mass spectrometry analysis. The identity of the proteins was determined either by MALDI-TOF MS or by ESI Q-TOF MS/MS fragment mass fingerprint (italic) in combination with a database search (MASCOT, BLAST).

Gel spot	Protein name	Accession number	Theor. pI	Theor. MW (kDa)	Peptide matches	Sequence coverage (%)
1 A/B/C/D	alpha-1-acid glycoprotein	Q_29014	5.83	20 899	2/2/2/2	12/12/12/12
2 A/B/C/D/E/F/G	alpha-1-antichymotrypsin 3	CAC06756	5.77	22 883	8/16/19/14/10/13/3	12/42/44/34/33/29/17
3 B	*alpha-1B glycoprotein (canis familiaris)*	*XP_541346*	*5.54*	*55 580*	*1*	*2*
4 A/B	alpha-2-HS-glycoprotein / fetuin A	P29700	5.74	38 400	1/2	6/6
5 A	complement component 3	NP_999174	6.09	188 229	13	11
6 A	ficolin	NP_999033	5.63	35 173	11	33
7 A	*haptoglobin precursor*	*Q8SPS7*	*6.93*	*38 457*	*1*	*4*
8 A/B/C/D/E/F/G	haptoglobin	NP_999165	6.93	38 457	10/9/10/11/10/10/8	34/23/35/33/34/35/27
9 D/E	hemoglobin, alpha-subunit	P_01965	8.76	15 087	8/8	64/64
10 B	*hemoglobin*	*1QPW_D*	*7.21*	*16 025*	*2*	*15*
11 A/B/C/D/E	hemopexin / hyaluronidase	NP_999118	6.59	52 071	2/15/16/16/10	8/29/26/32/23
12 A	Ig heavy chain variable region	CAJ45440	4.85	13 573	8	65
13 A	Ig lambda chain C region	P01846	6.75	11 168	4	65
14 A/B/C/D/E/F/G/H/I/J/K	inter-alpha trypsin inhibitor family heavy chain related protein	NP_001001537	6.42	102 254	15/16/18/18/19/25/24/31/24/21/19	15/20/19/19/23/28/26/34/30/24/26
15 D/E/F/G/H	properdin, B-factor	NP_001095294	7.45	87 179	18/16/18/11/11	31/21/24/18/15
16 A	*pulmonary surfactant-associated protein D*	*NP_999275*	*8.57*	*37 961*	*1*	*5*
17 B/C/D/E/F/G	transferrin	CAA30943	7.08	76 901	12/16/7/20/18/15	25/22/11/30/27/19

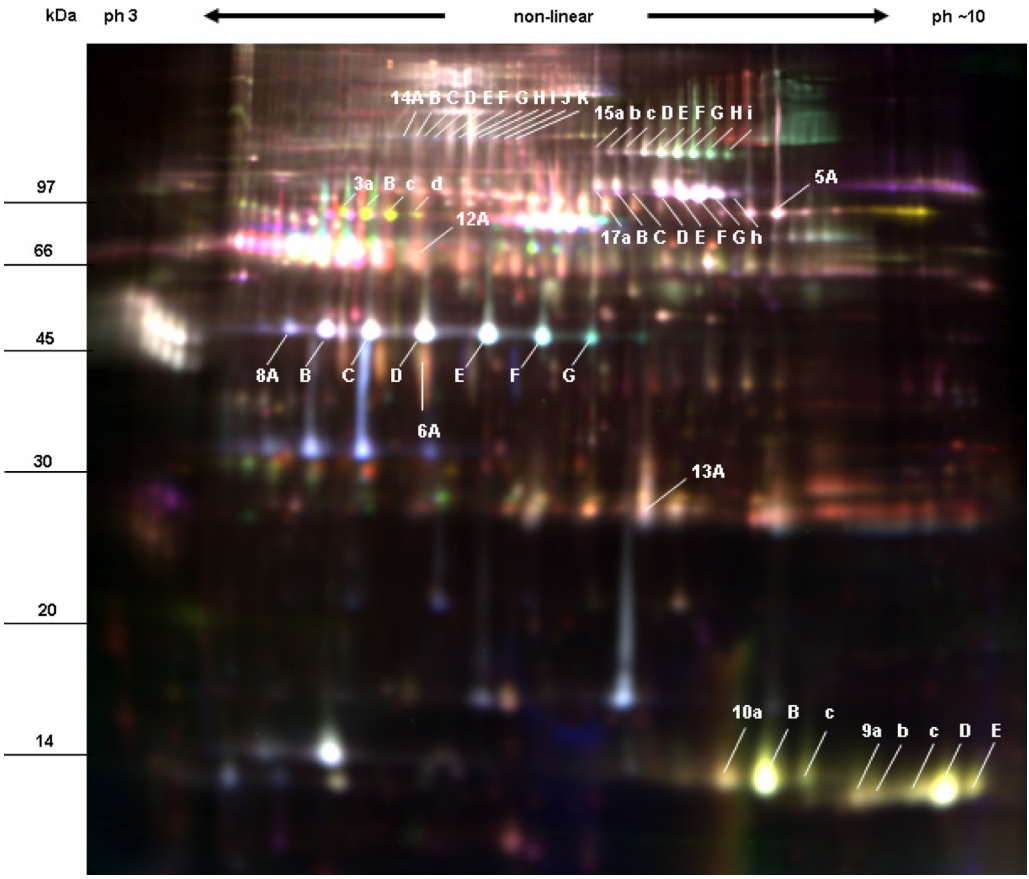

Figure 3. Breeding line-specific BALF glycoprotein expression on day 20 post infection by fluorescence based 2-D gelelectrophoresis (2-D DIGE). Glycoprotein fractions from three animals of each breed were pooled and labelled by different CyDyes before two dimensional separation: German Landrace = Cy2 (blue), Piétrain = Cy3 (green), and Hampshire = Cy5 (red). Proteins identified are indicated by a number/captial letter combination referring to Table I; potential isomers for which mass spectrometry gave no results are marked with lower case letters.

motrypsin 3, pulmonary SP-D, transferrin, and alpha-1B-glycoprotein; Tab. I). Relative breeding line-specific protein expression levels on the different days were quantified (Fig. 4). Neighbouring protein spots forming horizontal lines were considered to be isoforms of the same protein although a number of spots did not contain sufficient protein for MS identification but were visualized due to the high sensitivity of the CyDyes. On day −7 (uninfected pigs) fetuin A levels in pigs of the Hampshire breeding line were 1.8-fold higher than in pigs of the other two lines; in addition, levels of haptoglobin, haptoglobin precursor, and SP-D were 1.8- to 2-fold lower. Properdin, hyaluronidase, transferrin, and alpha-1B-glycoprotein levels were lower in pigs of the German Landrace breeding line in comparison to Piétrain pigs, and IHRP levels were increased. On day 4 post infection levels of haptoglobin precursor, hyaluronidase, and IHRP were comparatively lower in pigs of the Hampshire

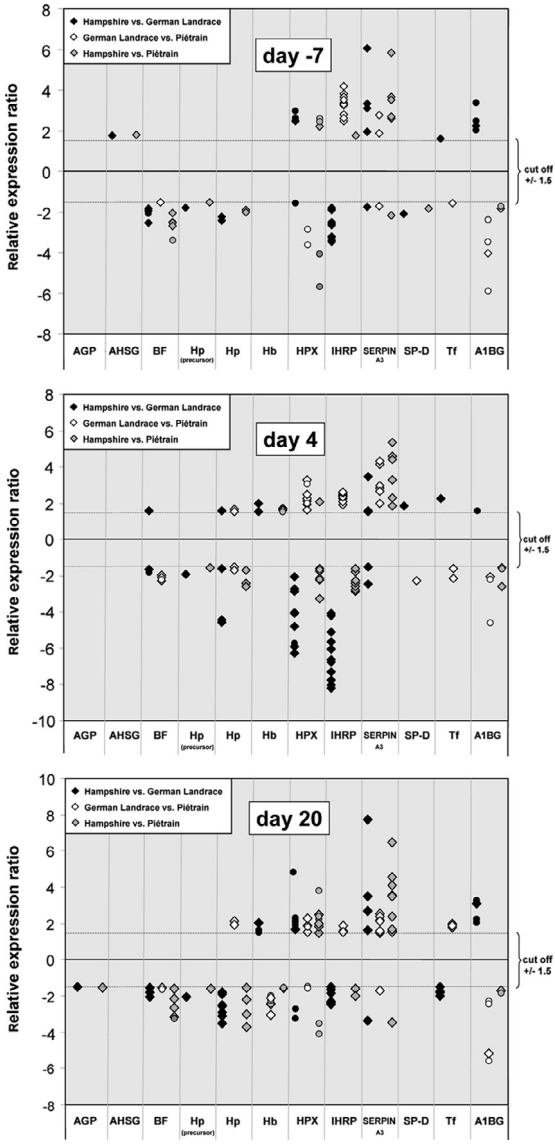

Figure 4. Differences in BALF glycoprotein expression between breeding lines on days −7, 4, and 20 post infection. Symbols indicate relative expression differences of individual proteins between Hampshire versus German Landrace (black), German Landrace versus Piétrain (white) and Hampshire versus Piétrain (grey). If the quotient was below 1, the negative inverse value is given. For proteins represented by more than one spot on gels, the expression of each isomer was compared separately between the different breeding lines. Identified proteins are marked by diamond symbols, potential isoforms of the respective proteins are represented by circles. AGP: alpha-1-acid glycoprotein, AHSG: fetuin A, BF: properdin B-factor, Hp: haptoglobin, Hb: hemoglobin, HPX: hyaluronidase/hemopexin, IHRP: inter-alpha-trypsin inhibitor family heavy-chain related protein, SERPINA3: alpha-1-antichymotrypsin 3, SP-D: pulmonary surfactant-associated protein D, Tf: transferrin, A1BG: alpha-1B-glycoprotein.

breeding line (which did not have lung lesions). Properdin, SP-D, transferrin, and alpha-1B-glycoprotein were lower in pigs of the German Landrace breeding line in comparison to Piétrain pigs, and IHRP and alpha-1-antichymotrypsin 3 were increased. On day 20 post infection alpha-1-acid glycoprotein, properdin, haptoglobin precursor, haptoglobin, and IHRP levels were lower in pigs of the Hampshire breeding line (which did not have lung lesions) in comparison to both other breeding lines. Properdin, hemoglobin, and alpha-1B-glycoprotein were lower in pigs of the German Landrace breeding line in comparison to Piétrain pigs, and haptoglobin, IHRP, and transferrin were increased.

4. DISCUSSION

The detection of biomarkers for respiratory health in pigs is of high interest to pork producers. Thus, current diagnostic procedures are based on serology, culture or PCR and, therefore, can only be used to confirm a specific pathogen-free status but do not detect pathogens newly emerging in the herd. In addition, these tests are hampered by a limited diagnostic sensitivity and specificity (serology). Suitable biomarkers can help to improve diagnostic information, as it has been shown recently for the cationic peptide PR-39 which has BALF-concentrations correlating with latent or chronic lung infection [20, 21].

Since glycosylated proteins are frequently involved in immune functions, in this study we focused on the analysis of these proteins in BALF. As it has been shown that different breeding lines differ with respect to their susceptibility to *A. pleuropneumoniae* infection [22] animals of three different breeding lines were investigated. The glycoprotein isolation was carried out by lectin chromatography with Concanavalin A [29], which binds to carbohydrate structures containing glucose and mannose groups. These sugar residues can be found on chains of N-linked glycans but are absent on O-glycans, resulting in an exclusion of solely O-glycosylated proteins like the mucin-type glycoproteins of the airway epithelium [26]. Since, however, solely O-glycosylated proteins form a minor part of the glycoproteome [2] and N-glycosylated proteins have been shown to be predominantly involved in infection response [46, 49], this work was focused on N-glycosylated proteins. Seventeen N-glycosylated proteins in the BALF glycoproteome could be identified; the MS-based identification of additional protein spots visible on the silver- or CyDye-stained gels was hampered by low protein abundance and incomplete annotation of porcine genome.

The selection of pigs for the sampling of BALF was based on the lung lesion score which has been shown to be a reliable indicator for the severity of infection [17]. For the German Landrace and Piétrain breeding lines pigs developing similarly severe lung lesions could be selected and, therefore, a direct comparison of the results appeared legitimate. In contrast, as described previously, no severely diseased Hampshire pigs could be obtained [22]. This finding is particularly relevant with respect to the interbreed-comparison of BALF of healthy pigs since Hampshire-specific expression profiles might be – at least in part – responsible for the increased resistance of these pigs and, therefore, present potential markers for breeding selection.

Concerning the breeding line-specific BALF glycoprotein regulation during the course of disease, individual pigs were followed up over a period of 11 or 27 days, respectively, in order to minimize the impact of inter-individual differences. A possible age-dependent BALF-protein expression as it has been observed when comparing juvenile and adult rats [47] was not investigated since all pigs were juvenile and varied in age by up to 7 days; in addition, it had been shown previously that PR-39 expression did not vary in juvenile pigs of the age span used in this study. During the course of acute infection pigs of the German Landrace breeding line showed an increased expression of alpha-1-acid glycoprotein and hyaluronidase. Alpha-1-acid glycoprotein is one of the major acute phase proteins derived from liver and alveolar cells and it is known to increase during inflammation or infection [32]. The presence of

hyaluronidase reflects increased hydrolysis of the extracellular matrix and enhanced permeability of the interstitial barrier for inflammatory cells. However, degradation of the interstitial barrier may also lead to a stronger infiltration of pathogens, possibly resulting in an increased immune response of the host [31]. Polymorphic neutrophil (PMN)-derived secretions of antibacterial enzymes such as serine proteases could cause the strong decline in the level of the elastase-sensitive protein SP-D which results in a decrease of the anti-inflammatory impact it has on the course of disease [9, 48]. In the chronic stage of infection, the expression of hyaluronidase was still increased in animals of the Germany Landrace and Piétrain breeding lines, indicating an ongoing convalescence and tissue repair process in the lung. Significantly decreased protein levels of fetuin A and haptoglobin indicate the occurrence of constant hemolysis and inflammation even on day 20 post infection. Thus, haptoglobin plays a major role as hem binding reactant and its expression is known to increase during the acute phase of *A. pleuropneumoniae* infections [18]; the significantly decreased haptoglobin levels in chronic diseased animals on day 20 may refer to severe hemolytic processes [54]. Fetuin A inhibits the production of pro-inflammatory cytokines such as IL-1β, TNF-alpha, and NO by participation in macrophage deactivation as opsonin [12, 51] and, thereby, participates in preventing excessive immune reactions detrimental to the host.

Acute infected pigs of the Piétrain breeding line exhibited a decrease of the proteins alpha-1-acid glycoprotein and alpha-1-antichymotrypsin 3 on day 4 compared to day −7. Alpha-1-antichymotrypsin 3 plays a significant role in the inhibition of serine proteases such as cathepsin G, which is released by granulocytes during inflammation [25]. It has been shown that cathepsin G regulates the ability of PMN to stimulate other immune cells, triggering the start of local inflammatory processes [41]. Thus, low levels of alpha-1-antichymotrypsin would lead to increased presence of cathepsin G and, presumably, increase inflammation. Unexpectedly, the level of alpha-1-acid glycoprotein was significantly decreased even though it represents one of the major proteins of the acute phase reaction with a half-life of several days [27]. This protein carries an extensive number of glycan residues which can be differently processed during the posttranslational protein maturation leading to a manifold functional diversity [14]. Former studies described that these modifications can also result in an altered affinity towards the lectin Concanavalin A [45]. Based on this fact, a higher presence of alpha-1-acid glycoprotein in the acute stage of infection cannot be excluded because the lectin-based glycoprotein purification of BALF possibly might hamper the detection of modified versions of this protein.

In pigs of the Hampshire breeding line – although they did not show lung lesions – a significant regulation of proteins was observed on day 20 post infection. Here, a depletion of SP-D and an increased expression of hyaluronidase indicate a delayed PMN infiltration. Additionally, this breeding line shows increased levels of fetuin A and alpha-1-antichymotrypsin 3 which have a negative impact on macrophages and neutrophil stimulation thereby preventing excessive immune reactions detrimental to the host.

Considerably altered levels of BALF-protein expression could be determined in the analysis of differences between the breeding lines. Here, comparably higher levels of fetuin A as well as lower protein levels of haptoglobin (and precursor) and SP-D in healthy animals (prior to infection) of the Hampshire breeding line compared to pigs of the German Landrace and Piétrain breeding lines indicate that these proteins are potential biomarkers for an increased resistance towards *A. pleuropneumoniae* infection. Thus, the increased resistance of Hampshire pigs might be due to a fetuin A-mediated reduction of the respiratory burst of macrophages with its potential tissue-damaging effect [10]. The consistently low levels of haptoglobin (and precursor) as well as of hyaluronidase and IHRP in Hampshire pigs confirm the absence of inflammatory processes. Hence, hyaluronidase limits the permeability of the respiratory barrier, and IHRP is considered to reduce the phagocytic activity of PMN by inhibiting the polymerization of cell surface actin [8].

In conclusion our findings demonstrate the potential value of glycoprotein expression

studies in porcine BALF. Thus, the increased expression of fetuin A on day 20 post infection in Hampshire pigs (no lung lesions) in comparison to pigs of the Piétrain and German Landrace breeding lines (severe lung lesions), implies that fetuin A may be applicable as a biomarker for chronic infections, and therefore, might be usable in support of the cationic peptide PR-39, a previously identified marker for chronically diseased animals [20]. The unanimously high expression of hyaluronidase in all breeding lines on day 20 indicates a potential suitability of this protein as a marker for animals having been exposed to respiratory pathogens. These findings are supported by the cultural examination where *A. pleuropneumoniae* was isolated from tonsils and lung tissue of pigs of German Landrace and Piétrain breeding lines on day 20 post infection but not from pigs of the Hampshire breeding line. Furthermore, the high level of fetuin A and low levels of haptoglobin (and precursor) and SP-D on day −7 in Hampshire pigs (no lung lesions upon infection) compared to pigs of the German Landrace and Piétrain breeding lines (significant lung lesions upon infection) indicates that these proteins are potential phenotypic markers applicable in a breeding selection for increased resistance towards *A. pleuropneumoniae* infection. Further studies have to be performed to validate the general use of these potential biomarkers with respect to applicability in different age groups and in lung infections caused by other respiratory tract pathogens such as *Mycoplasma hyorhinis*, *Actinobacillus porcitonsillarum* and viruses.

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