Original article

Differential proteomic analysis reveals increased cathelicidin expression in porcine bronchoalveolar lavage fluid after an *Actinobacillus pleuropneumoniae* infection

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Abstract – Accurate definition of respiratory health in pigs is an important problem for swine producers and veterinarians. In an approach to identify potential biomarkers, two-dimensional gel electrophoresis and mass spectrometry on bronchoalveolar lavage fluid (BALF)-derived proteins from pigs experimentally infected with *Actinobacillus pleuropneumoniae* were performed at different time points post infection. Mock-infected pigs were used as a control. It was shown that the antimicrobial peptides, prophenin-2 and PR-39, and the calcium-binding protein calgranulin C were reproducibly upregulated in BALF of pigs chronically infected with *A. pleuropneumoniae*. Concentrations of PR-39 were significantly ($p < 0.05$) increased in BALF (median of 4.8 nM) but not in serum (median of 2.5 nM) on day 21 after infection. A Receiver Operating Characteristics (ROC) plot showed that PR-39 in BALF is an accurate and easily accessible marker to detect clinically healthy pigs convalescent from an experimental *A. pleuropneumoniae* infection. These results imply that PR-39 might have a potential as a general biomarker to determine porcine respiratory health.

antimicrobial peptide / PR-39 / BALF / porcine respiratory tract

1. INTRODUCTION

Detection of respiratory diseases in pigs is of growing importance. Due to changes in liability laws, swine producers may be held responsible for disease-related losses unless it can be determined that the animals were healthy at the time they were sold.

Because several respiratory tract pathogens, such as *Mycoplasma hyopneumoniae*, *Haemophilus parasuis*, *Bordetella bronchiseptica*, *Pasteurella multocida*, and *Actinobacillus* (*A.*) *pleuropneumoniae,* can be responsible for respiratory disease in pigs and can persist for extended periods of time in convalescent animals, tests facilitating

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the detection of lung alterations independent of the pathogen would be highly relevant.

Clinically healthy individuals convalescent from respiratory disease are likely to still exhibit alterations in the respiratory tract for a prolonged period of time. These alterations are reflected in changes within the epithelial lining fluid (ELF; [14]), a thin layer covering the surface of the respiratory tract and presenting the first line of host defence against inhaled microbial invaders. Modifications in ELF have been exploited in humans to diagnose lung diseases based on measurements of phospholipid content, surface tension, cytological findings and protein profiles [25]. However, approximately 97% of ELF-proteins are serumderived [19] and, therefore, may be of limited use as a diagnostic marker for respiratory infections.

The use of proteomic technologies has intensified the search for lung disease markers in human ELF [25], and currently a list of approximately 1000 proteins identified in human bronchoalveolar lavage fluid (BALF) is available (http://w3.umh.ac.be/~biochim/proteomic.htm; [26, 36]). Furthermore, it was found that a broad spectrum of antimicrobial agents such as defensins, inducible nitric oxide synthetase and LL37/CAP-18, a member of the cathelicidin family, are found in ELF [10], which, in part, are present in increased concentrations during infection [29]. Most of these agents originate from emigrated phagocytes where some of them are stored as precursors in cytoplasmic granules [38]. Although porcine neutrophils lack defensins they are well endowed with cathelicidins, including protegrins, prophenins, PR-39 [28] and porcine myeloid antimicrobial peptides [31].

The definition of respiratory health in swine is limited by the difficulty of interpreting data collected from the respiratory tract. In addition to genetically determined resistance or susceptibility, many non-pathogenic and facultative pathogenic microorganisms as well as abiotic factors influence host immunity. Pathogenic microorganisms persisting on the respiratory epithelium of clinically healthy pigs have a high economic impact on swine production and can be spread into previously uninfected herds by carrier animals.

Our aim was to identify new diagnostic tools for the definition of respiratory health in swine, which are not restricted to bacteriological examinations of BALF. To identify suitable marker proteins in BALF we used an *A. pleuropneumoniae* infection model. Using two-dimensional gel electrophoresis and subsequent mass spectrometry, we detected reproducible changes in protein profiles between uninfected and convalescent animals 3-weeks post infection. Our findings identified three peptides that were up-regulated in convalescent animals and showed that one of them, the antimicrobial peptide PR-39, has a potential as a diagnostic marker to detect lung disease in convalescent pigs.

2. MATERIALS AND METHODS

2.1. Bacterial strains and media

Actinobacillus pleuropneumoniae serotype 7 clinical isolate AP76 [1] and *A. pleuropneumoniae* serotype 2 clinical isolate C5934 [34] were used for challenge experiments. Both strains were cultured and prepared for experimental aerosol infection studies as described previously [3].

2.2. Clinical study and lung lavage protocol

A total of 48 specific-pathogen-free pigs of both sexes, approximately 7- to 9-weeks old, were obtained from closed herds with a long record of respiratory health. All animals were tested for the absence of *A. pleuropneumoniae* antibodies using an ApxIIA-ELISA [24]. The animals were experimentally infected with an *A. pleuropneumoniae* serotype 7 (32 pigs) or a serotype 2 clinical isolate (8 pigs) in an aerosol infection model

[3]; eight pigs were used for a mock-infection. The challenge dose was titrated to induce infection but not fatal disease (10^2 to 10^3 bacteria per liter aerosol in the challenge chamber). BALF was collected as described previously [3] from all pigs prior to infection, from 28 pigs in the acute stage of infection (6 pigs infected with *A. pleuropneumoniae* serotype 2 on day 4 and 22 pigs infected with *A. pleuropneumoniae* serotype 7 on day 7 post infection), and from all 37 surviving pigs before necropsy on day 21 post infection. BALF was taken from control animals before and on day 7 and day 21 after mock-infection.

BALF samples were processed immediately for bacterial culture and for cytological and electrophoretical examinations. BALF cells were counted and cytospots were prepared and stained with 10% May-Grünwald/Giemsa solution for each differential count; 8% polymorphonuclear neutrophils (PMN) in the differential cell count was considered the cut-off value for healthy pigs [13]. Pigs with a higher cell count in the initial BALF were not included in the study (4 animals infected and 2 animals from the control group); two pigs not showing any pathomorphological lung alterations in combination with constant negative bacteriological results were excluded. Postmortem analyses as well as bacteriological and serological examinations of all pigs were performed as previously described [3, 17]. All animals were cared for in accordance with the principles outlined in the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (ETS123).

2.3. Selection of BALF-samples for two-dimensional gel electrophoresis

BALF samples from a subgroup of ten pigs (six pigs infected with *A. pleuropneumoniae* serotype 7 and four infected with *A. pleuropneumoniae* serotype 2) were selected for two-dimensional polyacrylamide gel electrophoresis (2-D PAGE). All pigs in the subgroup showed clinical signs such as depression and increased body temperatures after infection. Upon necropsy on day 21, all of these pigs had lung lesions, which were evaluated as previously described [17]. Briefly, each lung lobe was assessed a total possible lesion score of 5, resulting in a maximum score of 35 for the entire lung. Individual lesions were mapped on a simplified lung chart in which each lobe was subdivided into triangles. The number of affected triangles was counted and the score was calculated as a fraction of five for this lobe. All pigs in this study had lung lesion scores between 0.1 and 13.4. *A. pleuropneumoniae* was reisolated from pneumonic lesions, tonsils or lung lymph nodes in 9 of the 10 pigs.

BALF samples (taken before infection, 4 days (*A. pleuropneumoniae* serotype 2) or 7 days (*A. pleuropneumoniae* serotype 7) and 21 days after infection) were analysed in triplicate by 2-D PAGE. Briefly, BALF cells were removed by centrifugation ($5\,000 \times g$, 10 min), and proteins in the supernatant were precipitated overnight at 4 °C with trichloric acetic acid (TCA; 10% w/v final concentration) and harvested by centrifugation ($5\,000 \times g$, 10 min). The pellet was dispersed by sonication in ice-cold acetone, frozen for 2 h at –20 °C, and centrifuged again as described above. The pellets were air-dried and resuspended in rehydration solution (8 M urea, 2% CHAPS). Protein concentrations were determined (Micro BCA®, Uptima Interchim, France) and adjusted to 275 µg/mL in rehydration solution supplemented with dithiothreithol (DTT; 18 mM final concentration), IPG-buffer (pH 3–10, 0.5%; Amersham Biosciences, Uppsala, Sweden) and bromphenol blue (0.001%). Each sample (360 µL) was loaded on an Immobiline Dry Strip® (pH 3–10, 18 cm; Amersham Biosciences) and run in a Pharmacia Biotech IPGphor system (11 h rehydration followed by a multistep program of 2 h at 100 V, 1 h at 500 V, 1 h at 1000 V, 2 h at 4000 V, a linear gradient spanning from 4000 V to 8000 V in 2 h, and a final step at 8000 V for 6 h). After isoelectric focussing, the strips were stored at

–20 °C or processed directly for the second dimension. Briefly, the strips were placed in an equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 65 mM DTT) for 15 min followed by 15 min in an equilibration buffer containing iodoacetamide (244 mM) instead of DTT. Separation in the second dimension was carried out on sodium dodecyl sulphate (SDS) PAGE (12.7% acrylamide, 0.3% bisacrylamide). Gels were stained with colloidal Coomassie G-250 and with silver nitrate (Silver Staining Kit, Sigma, St. Louis, MO, USA) according to the manufacturer's instructions. Spot detection was performed using the 2-D PAGE software (Phoretix®, Nonlinear Dynamics, Newcastle, UK).

2.4. Identification of differentially expressed proteins by mass spectrometry

Prominent spots expressed in BALF from day 21 post infection samples were excised from gels, digested with trypsin and the resulting peptides were analysed by mass spectrometry (AgeLab Pharma GmbH, Hamburg, Germany). Peptide mass fingerprint analyses were performed using the MASCOT program (www.matrixscience.com), and significant matches were identified using the SwissProt database. Scores were calculated by the molecular weight search score algorithm (MOWSE) and are significant ($p < 0.05$) if greater than 69. Thus, a search result would be expected to occur at random with a frequency of less than 5% [27].

2.5. Western Blot analysis

BALF proteins were separated by discontinuous SDS-PAGE (10.8% acrylamide and 0.29% bisacrylamide) and blotted onto a nitrocellulose membrane using a Protean II Minigel system (Bio-Rad, Munich, Germany) following standard procedures [30]. Mouse monoclonal antibodies (mouse mAb) against PR-39, which recognise both mature PR-39 and its precursor (cathelin-containing PR-39; [32, 33, 39]), were used as primary antibodies. Blots were developed using a peroxidase-conjugate, and a chemiluminescent substrate (Super Signal Ultra®, Pierce, Illinois, USA).

2.6. Influence of PR-39 on bacterial growth and antigen expression

In order to investigate whether PR-39 was bactericidal against *A. pleuropneumoniae*, a growth inhibition zone assay on solid medium was performed essentially as described by Boman et al. [5]. Briefly, 100 μL of *A. pleuropneumoniae* AP76 suspension with an optical density at 660 nm (OD_{660}) of 0.1 in NaCl (150 mM) were plated onto supplemented PPLO-Agar, and filter paper discs saturated with different concentrations of PR-39 (0.024 nM to 8 μM) were placed onto the agar surface. Cultures were incubated overnight at 37 °C in a CO_2-incubator (5% CO_2) and bacterial growth around the paper discs was evaluated. In addition, minimal inhibitory concentrations (MICs) of PR-39 for *A. pleuropneumoniae* and *E. coli* (as the control) were determined by the broth microdilution method according to the NCCLS guidelines in document M31-A2 (2002) [7, 33]. PR-39 concentrations ranging from 1.0 to 40 μM were tested for their effect on bacterial growth ($2–8 \times 10^5$ CFU /mL) in cation-adjusted Mueller-Hinton-Boullion (MHBII), PPLO medium with Isovitalex (1%) and Tween 80 (0.1%) and Vast Fastidiuous Medium (VFM) in 96-well plates.

The influence of PR-39 on bacterial antigen expression was investigated by 2-D PAGE. Briefly, 100 mL of an *A. pleuropneumoniae* AP76 culture was grown (shaking at 200 rpm) to an OD_{660} of 0.3. This culture was split into two 50 mL aliquots, and PR-39 was added to one aliquot to a final concentration of 0.5 μM; for control cultures, an equal volume of NaCl (150 mM) was added. This PR-39 concentration was chosen based on the average PR-39 concentration in BALF from pigs 21 days post infection and an estimated dilution factor of BALF with respect to ELF of 6 to 40 [21].

Cultures were further incubated while shaking for 1 h, and grown to an OD_{660} of approximately 0.6. Harvesting and treatment of bacterial cells for 2-D PAGE was done as described previously [22].

2.7. PR-39 capture ELISA

A PR-39 capture ELISA was conducted essentially as previously described [39]. Briefly, 96-well microtitre plates (Maxi-iSorb™ Surface, Nunc, Roskilde, Denmark) were coated overnight at 4 °C with mouse mAb against PR-39 (1.4 µg/mL) in carbonate buffer (0.1 M; pH 9.8). After washing twice with PBST (phosphate buffered saline supplemented with 0.05% Tween 20; PBS is 10 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, 1.76 mM KH_2PO_4) plates were blocked for 2 h at 22 °C in PBST containing 1% bovine serum albumin (BSA). All BALF samples, antibodies and conjugates were used in a dilution buffer; the dilution buffer was PBS containing 1% BSA and 0.01% cetrimonium bromide (CETAB, Sigma, St. Louis, MO, USA). This capture ELISA was used to determine PR-39 concentrations in BALF using a reference-standard-method [6] based on serial two-fold dilutions of BALF (1:2 to 1:128) and synthetic PR-39 (12.5 ng/mL initial concentration). Briefly, PR-39 mouse mAb coated plates were incubated with BALF. After four washes, a rat polyclonal antibody to PR-39 (30 µg/mL) was added [33, 39]. After three washes, the plates were incubated with the conjugate (peroxidase-labelled goat-anti-rat Ab (Dianova, Hamburg, Germany)). All incubation steps were done for 2 h (shaking) at 22 °C. After three washes, the plates were incubated with substrate SeramunBlue® fast (3, 3', 5, 5' tetramethylbenzidine, 1.2 mM, and hydrogen peroxide, 3 mM; Seramun Diagnostika Gmbh, Wolzig, Germany) for 10 min at 22 °C followed by the addition of a stop solution (H_2SO_4, 2 M) according to the manufacturer's instructions. Colour development was determined at 450 nm using a microplate reader (SLT-Spectra, SLT Lab-instruments Deutschland GmbH, Crailsheim, Germany). Differences in PR-39 concentrations were evaluated using the Signed Rank test for Paired Samples (SAS® statistical software). The potential use of the PR-39 capture ELISA for diagnostics was investigated by constructing a Receiver Operating Characteristics (ROC) plot resulting from the calculation of sensitivities and specificities for different potential cut-off-values [40].

3. RESULTS

3.1. Clinical study

Within 2 days post infection 30 pigs showed an increase in body temperature above 40 °C and developed clinical signs such as inappetence and coughing. Three pigs (one infected with *A. pleuropneumoniae* serotype 2 and two infected with *A. pleuropneumoniae* serotype 7) died within the first 3 days post infection. BALF samples from ten pigs surviving until the end of the experiment were selected for further investigation (Tab. I).

3.2. Protein patterns in BALF of healthy and *A. pleuropneumoniae* infected pigs

Approximately 80 spots were found to be differentially expressed in BALF samples taken from individual pigs before as well as on day 4 or 7 and day 21 after infection. A comparison of individual BALF samples from day 4 or 7 after infection with BALF samples prior to infection did not show a consistent protein pattern among different pigs irrespective of the day or the serotype (data not shown). In contrast, BALF from day 21 after infection consistently contained increased amounts of 12 proteins in the triplicate gels; in each pig at least 8 spots were increased with each individual spot being present in at least 7 out of 10 pigs (Fig. 1). However, the severity of the lung lesions and the number of additional spots in the individual pigs were not correlated.

Table I. Clinical, pathological and microbiological parameters of pigs selected for 2-D PAGE of BALF samples.

Pig	Infection with (A. pp. serotype)	Average body temperature days 1–3 p. inf.	Lung lesion score[a]	PMN % acute (day 4/7 p. inf.)	PMN % day 21 p. inf.	PR-39 on day 21 p. inf. (nM)	A. pp. in BALF day 4/7 p. inf.[b]	A. pp. in BALF day 21 p. inf.[b]	A. pp. in lung tissue day 21 p. inf.[b]
1	2	40.0	0.3	7.5	16.5	18.6	0	0	0
2	2	39.8	0.5	11	8.7	0.4	0	0	3
3	2	39.3	1.1	7.3	4.5	0.5	2	0	3
4	2	40.3	0.1	10	5.5	1.1	2	0	1
5	7	40.8	3.15	12.2	2	2.4	1	0	3
6	7	40.8	5.21	7.5	22.8	30.3	1	0	3
7	7	40.0	12.99	11	42	34.7	0	0	1
8	7	40.2	13.38	8.2	52.2	75.9	1	0	1
9	7	40.4	7.49	15.7	36	19.6	0	3	3
10	7	40.7	12.5	6.3	15	66.8	0	3	3

[a] The scheme of lung lesion mapping and evaluation described by Hannan et al. [17] and stipulated in the European Pharmacopoeia for the testing of *A. pleuropneumoniae* vaccines was used.
[b] 0, no detectable colonies; 1, less than 100 colonies; 2, more than 100 colonies but no confluent growth; 3, confluent growth.

Table II. Protein identification by Q-TOF-mass spectrometry.

Spot number	Protein name	Accession number	Theoretical M_r[b]/IP[c]	Matching peptides	Coverage (%)	Matched peptides[a]
6	Calgranulin C	P80310	10608/5.67	2	27	LGHYDTLIK----------------- IFQNL DANQDEQVSF K
9	PR-39	P80054	19464/9.96	5	47	METQRASLCL GR---------- --------- ---LNEQSS EANLYRLLEL DQPPKADEDP GTPKPVSFTV K---------- ----E NGR---------- --------- - RRPRPPYLP RPRPPPFPP R--------- ---FPPRFPG K-
10	Prophenin-2 precursor	P51525	25839/10.56	2	21	LNEQSS EANLYRLLEL DQPPKADEDP GTPKPVSFTV K-------- RPP ELCDFKE NGR

[a] Mascot search results (www.matrixscience.com) based on de-novo-sequencing by Q-TOF mass spectrometry analysis of the excised protein spots shown in Figure 1. Dashes indicate missing amino acids. One-letter codes are used for amino acids.
[b] Relative molecular mass in Dalton.
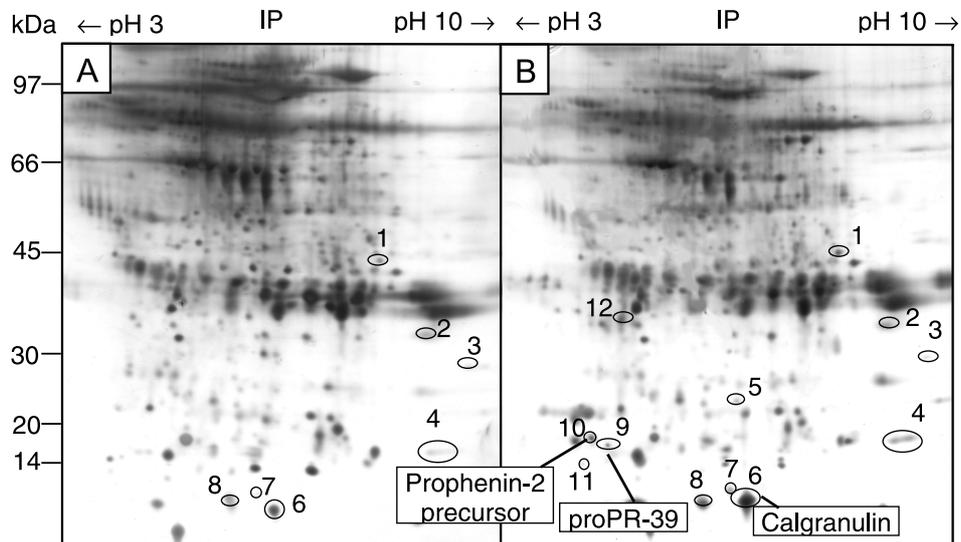[c] Isoelectric point.

Figure 1. Silver stained 2-D-PAGE of BALF from a representative pig prior to infection (**A**) and on day 21 post infection with *A. pleuropneumoniae* (**B**). Spots 3, 5 and 7 were present in seven pigs, spots 4, 6 and 8 in eight pigs, spots 1, 9, and 11 in nine pigs and spots 2, 10 and 12 consistently in all pigs. In addition to spots 6, 9 and 10, amino acid sequences were determined for spots 2, 5 and 12 using Q-TOF mass spectrometry and data base analyses (www. matrixscience.com), but no significant matches were detected.

3.3. Identification of differentially expressed BALF-proteins

The eight most prominent spots were analysed by mass spectrometry, and peptide mass fingerprint analysis revealed significant matches for three protein spots in the SwissProt database (Tab. II). The proteins were identified as prophenin-2 precursor and PR-39, both cathelicidin antimicrobial peptides, and calgranulin C, a member of the highly variable S100-protein family of calcium-binding proteins.

Western Blot analysis confirmed the differential expression of PR-39 that was observed in 2-D PAGE. Precursor and processed forms were detected reproducibly in BALF taken from pigs on day 21 after infection but not in BALF taken prior to infection (Fig. 2).

3.4. Influence of PR-39 on growth and antigen expression of *A. pleuropneumoniae*

Because PR-39 is a bactericidal peptide, we investigated whether the in vitro growth of *A. pleuropneumoniae* could be inhibited with concentrations of PR-39 that would be present in the ELF of infected pigs. Despite using up to 8 µM concentrations (more than 10 times the concentration calculated to be present in the ELF (0.5 µM)), no inhibition of growth was observed in the inhibition zone assay. Using the microdilution method, an MIC of 5 µM was determined for *A. pleuropneumoniae* in the VFM medium only; in *E. coli* the MIC was 1 µM with all media.

Because it was reported recently that virulence gene expression in *Salmonella* Typhimurium is regulated by cationic antimicrobial peptides, the protein profiles of

A. pleuropneumoniae cultured in the presence and absence of PR-39 were investigated. The PR-39 concentration used was 0.5 µM, which is within the concentration range in BALF from infected animals (0.4 to 75.9 nM) and concentrations in ELF are estimated to be approximately 6 to 40 fold higher. A comparison of protein patterns of bacterial whole cell lysates did not show clear differences, implying that PR-39 in the concentrations present in ELF of *A. pleuropneumoniae* infected pigs does not inhibit growth or regulate gene expression of the pathogen.

3.5. PR-39 is significantly increased in BALF of *A. pleuropneumoniae* convalescent pigs on day 21 post infection

Concentrations of PR-39 were increased ($p < 0.05$) in BALF on day 21 after infection when compared to concentrations before infection and in mock-infected control animals (Fig. 3). The increase of PR-39 in BALF from day 4 and 7 after infection was
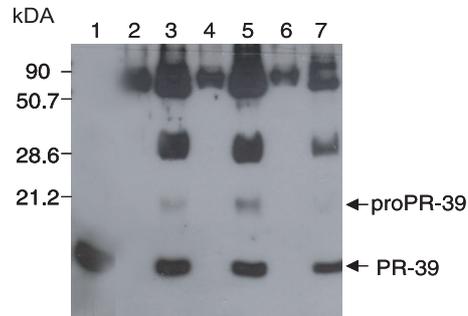

Figure 2. Western blot analysis of synthetic PR-39 (10 ng; lane 1), BALF-samples from pigs prior to infection (lanes 2, 4, 6) and 21 days post infection (lanes 3, 5, 7). The blot was developed using mouse anti-PR-39 mAb and a chemiluminescent peroxidase substrate. The high molecular weight bands also visible on the blot are caused by non-specific reactions of the peroxidase conjugate.

not significant and no difference in PR-39 concentrations was found between BALF of day 4 after infection with *A. pleuropneumoniae* serotype 2, BALF of day 7 after infection with *A. pleuropneumoniae* serotype 7,
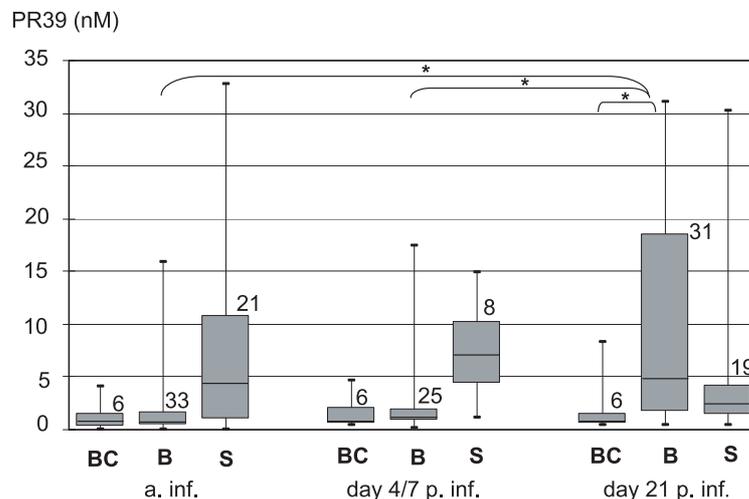

Figure 3. PR-39 concentrations in BALF (B) and serum/plasma (S) of infected pigs and in BALF of mock-infected pigs (BC). PR-39 concentrations were quantified using an ELISA. The box represents the 50% between 25% and 75% quartiles. The line inside the box indicates the median. The top and bottom lines denote maximum and minimum values. The numbers on the top of the boxes indicate the number of animals examined. * $p < 0.05$, Signed rank test for Paired Samples.
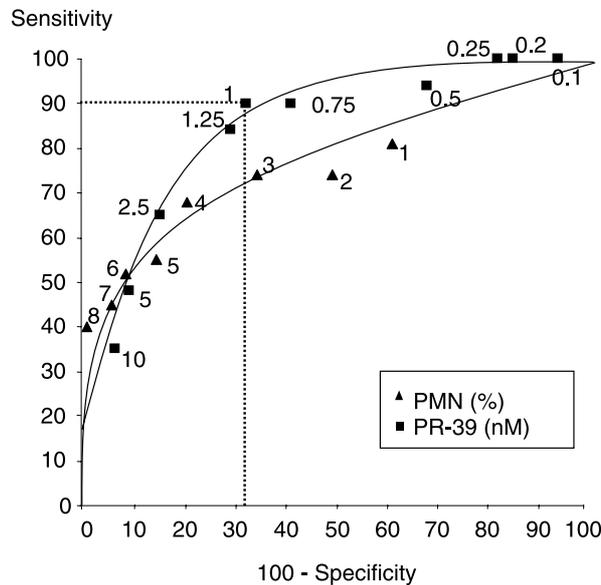
Figure 4. Receiver Operating Characteristic (ROC) plots for PR-39 and PMN on day 21 post infection. Numbered symbols in the curves are potential cut-off values for PR-39 (squares) and polymorphonuclear neutrophils (PMN, triangles) in BALF, which were positioned at their respective calculated sensitivities and 100-specificities. The more distant the potential cut-off value on the curve from a diagonal between the X- and Y- axes, the lower the combined number of false-positives and false-negatives. The dotted lines indicate the sensitivity and 100-specificity-values for the suggested cut-off value of PR-39 at 1 nM.

and BALF of day 7 after mock infection. Furthermore, the concentrations of PR-39 in serum/plasma remained unchanged (Fig. 3).

3.6. Potential use of PR-39 as a diagnostic marker for respiratory health in convalescent pigs

To investigate whether concentrations of PR-39 in BALF could be used as a diagnostic marker to detect convalescent pigs after an experimental *A. pleuropneumoniae* infection, the severity of the lung lesions, the relative number of PMN in BALF and the concentration of PR-39 in BALF were compared. Concentrations of PR-39 in BALF on day 21 after infection were correlated significantly to the lung lesion score (Spearman correlation coefficient 0.6, $p <$ 0.001). Furthermore, a comparison of PR-39 concentrations and the relative number of PMN in BALF, which is currently accepted as the sole diagnostic marker for respiratory health in pigs, revealed that the number of PMN was clearly increased on day 4 and 7 post infection but decreased again on day 21 post infection. To assess the possible diagnostic value of both concentrations of PR-39 and relative number of PMNs on day 21 post infection, a Receiver Operating Characteristic (ROC) plot was constructed (Fig. 4). Concentration of PR-39, using a cut-off value of 1 nM, identified convalescent pigs with lung alterations caused by *A. pleuropneumoniae* with 90% sensitivity and 70% specificity in the chronic stage of infection (21 days after infection). This is clearly superior to the relative number of PMN, which, at the recommended cut-off of 8%, has a sensitivity of only 40%.

4. DISCUSSION

An initial concern when proposing the use of BALF for diagnostic purposes in pigs is whether it is practical. However, methods such as the sampling of BALF with disposable materials have been established in recent years [8] and are now practical and cost-efficient, particularly when considering the high financial risks resulting from the introduction of respiratory tract pathogens into closed herds via convalescent animals. A second major concern for using this procedure is the high variability of BALF protein content [21]. However, the majority of proteins are serum-derived [19], and a BALF-based diagnostic scheme is considered practical if it is based on ELF-specific components [25]. Therefore, the 2-D PAGE-based approach was considered to be a reasonable screening method to identify possible ELF-specific marker proteins in the complex BALF samples.

In order to investigate changes in BALF protein samples from healthy and convalescent animals, we chose an established *A. pleuropneumoniae* infection model [3, 4]. This model closely mimics the natural infection and commonly results in a low mortality but high morbidity of infected pigs. Furthermore, numerous experiments have shown that lavaging pigs at weekly intervals does not influence respiratory health. The clinical data showed a typical course of *A. pleuropneumoniae* disease.

Because 2-D PAGE has been reported to be prone to methodological variation [15], we compared only protein profiles that were evident on triplicate gels. A comparison of protein patterns of BALF-samples from days 4 or 7 after infection to control BALF taken prior to infection showed no consistent differences. This finding likely reflects individual differences in the course of disease and in the acute local and systemic immune response commonly seen in groups of outbred animals. The differences seen in BALF taken from convalescent pigs suggests a consistent pattern in the late reaction to respiratory disease, which likely reflects the occurrence of consolidation and tissue repair processes. The finding that not all proteins observed to be upregulated were detected in the BALF of all animals, is likely due to individual differences and to the generally low concentrations of the proteins in question. The identification of only three of the ten proteins analysed by mass spectrometry is likely due to the lack of availability of the entire porcine genome sequence. All three proteins identified (PR-39, prophenin-2 and calgranulin C) are released by activated PMN. Both PR-39 and prophenin-2 belong to the cathelicidin family of antimicrobial peptides (reviewed by Zanetti [37]). The antibacterial properties of the amino terminal 60 residues of prophenin-1, primarily directed against gram negative organisms, have long been known [18, 23], and recently it has been shown that, even in the presence of a lung surfactant preparation, an 18-residue carboxy terminal fragment also possesses antibacterial activity [9, 35]. Calgranulin C belongs to a family of calcium- and zinc-binding proteins and also possesses antimicrobial as well as filariacidal activity [16]. Because PR-39 has been reported to be increased in serum after infection [39] and, because this antimicrobial peptide is involved in wound repair processes [12], we hypothesised that PR-39 might be an appropriate disease marker in BALF facilitating the detection of convalescent animals, which still might be carriers of the pathogen. Therefore, among the three proteins identified, we regarded PR-39 to be the most promising candidate for further investigation and performed alternative methods to confirm our 2-D PAGE findings. Western blot analysis (Fig. 2) and data from a PR-39 capture ELISA (Fig. 3) confirmed our proteomic data showing increased PR-39 in the BALF of pigs 21 days post infection. The finding that concentrations of PR-39 are not increased in serum, on the contrary to what had been reported previously for gastrointestinal infections in pigs with *Salmonella* Typhimurium [39], is likely due to the stage of infection. Thus, in

acute systemic *A. pleuropneumoniae* infection, serum concentrations of PR-39 also show a slight increase (Fig. 3). In chronic infection, however, the infection is localised in the lung and the involvement of PR-39 in wound repair processes might be its primary role [12] at this stage of disease.

The finding that PR-39 did not have bactericidal activity on *A. pleuropneumoniae* was unexpected since the bactericidal activity of PR-39 is well documented against other gram-negative bacteria [5, 23]. This lack of bactericidal activity was confirmed by the determination of the MIC-value which was found to be 5 times higher than the MIC-value of 1 µM determined for *E. coli* in accordance with previous reports [7, 33]. The apparent resistance of *A. pleuropneumoniae* against PR-39 was further confirmed by the results of the 2-D PAGE where no impact on gene expression could be found as shown recently in *Salmonella* Typhimurium using the synthetic cationic antimicrobial peptide polymyxin [2]. This resistance of *A. pleuropneumoniae* to PR-39 observed in vitro is in accordance with its ability to persist for extended periods of time on the respiratory epithelium of pigs [11, 20]. The cause for this resistance remains to be determined.

After confirming that PR-39 is increased in BALF taken from pigs 21 days after infection, we investigated whether it could be used as a disease marker by constructing a Receiver Operating Characteristic (ROC) plot. This would establish a quantitative diagnostic test to differentiate, at different cut-off values, between healthy and diseased individuals (Fig. 4). The optimal cut-off value determined for PR-39 (1 nM) would correspond to a concentration of approximately 40 nM in ELF; a value that is well below bactericidal concentrations [5].

In summary, our findings show for the first time that concentrations of PR-39 in BALF is a potential diagnostic marker allowing the detection of clinically healthy pigs convalescent from an experimental *A. pleuropneumoniae* infection. Because the introduction of respiratory tract pathogens into closed herds via convalescent pigs is a major risk to the swine industry, this method could become an important screening aid for swine producers. Further studies should be conducted to investigate the applicability of this approach to other respiratory pathogens, e.g. *Haemophilus parasuis, Mycoplasma hyopneumoniae*, and viruses. Moreover the influence of abiotic factors (dust, NH_3), which also impact respiratory health in pigs, should be evaluated.

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