

Characterisation of *Sarcoptes scabiei* antigens

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Abstract In pig herds, the status of *Sarcoptes scabiei* infections is routinely monitored by serodiagnosis. Crude antigen for ELISA is usually prepared from *S. scabiei* var. *canis* or other variations and may lead to variations in the outcome of different tests, making assay standardisation difficult. This study was performed to investigate the antigen profiles of *S. scabiei*, including differences between hydrophilic and more hydrophobic protein fractions, by Western blotting with sera from pigs with defined infection status. Potential cross-reactivity among *S. scabiei* (var. *canis*, *suis* and *bovis*), *Dermatophagoides farinae* and *Tyrophagus putrescentiae* was also analysed. Hydrophobic *S. scabiei* antigens were detectable in the range of 40–50 kDa, whilst the hydrophilic fraction showed no specific antigenicity. In the hydrophobic fractions of *D. farinae* and *T. putrescentiae*, two major protein fractions in a similar size range could be identified, but no cross-reactivity with *Sarcoptes*-positive sera was detectable. However, examination of the hydrophilic fractions revealed cross-reactivity between *Sarcoptes*-

positive sera and both the house dust mite and the storage mite in the range of 115 and 28/38 kDa. Specific bands in the same range (42 and 48 kDa) could be detected in blots from hydrophobic fractions of all three tested variations of *S. scabiei* (var. *canis*, *bovis* and *suis*). These results show that there are considerable differences in mange antibody reactivity, including reactions with proteins from free-living mites, which may interfere with tests based on hydrophilic antigens. Further refinement of antigen and the use of specific hydrophobic proteins could improve ELISA performance and standardisation.

Introduction

Sarcoptes scabiei, the causative agent of sarcoptic mange (scabies) in a range of species, is a parasite of global health significance in both humans and animals (Walton et al. 2004). This mite burrows in the stratum corneum of the skin, inducing clinical manifestations caused by inflammatory and allergy-like reactions to mite products (Walton and Currie 2007). In the infested host, scabies mites induced a cell-mediated as well as a circulating antibody response (Arlian et al. 1994a, b).

Whilst considered highly transmissible between individuals with close contact, the outcome of infestation is quite variable and ranges from mild to life-threatening (as reviewed by Walton et al. 2004). In pigs, where naturally acquired mange is probably most intensively studied due to its impact on economy and animal welfare (Davies 1995), both the numbers of mites and the serological responses vary greatly despite assumed identical exposure to infestation and are only poorly correlated (Davis and Moon 1990; Löwenstein et al. 2004). For routine testing, enzyme-linked immunosorbent assays (ELISAs) are used which employ

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homologous antigen (derived from *S. scabiei* var. *suis*) or heterologous antigen (*S. scabiei* var. *canis*, derived from foxes) dissolved in saline solutions. Although quick and easy to perform, the ELISA is not a suitable tool to evaluate the antibody response of single individuals (Löwenstein et al. 2004). The aims of this study were therefore to compare antigen patterns of different protein fractions of *S. scabiei*, to characterise mite-derived antigenic proteins bound by specific serum antibodies from infected pigs, and to evaluate potential cross-reactive antigens from non-parasitic mites with known allergenic potential, the house dust mite *Dermatophagoides farinae* and the mould mite *Tyrophagus putrescentiae*. Numerous studies describe the cross-antigenicity and allergenicity of several house dust mites and storage mites (e.g., Arlian et al. 1984; Thomas and Smith 1998; Gafvelin et al. 2001; Sidenius et al. 2001). Candidate allergens responsible for this belong to group 2 allergens with a sequence identity of >40% between proteins from storage mites (*Lepidoglyphus destructor* and *Tyrophagus* spp.) and *Dermatophagoides* spp. (van Hage-Hamsten and Johansson 1998). However, cross-reactivity of free-living mites with sera from pigs has not been investigated to date.

Materials and methods

Protein preparations

Sarcoptes mites were collected from the skin of animals with naturally acquired generalised sarcoptosis (*S. scabiei* var. *canis* from foxes, *S. scabiei* var. *suis* from pigs and *S. scabiei* var. *bovis* from cattle) using the migration method. The skin was removed and small pieces of 1×2 cm of dermis were placed into Petri dishes covered with damp filter paper. A black filter paper was chosen, facilitating collection of the light-coloured mites on the underside. After 24 h, the emigrated mites were collected from the filter paper by vacuum aspiration under the microscope, using a Pasteur pipette. A small piece of filter paper wrapped around the pipette was used as a collecting tray for the mites.

The house dust mite *D. farinae* and the mould mite *T. putrescentiae* were cultured at room temperature and a relative humidity of 90% in culture flasks with filter caps (TPP Techno Plastic Products AG, Trasadingen, Switzerland), fed with conventional fish feed (Tetra Min®, main feed for all ornamental fishes, Tetra GmbH, Melle, Germany) ad libitum.

House dust mites and mould mites were separated from their food before further processing by putting small portions of mites and food into Petri dishes, allowing the mites to migrate away from the food particles which were

subsequently removed by inverting the dishes. Mites were then suspended in phosphate-buffered saline (PBS; 3.1 mM potassium dihydrogen phosphate, 104.0 mM disodium hydrogen phosphate, 147.1 mM sodium chloride, pH 7.2), containing a protease inhibitor (Complete mini®, Roche Diagnostics GmbH, Mannheim, Germany) and homogenised on ice in a sterile glass tube with a Teflon pestle (Potter S, Braun, Melsungen, Germany). The efficacy of homogenisation was evaluated microscopically (absence of intact mites).

The homogenate was processed with an Ultrasonic homogeniser (Sonicator Ultrasonic Liquid Processor XL 2020, Labcare Systems Ltd., Clevedon, UK) for 3×3 min on ice, with 1 min intervals between the cycles (duty cycle 15%, output control 1–2). After centrifugation at 20,000×g for 30 min at 4°C, the supernatant (solution A, “hydrophilic fraction”) was removed. The remaining pellet was resuspended in lysis buffer (8 M urea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 40 mM Tris base), sonicated and centrifuged as above. The resulting supernatant (solution B, “hydrophobic fraction I”) was removed. Finally, the remaining pellet was resuspended in sodium dodecyl sulphate (SDS) buffer (0.626 M Tris–HCl pH 6.8, 0.5% SDS) and treated as above (supernatant: solution C; “hydrophobic fraction II”). Protein contents of solutions A and B were determined with a BioRad Protein Assay kit (BioRad Laboratories, Inc., Hercules, USA). Protein preparations were stored at –80°C until further use.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis and Western blotting

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on 12.5% gels with 0.8–1.2 µg protein for silver staining and 5–7 µg protein for blots under reducing conditions (reducing buffer, 0.0625 M Tris–HCl, 25% glycerol, 2% SDS, 0.01% bromophenol blue, 5% β-mercaptoethanol in deionised water). Gels were either stained with PlusOne Silver Staining Kit® (GE Healthcare Life Sciences, Munich, Germany) or blotted onto a Trans-Blot® Pure Nitrocellulose Membrane (BioRad Laboratories, Hercules, USA) in a semi-dry blotting system (NovaBlot®, Pharmacia Biotech Multiphor II, GE Healthcare Life Sciences, Munich, Germany). Blots were blocked with casein solution (Vector Laboratories, Inc., Burlingame, USA). Positive sera were derived from pigs positive in skin scrapings and serology (*Sarcoptes*-ELISA 2001, VM-Pro GmbH, Luckenwalde, Germany). Negative sera were obtained from *Sarcoptes*-free pig farms (herds negative in skin scrapings and ELISA) as described previously by Löwenstein et al. (2004).

Negative and positive control sera were used as pools or separately (see below) in dilutions of 1:300. Western

blotting was carried out with an ABC kit (Vectastain®, Vector Laboratories Inc., Burlingame, USA) according to the manufacturer's instructions. Biotin-labelled goat-anti swine IgG (Vector Laboratories, Inc., Burlingame, USA) was used as secondary antibody in a 1:600 dilution. Visualisation was obtained using a DAB Peroxidase substrate kit (Vector Laboratories Inc., Burlingame, USA).

Lectin blotting

SDS-PAGE was carried out as described above for immunoblots. After transfer, membranes were blocked with PBS containing 1% bovine serum albumin (Sigma-Aldrich, St. Louis, USA). Blotted proteins were stained using a panel of biotinylated lectins (Vector Laboratories, Inc., Hercules, USA) according to the manufacturer's instructions.

Concanavalin A (Con A), *Dolichos biflorus* agglutinin (DBA), peanut agglutinin (PNA), *Ricinus communis* agglutinin 120 (RCA₁₂₀), soybean agglutinin (SBA), *Ulex europaeus* agglutinin I (UEA I) and wheat germ agglutinin (WGA) were used. Specificity of the lectin binding was checked by using the appropriate specific sugars as inhibitors, as described in the manufacturer's instructions.

Preparative isoelectric focusing

For determination of the isoelectric point (pI), preparative isoelectric focusing (IEF) was performed in a Mini-Rotofor® System (BioRad Laboratories, Hercules, USA). Solution B containing 4.6 mg protein in lysis buffer (8 M urea, 4% CHAPS, 40 mM Tris base) and 1.5% of ampholyte solution pH 3–10 (Bio-Lyte3/10 Ampholyte®, BioRad Laboratories, Hercules, USA) were focused at 12 W for about 3 h until a 30-min voltage plateau was reached. After the run, the proteins were recovered in 20 fractions, the pH of each fraction was determined and proteins were precipitated with trichloroacetic acid overnight at 6°C. After centrifugation at 20,000×g, the pellets were resuspended in 40 µl of the reducing buffer (see above) and used for SDS-PAGE (3 µl) or Western blot (15 µl). SDS-PAGE and Western blot analyses were performed as described above.

Two-dimensional electrophoresis

Conventional two-dimensional electrophoresis (2-DE) under denaturing and reducing conditions was performed according to existing protocols (Anonymus 2006). Protein (35–70 µg) per sample was applied by in-gel-rehydration in urea/CHAPS/dithiothreitol/carrier ampholytes for 7–8 h to non-linear immobilised pH gradients (IPGs) pH 4–10 of 10 cm length (Gianazza et al. 1985). Strips were run on a Multiphor® system (GE Healthcare Life Sciences, Munich, Germany) until 15 kVh were reached. After equilibration,

strips were transferred to an SDS-PAGE gel ($T=10\text{--}15\%$ linear gradient, $C=2.7\%$) for the second dimension in a Hoefer SE 600 vertical electrophoresis chamber (Hoefer Scientific Instruments, San Francisco, USA). Protein patterns were detected by acidic silver nitrate staining (modified Heukeshoven method) described by Miller and Gemeiner (1992). For mass spectrometry-compatible staining, glutaraldehyde was omitted in the sensitising solution and formaldehyde in the impregnating solution.

For immunoblotting experiments, the 2-DE run was followed by semi-dry blotting onto nitrocellulose (Hybond ECL, GE Healthcare Life Sciences, Munich, Germany). The membrane was stained for total protein pattern with ruthenium (II) tris (bathophenanthroline disulfonate) (Miller et al. 2006). After scanning on a Typhoon 9400 (GE Healthcare Life Sciences, Munich, Germany; excitation 488 nm, emission filter 610 nm), the blots were probed with pooled positive sera as described for one-dimensional SDS-PAGE (see above).

Imaging and analysis of gels and blots

Gels and blots were scanned and digitised with a Sharp JX-330 Scanner and analysed with Pharmacia Biotech Image Master 1D Software, Version 2.0 (GE Healthcare Life Sciences, Munich, Germany, Uppsala, Sweden).

Protein identification by mass spectrometry

The respective spots were excised from 2-DE gels stained with mass spectrometry-compatible silver staining. MS analysis was performed using nano-electrospray ionisation liquid chromatography-mass spectrometry and liquid chromatography with matrix-assisted laser desorption/ionisation (for details see Supplement)

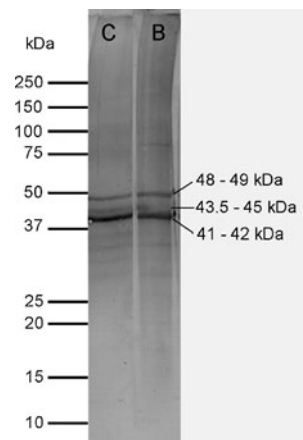
Results

Western blotting of *S. scabiei* var. *canis* protein fractions

SDS-PAGE revealed multiple bands in the different fractions. In the hydrophilic fraction, specific antibody binding with positive sera was not observed. In hydrophobic fraction B of *S. scabiei* var. *canis*, three bands in the range of 40–50 kDa were detected. The main bands stained in SDS-PAGE were located at 43.5–45 kDa, in Western blotting at 41–42 kDa. Hydrophobic fraction C revealed the same banding pattern in the blots, most likely because with this buffer, some of the material which could not be dissolved in the previous buffer was carried over (Fig. 1).

After preparative IEF, these three immunoreactive bands were detected in SDS-PAGE and Western blot in the

Fig. 1 Western blots of hydrophobic fraction B and hydrophobic fraction C of *Sarcoptes scabiei*: reactivity with *Sarcoptes*-positive pig sera



fractions with pH 5.5–8.5. In Western blots particularly, the first band in the range of 42 kDa was found in fractions 6–15 (pH 5.5–8.5), whilst the two bands between 44 and 48 kDa were located in fractions 11–15 (pH 7–8.5) (Fig. 2).

Western blots using different individual and pooled sera of *Sarcoptes*-positive or negative animals showed specific binding with a distinct band in the range of 42 kDa. Two sera also stained a band in the range of 48 kDa, which showed only weak or no reaction with the other sera (Fig. 3a).

Examinations of *S. scabiei* (hydrophobic fraction B) from different host species (*S. scabiei* var. *canis*, *S. scabiei* var. *bovis*, *S. scabiei* var. *suis*) revealed the specific bands of

42 and 48 kDa (Fig. 3b) in Western blots with positive but not with negative pig sera.

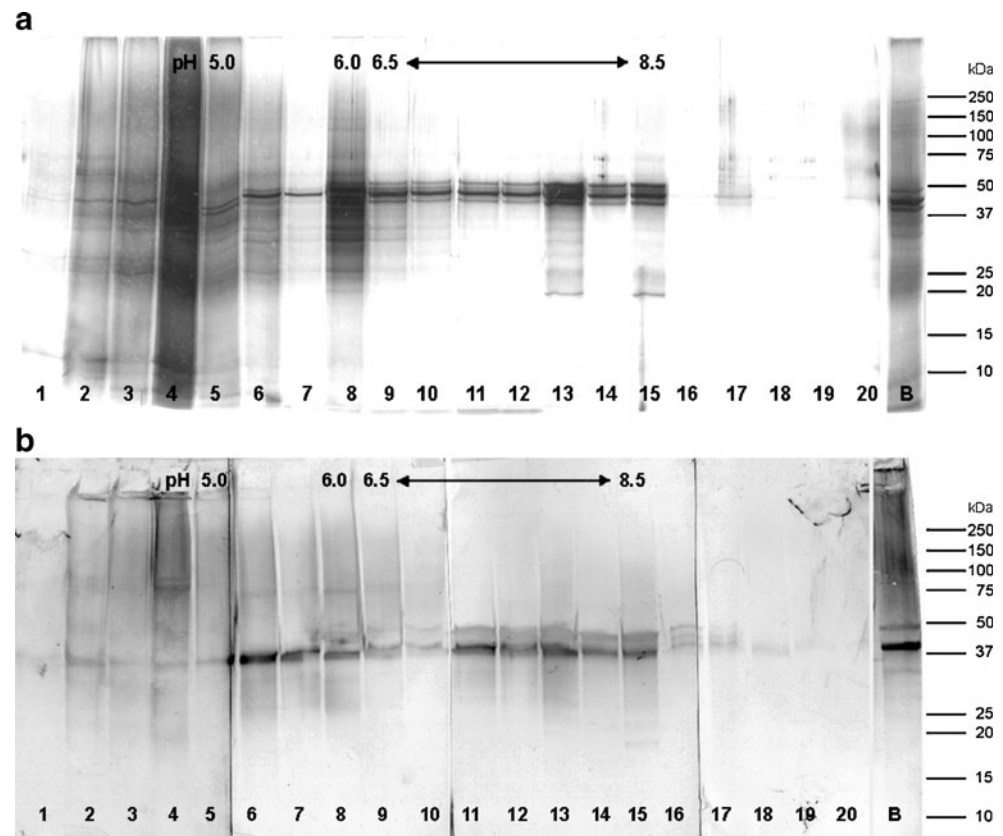
Analysis of the hydrophobic fraction B of the house dust mite and the mould mite by SDS-PAGE showed two bands at 38.5 and 41 kDa, but in Western blotting with *Sarcoptes*-positive sera, no binding was detected (details not shown). In SDS-PAGE of the hydrophilic fraction A of the house dust and the mould mite, numerous bands between 25 and 250 kDa were detected. In Western blotting with *Sarcoptes*-positive sera, antibody binding was seen at 115 and 28 kDa for the house dust mite and at 115 and 38 kDa of the mould mite, whilst *Sarcoptes*-negative sera showed no antibody binding (Fig. 4a, b).

To evaluate the variability of antigen binding in these preparations, 22 *Sarcoptes*-positive and 22 negative pig sera (for definition of positivity, see “Materials and methods”) were screened with Western blots of the hydrophilic solution of the house dust mite. Of the positive sera, 15 sera showed a negative and seven a positive result. Of the *Sarcoptes*-negative sera, 17 showed a negative and five a positive result. The examination of the hydrophilic solution of the mould mite gave similar findings (Table 1).

Lectin blotting

In the lectin blots of *S. scabiei* hydrophobic fraction B with Con A, WGA, UEA I, PNA or RCA₁₂₀, no specific bands

Fig. 2 Silver stained SDS-PAGE (a) and Western blot (b) of the hydrophobic fraction B before (a) and after separation by preparative IEF (fractions 1–20)



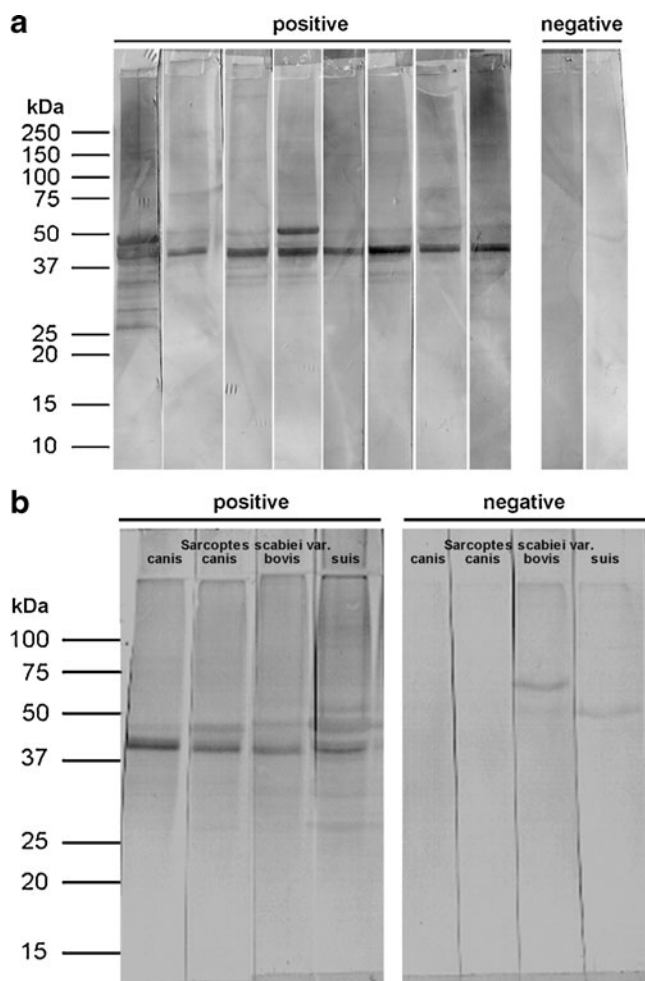


Fig. 3 Western Blot of **a** *S. scabiei* var. *canis*, **b** *S. scabiei* var. *canis*, *bovis* or *suis*: reactivity with different individual and pooled sera of *Sarcophaga*-positive or negative animals (marked as *positive* or *negative*, respectively)

between 40 and 50 kDa were detectable. In contrast, for SBA and DBA, specific bands could be found in this area, corresponding in size with the positive serum control and not detectable with negative serum or after blocking with N-acetylgalactosamine (details not shown).

2D-electrophoresis

With the total protein staining, three spot chains were detectable in the range of 40–50 kDa at a pH value of 7–8.5, with only a weak spot chain at 42 kDa. Additionally, at a pH value of 5, distinct spots in the range of 44 kDa were seen (Fig. 5a). The three spot chains with neutral or basic pI were also stained in Western blotting, particularly the 42 kDa spot chain whose most acidic spots reached below pI 5.0 (Fig. 5b). These results not only confirmed findings of SDS-PAGE and Western blotting but also resolved the isoform pattern of the respective protein(s).

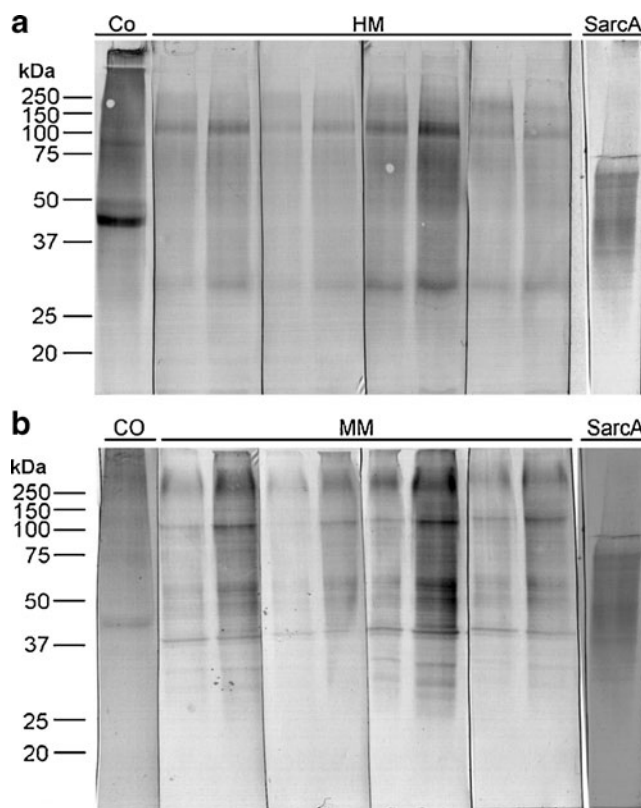


Fig. 4 Western blot of hydrophilic proteins of house dust mites (**a**) and mould mites (**b**) compared to *Sarcophaga* (SarcA) using *Sarcophaga*-positive swine serum. Co: positive control using hydrophobic fraction I of *S. scabiei*

Protein identification

The three immunoreactive spots indicated in Fig. 5 were cut out of the silver-stained gels and subjected to mass spectrometric analysis. Due to their low protein staining, spots 1 and 2 were pooled and only spot 3 was analysed separately. In all samples, peptides similar or identical to two parts of the protein sequence of DFP1 (Accession No. gi|37785880) of the house dust mite *D. farinae* were detected in the database (<http://www.ncbi.nlm.nih.gov/>). In one region, peptides showed an 88% similarity over a stretch of 18 amino acids, in the other, closely adjacent, 100% identity over a range of 14–19 amino acids (Table 2). The examined protein spots are presumably different protein modifications (e.g. with altered glycosylation) of the same protein.

Discussion

In the present study, protein was sequentially extracted from *S. scabiei* var. *canis* with buffers of increasing solubilising power, and resulting extracts were used as antigens for Western blots with pig serum. Commonly,

Table 1 Results of the Western blot screening of the hydrophilic fraction of mould mite and house dust mite homogenates with *Sarcoptes*-positive ($n=22$) and *Sarcoptes*-negative ($n=22$) pig sera

Serum	Mould mite		House dust mite	
	<i>Sarcoptes</i> -positive	<i>Sarcoptes</i> -negative	<i>Sarcoptes</i> -positive	<i>Sarcoptes</i> -negative
Positive	7	5	8	9
Negative	15	17	14	13

hydrophilic extracts with PBS are used to evaluate serum antibody binding (Arlian et al. 1994a; Beck and Hiepe 1997; Arlian and Morgan 2000; Lerch 2001; Rambozzi et al. 2007). However, it could be demonstrated here that a group of PBS-insoluble antigens was recognised by serum antibodies from scabies-infested swine, whilst PBS-soluble antigens showed only weak reactions in Western blotting. This raises the question as to whether the lack of reactivity of sera in ELISA tests is due to the relatively low antigenicity of the used antigen preparations (Löwenstein et al. 2004). During the course of infection, the recognition patterns for swine sera also changes over time (Rambozzi et

al. 2007). Lack of specificity of antigen recognition, as described for human scabies patients allergic to house dust mites (Walton et al. 2004), is a problem when an infestation is to be excluded from a herd, and single recombinant antigens have been proposed to be more useful than crude homogenates for serodiagnosis in animals and humans (Casais et al. 2007; Kuhn 2008). The immunodominant antigens of *S. scabiei* recognised by positive swine sera were between 40 and 45 kDa in size and had different pIs. Both the IEF and 2-DE patterns and the lectin blotting results suggest a certain degree of glycosylation, which most likely precludes these antigens from expression as recombinant antigens in *Escherichia coli* for large-scale production.

Despite the finding that the detected antigens show a high similarity to a protein from *D. farinae*, no cross-reactive antigens could be defined in the present study; however, sera from infected pigs recognised proteins from the hydrophilic fraction of house dust, and this could further contribute to the sometimes poor specificity of ELISA tests for scabies serodiagnosis in pigs (Löwenstein et al. 2004).

In human patients, IgE has been identified as the main immunoglobulin involved in the antibody response to *S. scabiei* (Arlian et al. 1994a). For swine, however, there are no anti-IgE antibodies available, therefore IgG, which is also elevated during infestation (Arlian et al. 1994a; Rambozzi et al. 2007), was detected instead.

Cross-reactions between antibodies from canine patients allergic to house dust mites with antigen of *Sarcoptes* have been described previously. Arlian and coworkers demonstrated cross-antigenicity between *D. farinae* and *T. putrescentiae* (Arlian et al. 1984) and also showed that *S. scabiei* var. *canis* had a high degree of cross-reactivity with the house dust mites *D. farinae* and *Dermatophagoides pteronyssinus* (Arlian et al. 1988; Arlian et al. 1991).

Table 2 Overlapping protein fragments with high similarities to DFP1 of *D. farinae*

Analysed spots	Amino acid sequence	Similarity (%)
Spots 1+2, 3	ITQELKPVQETIHQILPR	88
Spots 1+2, 3	QDIVKPVVQDVHETIVPFR	100
Spots 1+2	PVVQDVHETIVPFR	100
Spot 3	RITQELKPVQETIHQILPR	100

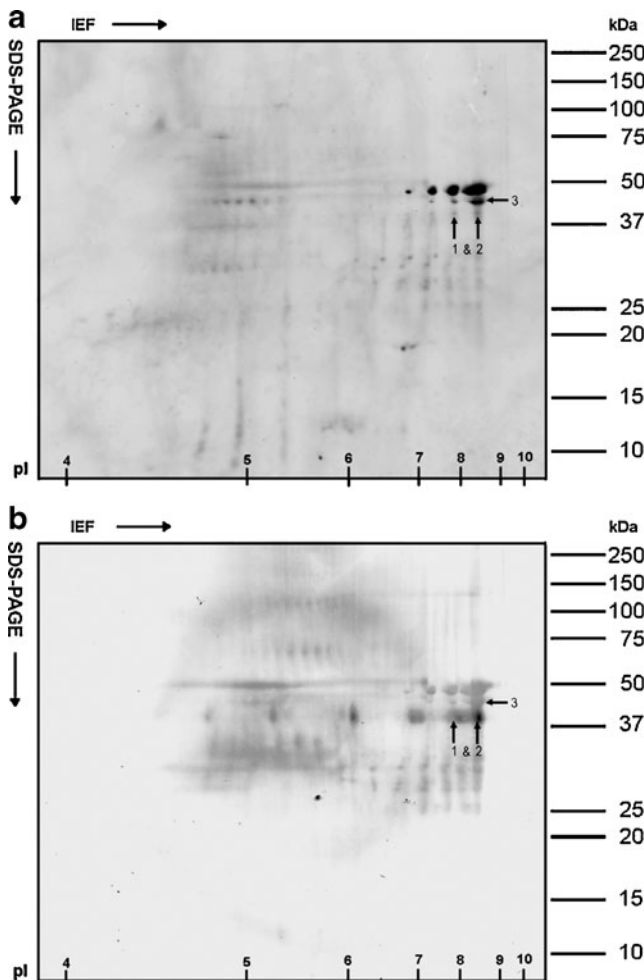


Fig. 5 2D-electrophoresis of *Sarcoptes*-protein preparation (hydrophobic fraction I). **a** Silver staining, **b** Western blot. The three indicated spots (1–3) were subjected to tryptic digestion and mass spectrometric analysis (for results, see Table 2)

Immunisation of hosts with an extract of *D. farinae*/*D. pteronyssinus* resulted in reduced levels of scabies infestation in 71% of the canine hosts when they were challenged with scabies (Arlian et al. 1995).

In summary, in this study, several more hydrophobic proteins of *S. scabiei* could be identified as specific antigens whilst hydrophilic proteins only gave weak signals. This finding might explain the different sensitivities of commercial ELISA tests (Löwenstein et al. 2004). With respect to the reactions of porcine sera to storage mite proteins, it is assumed that this was due to reactions to the mites itself, not to cross-reactivity to *S. scabiei*. This might further explain variations in the ELISA tests in scabies diagnosis. Additional studies should investigate the usefulness of the more hydrophobic *Sarcoptes* proteins for ELISA in large-scale serodiagnosis.

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