Characteristics of *Staphylococcus hyicus* strains isolated from pig carcasses in two different slaughterhouses

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Abstract

In a previous study, we showed that coagulase positive staphylococci, which are often used as indicators for *Staphylococcus aureus*, are frequently found on pig carcasses. Further characterization of the strains identified only a minor part as *S. aureus*. Selected non-*S. aureus* strains were all identified as *Staphylococcus hyicus*. However, two studies described in this species strains that produce staphylococcal enterotoxins. The aim of the present study was to further characterize such coagulase positive *S. hyicus* strains isolated from pig carcasses and to assess the results for their food safety relevance. A total of 189 strains from two abattoirs were characterized. Phenotypically, 98.9% showed non-pigmented colonies, 99.5% no haemolysis and 67.7% were egg yolk-positive. DNase activity was found in all but one isolate. Only five of the 189 strains were resistant to the antimicrobials tested. One strain harboured the *mecA* gene. Exfoliative toxin genes were detected in 31 (16.4%), *S. aureus* enterotoxin genes in none of the strains.

The PFGE genotyping results show only a limited number of clusters. Cluster I included more than 50% of the strains. The fact that similar or closely related PFGE patterns of *S. hyicus* can be found on carcasses after bleeding in both abattoirs indicates the occurrence of widespread strains in the Swiss pig population. Moreover, the genotyping results revealed a remarkable homogeneity in *S. hyicus* strains isolated from different process stages in abattoir B, which could indicate a recontamination problem with persisting strains.

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1. Introduction

Coagulase positive staphylococci (CPS) are often used as indicators for *Staphylococcus (S.) aureus*, which are worldwide the most important cause of food-borne intoxications. In a previous study, we showed that CPS are frequently found on pig carcasses from healthy animals (Spescha, Stephan, & Zweifel, 2006). However, further characterization of the CPS identified only a minor part as *S. aureus* (Nitzsche, Zweifel, & Stephan, 2007). Selected non-*S. aureus* strains were all identified as *Staphylococcus hyicus*. The coagulase-variable species *S. hyicus* is mainly involved in exudative epidermitis in pigs (Wegener, Andersen, & Bille-Hansen, 1993). Moreover, *S. hyicus* has been occasionally isolated from subclinical mastitis in cows (Capurro, Concha, Nilsson, & Ostensson, 1999; Roberson, Fox, Hancock, Gay, & Besser, 1996; Waage et al., 1999), from skin infection in horses (Devriese, Nzuambe, & Godard, 1985), from chickens with exudative dermatitis or tenosynovitis (Kibenge, Rood, & Wilcox, 1983) and from a human wound infection after a donkey bite (Osterlund & Nordlund, 1997). In *S. hyicus* four different exfoliative toxins ETs (ExhA, ExhB, ExhC and ExhD), which specifically cleave a single peptide bond in the extracellular region of swine desmoglein 1, are described (Ahrens & Andresen, 2004). However, two studies (Hoover, Tatini, & Maltais, 1983; Valle et al., 1990) have also found *S. hyicus* strains producing staphylococcal enterotoxins (SE), which are normally found in *S. aureus* and are responsible for food-borne intoxications. In contrast to *S. hyicus* strains isolated from clinical cases, very little genotypic
characterization data are available for strains isolated from healthy pigs. Consequently, it is important to further characterize \textit{S. hyicus} isolated from pig carcasses in order to evaluate a potential food safety risk.

The aim of the present study was therefore (i) to identify \textit{S. hyicus} strains among coagulase positive staphylococci (CPS) collected from pig carcasses at two abattoirs during slaughter, (ii) to characterize the isolated \textit{S. hyicus} strains by phenotypic and genotypic traits, (iii) to assess these results in light of their food safety relevance and (iv) to further evaluate the epidemiological relationship of these strains.

2. Materials and methods

2.1. Isolates

In a previous study 200 pig carcasses from two abattoirs were examined at sequential steps of slaughter (scalding, dehairing, singeing, polishing, trimming, washing, chilling) for CPS by the wet–dry double swab technique at the neck, belly, back and ham (Spescha et al., 2006). The origin of the animals (each of about 100 kg) was distributed throughout Switzerland. Of the isolated 337 CPS, 142 have been identified as \textit{S. aureus} and were further characterized (Nitzsche et al., 2007). The remaining 195 CPS isolates were used in the present study.

According to the frequency of CPS on the carcasses at the different stages, isolates from abattoir A originated from the beginning of the slaughter process (after bleeding), whereas isolates from abattoir B were distributed throughout the slaughter process (after bleeding, after scalding, after dehairing/singeing, after polishing, after trimming and after washing).

2.2. \textit{S. hyicus} identification and further biochemical characterization

For the identification of \textit{S. hyicus}, total cellular DNA was extracted with QIAGEN DNeasy Tissue Kit (Qiagen, Basel, CH) according to the manufacturer’s protocol. The extracted DNA was then evaluated by PCR for species-specific sequences of superoxide dismutase A encoding gene \textit{sodA} (Table 1). Randomly selected strains were additionally identified by 16S rRNA sequencing.

To evaluate colour and haemolysis of the colonies, the \textit{S. hyicus} isolates were cultivated on sheep blood agar (Difco Laboratories, Becton Dickinson; 5% sheep blood, Oxoid Ltd.) at 37 °C for 24 h. Strains were further phenotyped by appraising the egg yolk reaction on Baird–Parker agar (BP agar, Oxoid Ltd.). DNase activity was assayed on DNase test agar (Difco DNase Test Agar).

2.3. Antimicrobial susceptibility testing of \textit{S. hyicus} strains

Susceptibility to seven antibiotics was determined using the Sensititre NLV 23 system according to the manufactur-
denaturation at 94°C for 30 s followed by 35 cycles (120 s at 94°C, 120 s at 57°C, 60 s at 72°C), and a final extension at 72°C for 420 s; (ii) for the detection of *seg* and *sei*, initial denaturation at 94°C for 240 s followed by 30 cycles (120 s at 94°C, 120 s at 55°C, 60 s at 72°C), and a final extension at 72°C for 420 s; (iii) for the detection of *sej*, initial denaturation at 94°C for 120 s followed by 30 cycles (60 s at 94°C, 60 s at 62°C, 60 s at 72°C), and a final extension at 72°C for 120 s. SE positive strains obtained from a previous study were used as positive controls (Scherrer et al., 2004).

2.5.3. Detection of the genes encoding exfoliative toxins

Primers described in the work of Andresen and Ahrens (2004) were used to detect the exfoliative toxins ExhA, ExhB, ExhC, ExhD. The DNA was amplified by an initial denaturation at 94°C for 180 s followed by 30 cycles of 60 s at 94°C, 60 s at 56°C and 60 s at 72°C. The PCR reaction was completed by a 10 min incubation at 72°C to ensure full extension of the PCR products.

2.6. Macrogenetic analysis by PFGE

*S. hyicus* isolates were grown aerobically in brain heart infusion broth at 37°C for 18–24 h. The cells were harvested and resuspended in TE buffer (5 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0). First, 240 μl of the suspension was mixed with 6 μl of Lysozyme (50 mg/ml) for 10 min at 37°C, this was then mixed with 300 μl of 1.2% Certified Megabase Agarose (Bio-Rad Laboratories), 30 μl 10% SDS (sodium dodecyl sulfate) and 7.5 μ Proteinase K (20 mg/ml) before dispensing into plugs. The plugs were incubated overnight at 37°C in lysis buffer II (1 M NaCl, 10 mM Tris, pH 8.0, 200 mM EDTA, pH 8.0, 0.5% N-lauroylsarkosine, 0.2% deoxycholic acid) with 20 μl (50 mg/ml) lysozyme and lysostaphin and 3.2 μl (50 mg/ml) tolylsarkosine, 0.2% desoxycholic acid) with 20 μg (20 mg/ml) before dispensing into plugs. The plugs were stored in TE buffer at 4°C. The overnight incubation was followed by a second incubation at 53°C for 2 h. The lysis buffer was then removed, the plugs were washed twice in double-distilled water and twice in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA) at 53°C, each for 15 min. The plugs were then stored in TE buffer at 4°C.

Plugs were digested with 40 U Smal (Roche Diagnostics, Mannheim, Germany) according to the instructions of the manufacturer. Digested DNA was separated in 1% Pulsed Field Certified Agarose (Bio-Rad Laboratories) with a CHEF DR III (Bio-Rad Laboratories, Hercules, CA) pulsed-field electrophoresis system in 0.5× Tris–borate–EDTA (1× Tris, 0.01 M EDTA, 1× boric acid). Running parameters were as follows: 3 s to 33 s ramping for 20 h; 6 V/cm; 120° angle; 12°C. Gels were stained with ethidium bromide (0.5 μg/ml) for 1 h. The patterns were visualized using a UV transilluminator and then photographed. The Lambda Ladder PFGE Marker (New England Bio Labs) was used as a molecular size marker. DNA restriction bands were analyzed using GelCompar II (Applied Maths, Sint-Martens-Latem, Belgium). Similarity coefficients were calculated and dendograms were constructed using the Dice coefficient and the un-weighted pair similarity coefficient (GelCompar II).
group method with arithmetic averages (UPGMA), respectively, with an optimisation value of 1.0% and a position tolerance of 3%. Clusters were designated as greater than 80% similarity with subgroups designated as greater than 90% similarity. Isolates with indistinguishable banding patterns (i.e., 95–100% similarity) were assigned to the same pulsotype.

3. Results

3.1. S. hyicus identification and further biochemical characterization

According to the PCR results, 189 of the CPS isolates were identified as S. hyicus. Randomly selected strains were additionally confirmed by sequencing the 16S rRNA. Of the S. hyicus strains, 36 originated from abattoir A (after bleeding, n = 31; scalding water, n = 4; after polishing, n = 1), and 153 from abattoir B (after bleeding, n = 21; after scalding, n = 1; after dehairing/singeing, n = 46; after polishing, n = 32; after trimming, n = 27; after washing, n = 26). On sheep blood agar, 187 of the 189 isolates showed no pigment and two isolates gave yellow-pigmented colonies. The appraisal of haemolysis revealed that all but one strain showed no hemolysis. In total, 128 (67.7%) strains were egg yolk-positive. DNase activity was found in all but one isolate.

3.2. Antimicrobial susceptibility

Resistances to antimicrobials were found in only 5 (2.6%) S. hyicus strains. None of the strains were resistant to amoxicillin/clavulanic acid, gentamicin, or kanamycin.

Among strains showing resistances, one strain displayed a double resistance to ampicillin/penicillin, and for one strain each a resistance to lincomycin, cefoperoxazone and clloxacinill was detected. One strain harboured the mecA gene, but showed no other resistances against the antimicrobials tested. The PCR product of this strain was sequenced and showed a 100% homology to the mecA gene, but showed no other resistances against the antimicrobials tested. The PCR product of this strain was sequenced and showed a 100% homology to the mecA gene reference sequence (accession number GI 156978331).

3.3. Detection of ET genes

ET genes (exhA, exhB, exhC, exhD) were detected in 31 (16.4%) strains, isolated in both slaughterhouses. Six strains harboured the exhA and 25 strains the exhD gene. One strain was positive for the combination of exhA and exhD genes. None of the strains harboured genes for exhB or exhC.

3.4. Detection of S. aureus enterotoxin

SE genes (sea to sed, seg, sei, sef) were not detected in any of the 189 strains tested. Neither were SEs detected by the Vidas Staph enterotoxin II (bioMérieux), which was performed on 55 randomly selected strains.

3.5. Macrorestriction analysis

PFGE of the 189 S. hyicus DNAs digested with Smal detected 3–10 fragments and formed 60 individual pulsotypes (PT) distinguished by at least one band difference. The PT profiles formed fourteen clusters at a similarity level of 80% and 24 subgroups at 90% similarity. Clusters II and IX each contained 12 PT among 2 subgroups; cluster I contained 8 PT among 4 subgroups; cluster X contained 5 PT among 3 subgroups; clusters III and XII each contained 4 PT in 2 subgroups, while V also contained 4 PT. Clusters VI, VIII, XI and XIII all contained 2 PT with those in cluster VI divided as 2 subgroups. Clusters IV, VII and XIV contained 1 PT. Out of the 189 S. hyicus strains, 97 (52.2%) belonged to cluster I, 27 (14.5%) to cluster II, 19 (10.2%) to cluster IX and 11 (5.9%) to cluster III. Each of the 10 additional clusters included only two to five strains. The patterns of the exfoliative producing strains grouped into 6 of the 14 clusters. It was remarkable that 10 (52.6%) of the 19 strains in cluster IX harboured exfoliative toxin genes.

A PFGE rendered tree of the S. hyicus strains based on a Dice coefficient with 1.00% optimisation and a branch similarity cut-off at 90% to illustrate subgroups are given in Fig 1, indistinguishable pulsotypes shared terminal nodes at 95% similarity.

4. Discussion

To our knowledge, this is the first study providing comprehensive phenotypic and genotypic characterization data of S. hyicus strains isolated from pig carcasses. In general, characterization data of S. hyicus originating from healthy animals are very limited (Andresen, 2005; Futagawa-Saito, Ba-Thein, Higuchi, Sakurai, & Fukuyasu, 2007; Tanabe et al., 1996).

In this study, a minority (2.6%) of S. hyicus isolated from pig carcasses were resistant to the antibiotics tested. One strain harboured the mecA gene. To our knowledge, this is the first study that describes a mecA positive S. hyicus. Comparable data are not available as the two previous studies dealing with antimicrobial susceptibility of S. hyicus were based on strains isolated from pigs with exudative epidermitis (Aarestrup & Jensen, 2002; Wegener, Watts, Salmon, & Yancey, 1994). However, the favourable resistance results in S. hyicus are in accordance with the situation in S. aureus strains isolated from pig carcasses in Switzerland (Nitzsche et al., 2007).

Exfoliative toxin genes were detected in 31 (16.4%) strains. Six strains harboured the exhA and 25 strains the exhD gene. A variable prevalence of toxin types among S. hyicus isolated in different countries has been reported. Among the isolates from Russia, Belgium, Germany and Slovenia, exhD-positive were the most predominant (Andresen, 2005; Kanbar et al., 2006). In Denmark, ExhA-, ExhB-, ExhC- and ExhD-producing S. hyicus were isolated respectively from 20%, 33%, 18% and 22% of pigs with
exudative epidermitis (Andresen & Ahrens, 2004). Whereas, in a recently published study from Japan, the corresponding genes were present in 42.9%, 23.6%, 0.6% and 20.5% of 161 S. hyicus strains from diseased pigs (Futagawa-Saito et al., 2007). Moreover, these authors found no significant differences between strains from diseased and healthy pigs with regard to the carriage of toxin types. However, the isolation rate of toxigenic S. hyicus was four times higher in the pigs with exudative epidermitis than the healthy pigs (87.6% versus 19.6%). Similar results have been described by Tanabe et al. (1996). Nevertheless, two studies (Hoover et al., 1983; Valle et al., 1990) reported S. hyicus strains producing staphylococcal enterotoxins (SE). However, the two Enterotoxin C-producing strains isolated from healthy goats described by Valle et al. (1990) were coagulase negative, and the four S. hyicus strains described by Hoover et al. (1983) gave typical enterotoxigenic responses in monkey-feeding tests but were negative for enterotoxins A through to E in a microslide gel double diffusion assay.

To evaluate the genetic relationship between strains, S. hyicus were typed by PFGE analysis, which has been used successfully in epidemiological studies. The genotyping results show, that only a limited number of dominant clusters were found. In each of these clusters strains from both abattoirs were grouped. Cluster I included more than 50% of the investigated S. hyicus strains. Of these 97 S. hyicus strains, which were isolated over a period of five months, 10 originated from abattoir A (all after bleeding) and 87 from abattoir B (after bleeding, n = 6; after dehairing/singeing, n = 30; after polishing, n = 19; after trimming, n = 18; and after washing, n = 14). The difference in the numbers of isolates from abattoir A and abattoir B is based on differences in the slaughter technology (Spescha et al., 2006). While CPS were consistently found in both abattoirs at the beginning of slaughter, and scalding reduced detection rates and counts considerably, striking differences between the abattoirs were evident at the subsequent stages. At abattoir A, the low CPS results obtained after scalding remained constant during the slaughter process. In contrast, at abattoir B reductions obtained by scalding were offset by the combination of dehairing/singeing, and results remained at a high level during the remaining processes.

The fact that similar PFGE patterns of S. hyicus could be found on carcasses after bleeding in both abattoirs indicates the occurrence of closely related strains in the Swiss pig population, which is comparable to previous investigations characterizing S. aureus strains from pig carcasses (Nitzsche et al., 2007). Moreover, the genotyping results revealed a remarkable homogeneity in S. hyicus strains isolated after the dehairing/singeing step from different slaughter stages in abattoir B. This may indicate a recontamination of the pig carcasses at the dehairing/singeing step with persisting strains. Some pulsotypes could be found over the whole sampling period on the pig carcasses.
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References


