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Exudative epidermitis in pigs caused by toxigenic *Staphylococcus chromogenes*

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Abstract

Staphylococcus chromogenes is closely related to *Staphylococcus hyicus*, which is recognised as the causative agent of exudative epidermitis (EE) in pigs. *S. chromogenes* is part of the normal skin flora of pigs, cattle and poultry and has so far been considered non-pathogenic to pigs. A strain of *S. chromogenes* producing exfoliative toxin type B, ExhB, was identified by the use of a multiplex PCR specific for the exfoliative toxins from *S. hyicus*. The exfoliative toxin from *S. chromogenes* reacted in immunoblot analysis with polyclonal and monoclonal antibodies specific to ExhB from *S. hyicus* and had an apparent molecular weight of 30 kDa. Sequencing the gene encoding the exfoliative toxin from *S. chromogenes* revealed that the molecular weight of the toxin with the signal peptide and the mature toxin was 30,553 and 26,694 Da, respectively. Comparison of the *exh*B genes from *S. chromogenes* strain VA654 and *S. hyicus* strain 1289D-88 showed differences in seven base pairs of the DNA sequences and in two amino acid residues in the deduced amino acid sequences. Pigs were experimentally inoculated with *S. chromogenes* strain VA654. By clinical observations and histopathological evaluation of the skin alterations, all pigs revealed development of generalized exudative epidermitis. No toxin producing *S. hyicus* was isolated from the pigs and all ExhB-positive bacterial isolates were identified as *S. chromogenes*. This confirmed that the disease-causing agent was the inoculated *S. chromogenes* strain VA654. The results of this study show that *S. chromogenes* may cause exudative epidermitis in pigs.

Keywords: *Staphylococcus chromogenes*; Exfoliative toxin; Exudative epidermitis; Experimental infection; Pig-bacteria

1. Introduction

The bacterial species *Staphylococcus chromogenes* and *Staphylococcus hyicus* are part of the skin flora of pigs and have also been isolated from cattle, poultry (Devriese et al., 1985; Shimizu et al., 1992) and goats (Valle et al., 1991). Both species were previously subspecies of the species *S. hyicus*, which was a new combination of *Micrococcus hyicus*, coagulase-positive *S. epidermidis* biotype 2, and *Staphylococcus aureus* avian biotype 2 (Devriese et al., 1978). Elevation of *S. hyicus* ssp. *chromogenes* to *S.*

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chromogenes was suggested by Hájek et al. (1986) based on several criteria, including DNA–DNA hybridisation studies, phenotypic characteristics, differences in penicillin-binding proteins and phage host range, which separated the two subspecies of *S. hyicus* from one another. Consequently both *S. hyicus* ssp. *hyicus* and *S. hyicus* ssp. *chromogenes* were raised to species status (Annon., 1987). A few characteristics, which are useful in diagnostic laboratories, can easily differentiate *S. chromogenes* and *S. hyicus*. The former lacks heat-stable nuclease, hyaluronidase, and does not possess lipase activity that hydrolyse Tween 80, whereas *S. hyicus* shows positive reactions for these enzymes. In addition, *S. chromogenes* most often appears with orange or creamy colonies in contrast to the white colonies of *S. hyicus*. Isolation of non-pigmented *S. chromogenes* from pigs has, however, been reported (Saito et al., 1996).

S. hyicus is recognized as the causative agent of exudative epidermitis in pigs, which is a generalized skin infection (Wegener and Skov-Jensen, 1999). Exfoliative toxins that are produced by certain strains of *S. hyicus* cause the characteristic histopathological signs of EE (Andresen et al., 1997). Both toxigenic and non-toxigenic *S. hyicus* can be isolated from diseased animals (Andresen, 1998). The human disease staphylococcal scalded skin syndrome (SSSS), caused by exfoliative toxin-producing *S. aureus* (Ladhani et al., 1999), has several comparative aspects to EE in pigs including histopathology, i.e., blister formation and exfoliation of the skin caused by splitting of the skin at the granular layer of the epidermis. *S. aureus* from cases of SSSS and bullous impetigo in humans produces exfoliative toxins, designated ETA, ETB and ETD (Hanakawa et al., 2002; Yamaguchi et al., 2002). These toxins have been shown to specifically cleave the desmoglein-1, which is a desmosomal cadherin-like molecule involved in cell-to-cell adhesion (Hanakawa et al., 2002). Recently, it was shown that the amino acid sequences of the exfoliative toxins from *S. hyicus* and *S. aureus* share similarities both between and within the two species (Ahrens and Andresen, 2004). On the basis of the DNA sequences of the exfoliative toxins and available reference strains for the exfoliative toxins ExhA, ExhB, ExhC and ExhD from *S. hyicus* a multiplex PCR was recently developed (Andresen and Ahrens, 2004).

In dairy cattle, *S. chromogenes* is a well-known pathogen associated with mastitis (Devriese et al., 2002) and has also been reported as the cause of skin infection in a goat (Andrews and Lamport, 1997). *S. chromogenes* has been isolated from the skin of both diseased and healthy pigs (Devriese et al., 1978; Saito et al., 1996) but in those studies was not established as an ethiological agent of disease. *S. chromogenes* from chickens and bovine udders was not pathogenic to pigs in inoculation experiments (Devriese and Oeding, 1975), and consequently, *S. chromogenes* has been regarded as non-pathogenic in pigs (Hájek et al., 1986). In the present study, we have identified and characterised an exfoliative toxin from *S. chromogenes* similar to the exfoliative toxin ExhB from *S. hyicus*. By experimental infection studies it was demonstrated that *S. chromogenes* may provoke EE in pigs.

2. Materials and methods

2.1. Bacterial strains and culture conditions

S. chromogenes investigated in the present study comprised 15 from Belgium (13 porcine and 2 bovine), all originating from different farms and 4 strains from bovine mastitis isolated in Norway (see Table 1). The type strains of *S. chromogenes*, NCTC10530 (Hájek et al., 1986) and *S. hyicus*, NCTC10350 (Devriese et al., 1978) were used for comparison and as control strains in the identification procedures. *S. hyicus* strains NCTC10350^{T}, 1289D-88, 842A-88 (Wegener et al., 1993) and A2869C (Amtsberg, 1979) were used as reference strains with regard to the exfoliative toxins ExhA, ExhB, ExhC and ExhD, respectively, and the corresponding genes. Additionally, four ExhB-positive field strains of *S. hyicus* isolated from pigs with EE in Denmark in 1996, 1997, 2003 and 2004, respectively, were used for sequencing of the *exh*B gene. The strains were grown aerobically at 37 °C for 17–24 h on Columbia agar base (Oxoid, Unipath Ltd., Basingstoke, UK) supplemented with 5% bovine blood (C-blood agar). Liquid growth medium consisted of 30 g l^{-1} trypticase soy broth (Becton Dickinson and Co., Cockeysville, MD) supplemented with 10 g l^{-1} yeast extract (Oxoid), pH was adjusted to 7.2 before autoclaving the medium. The liquid growth medium was supplemented with

Table 1
Strains of *Staphylococcus chromogenes* and *Staphylococcus hyicus* used in present study

Species	Strain designation	Origin	Year of isolation, country	Reference[a]	Exfoliative toxin produced
S. chromogenes	NCTC10530^T		–, England	1	
	VA103, VA104, VA110, VA401, VA404, VA650	Pig, healthy skin	1979, Belgium	2	
	VA654	Pig, healthy skin	1979, Belgium	2	ExhB
	VAC 44	Pig, healthy skin	1989, Belgium	–	
	G91	Pig, joint	1984, Belgium	–	
	G92	Pig, skin	1984, Belgium	–	
	G248, G254	Sow, endometritis	1990, Belgium	–	
	Bo59, Bo81	Cow, lesion	1977, Belgium	2	
	6105-4, 6042-3, 6087-2, 6128-3	Cow, mastitis	1997–1998, Norway	–	
S. hyicus	NCTC10350^T	Pig with EE, skin	1949, Denmark	3	ExhA
	1289D-88	Pig with EE, skin	1988, Denmark	4	ExhB
	842A-88	Pig with EE, skin	1988, Denmark	4	ExhC
	A2869C	Pig with EE, skin	1970, Germany	5	ExhD
	9606363-1	Pig with EE, skin	1996, Denmark	–	ExhB
	9716142-1	Pig with EE, skin	1997, Denmark	–	ExhB
	7513260-1	Pig with EE, skin	2003, Denmark	–	ExhB
	7611103-1	Pig with EE, skin	2004, Denmark	–	ExhB

[a] 1, Baird-Parker (1962); 2, Devriese et al. (1978); 3, Sompolinsky (1950); 4, Wegener et al. (1993); 5, Amtsberg et al. (1979). Strains with no reference indicated (–) have not previously been published.

0.5 mM $CoCl_2$ and 0.5 mM $ZnSO_4$ after sterilization. Cultures were grown in 10-ml tubes containing 5 ml liquid growth medium inoculated with a single colony from an overnight culture on C-blood agar. Liquid cultures were incubated for 24 h at 37 °C with shaking at 130 rpm.

2.2. Identification

Characters that were used for distinguishing between *S. chromogenes* and *S. hyicus* were pigmentation of the bacterial colonies on C-blood agar, lipase activity tested for by the use of a selective and indicative agar-medium previously described by Devriese (1977), hyaluronidase activity (Devriese et al., 1985) and heat-stable nuclease (Lachica et al., 1971). Additionally, the strains were non-haemolytic on C-blood agar plates, positive for catalase and negative for oxidase.

2.3. Detection of exfoliative toxin

Strains were screened for the presence of genes encoding the exfoliative toxins ExhA-D using a multiplex PCR as previously described (Andresen and Ahrens, 2004). Expression of exfoliative toxin was detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting as previously described (Andresen, 1999) using the monoclonal antibody MabEXH5.1 and polyclonal rabbit antibodies specific for ExhB (Andresen et al., 1997) as primary antibodies.

2.4. DNA sequencing

Genes encoding 16S rDNA were sequenced as previously described (L'Abée-Lund et al., 2003) by PCR amplification, purification of the PCR-products and sequencing on an ABI 377 automatic sequencers using the Prism BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA). The DNA sequence of the gene encoding the exfoliative toxin from *S. chromogenes* strain VA654 was obtained by inverse PCR. Initially, a part of the toxin gene was PCR amplified using the degenerate primers, NN1 and NN2, with general *S. hyicus* exfoliative toxin specificity (Ahrens and Andresen, 2004). The amplicon was sequenced and the flanking regions were

Table 2
Primers used for sequencing *exh*B in this study

Gene	Primer name	Oligonucleotide sequence
*exh*B	315FU	5′-TCCATACAATACTGTGCAAG-3′
*exh*B	315RU	5′-CAACAGATTGATAAGGATATTC-3′
*exh*B	chrom36F	5′-AAAGTCACCAATTAATGATG-3′
*exh*B	chrom1360R	5′-TAAGAATTTCATAAAGTTGCC-3′
S. hyicus exfoliative toxins	NN1	5′-CCWTATCAAKCWGTWGGC-3′[a,b]
S. hyicus exfoliative toxins	NN2	5′-ATNSSWGAKCCTGAATTWCC-3′[a,b]
*exh*B	V5F	5′-TCATTGTTAATCCCAGCTAC-3′
*exh*B	V5R	5′-CCTCTATCTTTTATACCTGC-3′

[a] K = G or T; N = A, C, G or T; S = C or G; W = A or T.
[b] Ahrens and Andresen (2004).

amplified by inverse PCR using the primers 315FU and 315RU (Table 2). For inverse PCR, *S. chromogenes* DNA was digested with the restrition enzymes *Eco*RI, *Hin*dIII and *Xba*I, ligated with T4 ligase and amplified with the above-mentioned primers. The amplicons were sequenced using primers listed in Table 2. The *exh*B sequence obtained from *S. chromogenes* strain VA654 is available in GenBank under the accession number AY691553. Genes encoding the exfoliative toxin ExhB from *S. hyicus* were sequenced using primers specific for the *exh*B gene (GenBank accession number AF515454) indicated in Table 2.

2.5. Sequence analysis

Sequence comparison was performed with GeneDoc (Nicholas, K.B., Nicholas Jr., H.B., Deerfield II, D.W., 1997, www.psc.edu/biomed/genedoc). Prediction of putative protoxin cleavage site was performed with the SignalP tool at www.cbs.dtu.dk (Nielsen et al., 1997). Molecular weights were calculated with the Compute p*I*/M_w tool at www.expasy.ch.

2.6. Experimental infection in pigs

Experimental infection with *S. chromogenes* strain VA654 was performed in six females, cross-bread (Danish Landrace, Danish Yorkshire and Duroc) pigs at the age of 3 weeks obtained from a pig herd with no history of EE. The animals were housed individually with no possibility of direct contact with each other and fed antibiotic free feed and sow milk replacement ad libitum. Immediately before inoculation of the pigs, swab samples from the skin behind the right ear were collected from each of the experimental animals for microbiological examination. On day 0, two groups of three pigs each were inoculated with 10^9 CFU (pigs I–III) and 10^{10} CFU (pigs IV–VI), respectively, of *S. chromogenes* strain VA654 in 1 ml of 0.9% NaCl. Inoculations were made subcutaneously in the neck just behind the right ear. The pigs were observed clinically once each day during 12 days and signs of disease were recorded. Pigs that developed severe symptoms of disease were euthanized in order to avoid unnecessary suffering. The remaining pigs were euthanized at the end of the experiment on day 12 post inoculation (p.i.). The experimental infection of pigs was performed in accordance with a licence from the Danish Animal Experiments Inspectorate.

2.7. Microbiological and pathological examination

Swab samples from the skin of the experimental animals were collected by rubbing a sterile cotton swab soaked in sterile 0.9% NaCl over an area of approximately 10 cm^2 of the skin. The cotton swabs were then transferred to tubes containing 1.0 ml sterile 0.9% NaCl.

Necropsy was performed immediately after euthanasia and gross pathological lesions were recorded. Tissue samples for histopathological examination and samples for microbiological examination were collected from affected skin, liver, spleen and kidney from each of the pigs. From the joints of the hind legs a pool of synovial fluid was collected for microbiological examination. Swab samples and synovial fluid were in appropriate dilutions in 0.9% NaCl plated on selective and indicative medium (Devriese, 1977) and *S. chromogenes*-like and *S. hyicus*-like colonies were

sub-cultivated on C-blood agar. Non-haemolytic *S. chromogenes*-like and *S. hyicus*-like bacteria were then subjected to multiplex PCR for exfoliative toxins and those that were toxin-positive were identified further as described above.

2.8. Histopathological examination

Tissue samples from skin, liver, spleen and kidneys were fixated in 4% neutral buffered formaldehyde and embedded in paraffin wax. Sections were cut 5–6 μm, stained with hematoxylin and eosin and examined by light microscopy.

3. Results

3.1. Identification of the ExhB-producing S. chromogenes strain VA654

Screening a collection of 19 *S. chromogenes* strains with the multiplex PCR specific for the exfoliative toxins ExhA-D from *S. hyicus* showed a positive reaction corresponding to *exh*B for strain VA654 (Fig. 1A) and negative reactions for the other strains (not shown). Strain VA654 was subsequently subjected to further investigations including tests for identification and discrimination between *S. chromogenes* and *S. hyicus*, and sequencing of the 16S rDNA. Strain VA654 had creamy coloured colonies on C-blood agar and reacted identically to the reactions of *S. chromogenes* strain NCTC10530^T in all the identification tests. In addition, the 16S rDNA sequences of strains VA654 and NCTC10530^T were identical and clearly distinct from that of *S. hyicus* type strain (GenBank accession number D83368). These results confirmed that strain VA654 was *S. chromogenes*.

3.2. Characterization of ExhB from S. chromogenes strain VA654

Strain VA654 was cultured in liquid growth medium and the supernatant from the culture was by immunoblotting analysed for the presence of exfoliative toxin using a monoclonal antibody, MabEXH5.1 (Fig. 1B), and polyclonal rabbit antiserum specific for the exfoliative toxin ExhB. The monoclonal antibody and the polyclonal rabbit antiserum reacted with the exfoliative toxin from the reference strain for ExhB, *S. hyicus* strain 1289D-88, and a protein of the same size from *S. chromogenes* strain VA654 indicating that strain VA654 was producing ExhB. The molecular weight of the ExhB-protein was approximately 30 kDa, as calculated from the molecular weight marker in Fig. 1B. The DNA sequence of the gene encoding exfoliative toxin from *S. chromogenes* strain VA654 was obtained as described above. Comparison of the DNA sequence and the deduced amino acid sequences of the *exh*B genes from *S. chromogenes* stain VA654 and *S. hyicus* strain 1289D-88 showed differences in seven bases in the DNA sequences of the functional gene and two amino acid residues in the amino acid sequences. The amino acid substitutions were asparagine to threonine at position 74 and serine to glycine at position 266. Both amino acid substitutions were in the mature toxin. Additionally, 10 single nucleotide differences were observed outside the functional gene of ExhB in the DNA-sequence from *S. chromogenes* strain VA654 (GenBank accession no. AY691553) when compared to the DNA-sequence from *S. hyicus* strain 1289D-88

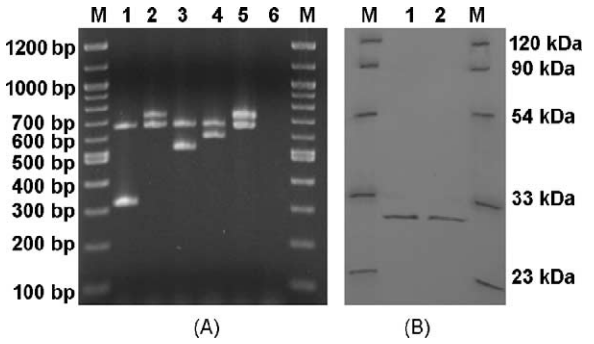

Fig. 1. Demonstration of ExhB in *Staphylococcus chromogenes*. (A) Multiplex PCR for genes encoding exfoliative toxins ExhA-D. The multiplex PCR generates a specific amplicon with each of the toxin genes and as an internal control a 662 bp-amplicon from 23S rDNA appears in samples containing chromosomal DNA from bacteria (Andresen and Ahrens, 2004). Lane M, 100-bp ladder molecular weight marker (New England BioLabs, Beverly, MA); (1) *S. hyicus* strain NCTC 10350^T, ExhA$^+$; (2) *S. hyicus* strain 1289D-88, ExhB$^+$; (3) *S. hyicus* strain 842A-88, ExhC$^+$; (4) *S. hyicus* strain A2869C, ExhD$^+$; (5) *S. chromogenes* strain VA654, ExhB$^+$; (6) sample without bacterial DNA. (B) Immunoblot analysis of exoproteins with the monoclonal antibody MabEXH5.1 specific for ExhB as primary antibody. Lane M, protein molecular weight markers; (1) culture supernatant from *S. hyicus* strain 1289D-88; (2) culture supernatant from *S. chromogenes* strain VA654.

(GenBank accession no. AF515454). The molecular weight of the deduced amino acid sequence of the ExhB with the signal sequence and the mature ExhB from *S. chromogenes* strain VA654 was 30,553 and 26,694 Da, respectively. The *exh*B genes of the four *S. hyicus* field strains 9606363-1, 9716142-1, 7513260-1, 7611103-1 were sequenced in order to clarify if the minor differences between ExhB-sequences from *S. hyicus* strain 1289D-88 and *S. chromogenes* strain VA654 reflected a natural variation in the *exh*B gene and ExhB protein. The DNA-sequences of *exh*B from the four *S. hyicus* strains were all found identical to *exh*B from strain 1289D-88.

3.3. Experimental infection of pigs and clinical observations

In the experimentally infected animals the first clinical signs were exfoliation of an area of approximately 3 cm^2 behind the left ear of pig IV on day 2 post inoculation and of pigs I–III and V on day 3 p.i., respectively (Fig. 2A). On day 4 p.i, pig VI showed exfoliation on the inside of the right hind leg, and it was possible to produce a positive Nikolsky sign (loosening of the skin when gently stroked) (Fig. 2B). During the following days (days 3–6 p.i.) the lesions of all six pigs expanded and exfoliations developed behind both ears, and on other parts of the body, e.g., the abdominal skin and the inside of the legs. The clinical signs of disease progressed with crust formation at exfoliated sites, erythema of the skin all over the body and the general appearance of the pigs deteriorated. On day 6 p.i., pigs I and III showed signs of lameness and pig VI was unthrifty and depressed. These three pigs were euthanized. During days 6–12 p.i., the lesions of the remaining pigs intensified with exudation and crust formation and expanded to the abdominal and facial skin. The lesions were more pronounced on pigs IV and V showing thick crust on both sides of the neck. At the autopsy of pigs

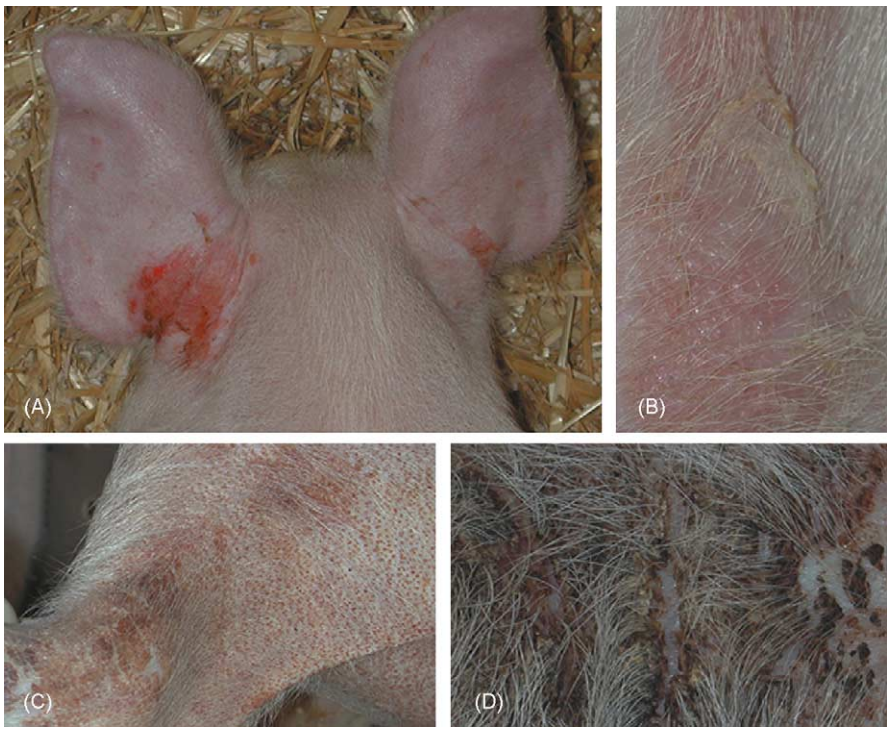

Fig. 2. Skin lesions caused by infection with *Staphylococcus chromogenes* strain VA654 in pigs. These gross skin alterations are typical for exudative epidermitis. (A) Exfoliation of the skin behind the left ear three days post inoculation (p.i.). (B) Exfoliation of the skin on the inside of hind leg as a result of stroking the skin, a positive Nikolsky sign. (C) Tiny brown spots of exudate and crust. (D) Skin with crust and deep fissures.

II, IV and V on day 12 p.i. the disease was generalized. Tiny brown spots of exudate were observed all over the abdominal skin (Fig. 2C). Pigs IV and V had a thick dark crust with deep cracks on the neck at the site of injection (Fig. 2D).

3.4. Microbiological examination

Examination by multiplex PCR of between 4 and 20 *S. hyicus*-like and *S. chromogenes*-like bacterial isolates from swab samples collected from each of the six experimental animals before they were inoculated showed no colonization with exfoliative toxin-producing *S. hyicus* or *S. chromogenes*.

Swab samples were taken from the exfoliations behind the left ear of pigs I–V and from the exfoliated area of the right hind leg of pig VI on day 4 p.i. PCR-testing of between 1 and 10 *S. hyicus*-like and *S. chromogenes*-like bacterial isolates from each lesion and further microbiological examination of the *exh*B-positive isolates showed that *exh*B-positive *S. chromogenes* was at this point isolated from the lesions of pig I and pig IV, only.

Table 3 summarizes the microbiological examination and testing by multiplex PCR of *S. hyicus*-like and *S. chromogenes*-like isolates from the pigs post-mortem. Toxigenic *S. chromogenes* could be isolated from different sites of all the pigs except from pig VI. None of the *S. hyicus*-like organisms isolated from the experimental pigs before the experimental inoculation, during the animal experiment, and post-mortem that were tested in the multiplex PCR were positive for exfoliative toxin.

Table 3
Microbiological examination post-mortem. Number of *exh*B-positive *S. chromogenes* found among *S. chromogenes*-like and *S. hyicus*-like isolates tested by multiplex PCR

Site of isolation	Pig I	Pig II	Pig III	Pig IV	Pig V	Pig VI
Skin	2/14[a]	12/15	1/14	7/12	0/12	0/7
Liver	1/4	–	4/5	–	–	0/1
Spleen	–[b]	1/1	–	–	7/7	0/8
Kidney	0/2	10/10	0/1	–	–	0/2
Synovial fluid	1/3	–	–	0/1	–	0/8

Pigs I, III and VI were euthanized on day 6 post inoculation (p.i.) and pigs II, IV and V were euthanized day 12 p.i. No *S. hyicus*-like isolates were toxigenic.

[a] Number of *exh*B-positive *S. chromogenes*/number of isolates tested.

[b] (–) No *S. hyicus*-like or *S. chromogenes*-like isolates were found in the sample.

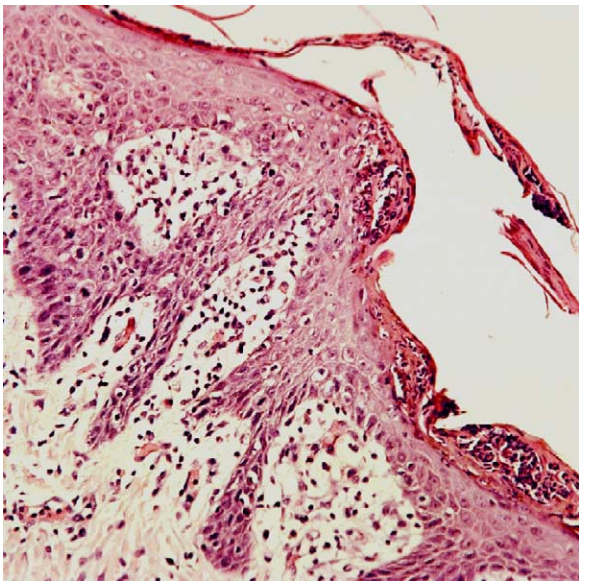

Fig. 3. Histopathology. Skin alterations caused by infection with *S. chromogenes* strain VA654, intraepidermal pustulat dermatitis with exocytosis, exudation, hyperkeratosis, acantosis and perivascular infiltration in the dermis (25×).

3.5. Histopathology

Histopathological evaluation of the gross skin alterations revealed an intraepidermal pustular dermatitis with exocytosis, exudation (crust formation), erosion, hyperkeratosis and acanthosis together with perivascular cellular infiltrations in dermis (Fig. 3). There were no histomorphological differences between the two groups (infection doses of 10^9 CFU and 10^{10} CFU, respectively), neither between the individual pigs of each group. Histopathological evaluation of tissue samples from liver, spleen and kidneys revealed no variation from normal conditions.

4. Discussion

In the present study, we have shown that the exfoliative toxin ExhB can be found in the species *S. chromogenes*. The ExhB-positive strain VA654 was originally isolated from the skin of a healthy pig and

published as belonging to the *S. hyicus* group B strains investigated by Devriese et al. (1978) and was in that publication categorized as *S. hyicus* ssp. *chromogenes*. The results of the present study support that strain VA654 is *S. chromogenes*. Recently, Sato et al. (2004) published the purification and characterization of a 30-kDa exfoliative toxin from *S. chromogenes*. However, characterization at sequence level and experimental inoculation with toxigenic *S. chromogenes* in pigs was not included in that study.

ExhB from *S. chromogenes* strain VA654 differs form ExhB from *S. hyicus* strain 1289D-88 in two amino acid residues and at the DNA-level in seven base pairs. These differences seem to be limited to ExhB from strain VA654 as the DNA sequences of *exh*B from the four field strains of *S. hyicus* investigated were identical to the DNA sequence of *exh*B from *S. hyicus* strain 1289D-88. The four *S. hyicus* isolates and strain 1289D-88 represent a time span of 16 years indicating that the *exh*B gene is very stable in *S. hyicus*. However, only a few *S. hyicus* strains from Denmark were investigated in the present study. Further studies may give a more comprehensive description of the possible variability of *exh*B. The two amino acid residue substitutions in the variant of ExhB from *S. chromogenes* strain VA654 have apparently no significant impact on the activity of the toxin and were situated on each side of the central part of the amino acid sequence, which have higher homology between the exfoliative toxins from *S. aureus* and *S. hyicus* compared to the more variable C- and N-terminal parts (Ahrens and Andresen, 2004).

The molecular weight of approximately 30 kDa for ExhB from strain VA654 was most probably for the mature toxin without the signal sequence because the protein analysed was from the culture supernatant and it was the same as for ExhB from *S. hyicus* strain 1289D-88 (Fig. 1B), which previously was also determined to be 30 kDa by SDS-PAGE (Andresen et al., 1997). However, it was slightly higher than the theoretical molecular weight of 26,694 Da calculated from the deduced amino acid sequence of the ExhB-protein without the signal-peptide.

In the experimental infection in the present study, the bacterial culture was inoculated subcutaneously behind the right ear. However, the first clinical signs of EE in the pigs were exfoliation of the skin behind the left ear (Fig. 2A), and in one case on the right hind leg (Fig. 2B). Toxigenic *S. chromogenes* were present in swab samples from only two out of six of these initial lesions. In resemblance to descriptions of SSSS (Ladhani, 2003), the present results indicate the possibility of circulation of the exfoliative toxin from *S. chromogenes* and that the exfoliative toxin can be produced at a site distal from the skin lesions.

The microbiological examination and multiplex PCR-testing of the *S. hyicus*-like organisms isolated from the pigs before inoculation with *S. chromogenes* strainVA654, during infection, and at necropsy, showed that none of them were toxigenic. Thus, the characteristic clinical and pathological signs of EE in the experimental pigs could not be attributed to infection with toxigenic *S. hyicus*. The observation that all the *exh*B-positive isolates from the experimental animas were identified as *S. chromogenes* supports that the inoculated strain VA654 was the disease-causing agent. Even though one pig, pig VI, that did develop clinical EE and showed histopathology typical for EE, toxin-producing *S. chromogenes* was not isolated from this animal at necropsy (Table 3). This could be because the focus of infection and production of exfoliative toxin was located at a site, which was not included in the microbiological examination of pig VI. The microbiological examination showed that ExhB-producing *S. chromogenes* could be isolated from the skin and/or from internal organs of all the other pigs. *S. hyicus* has also been isolated from liver, spleen and kidney of spontaneous cases of EE (Wegener, H.C., unpublished). These observations show that the disease causing agents of EE not only colonise the skin of the pigs, but may also be isolated from the internal organs, in some cases with even more success (Table 3). Pigs I and III showed lameness but had other bacteria than *S. chromogenes* as the dominating bacterial flora in the synovial fluid when examined post-mortem (not shown). However, these other bacteria could be due to contamination during sampling.

The histopathological examination of the skin lesions showed that the alterations of the skin corresponded to previous descriptions of both experimental and spontaneous cases of EE in pigs (Sompolinsky, 1950; Obel, 1968) and to the alterations caused by concentrated culture supernatant and partially purified exfoliative toxin from *S. hyicus* (Andresen et al., 1993).

From the clinical observations and the results of the microbiological and histopathological examinations it is evident that *S. chromogenes* strain VA654 may cause EE in pigs. ExhB from *S. chromogenes* was, however, only found in 1 out of 19 *S. chromogenes* strains investigated. Therefore, more *S. chromogenes* strains from pigs need to be investigated before the significance of exfoliative toxin producing *S. chromogenes* in relation to EE in pigs can be quantitatively established.

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