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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 (CPS), which are often used as indicators for *S. aureus,* are frequently found on pig carcasses at slaughter. Further characterization of the CPS identified only a minor part as *S. aureus*. Selected non-*S. aureus* strains were all identified as *S. hyicus,* However, two studies have described also in this species strains that produce staphylococcal enterotoxins. The aim of the present study was therefore to further characterize such coagulase positive *S. hyicus* strains isolated from pig carcasses and to assess the results also in view of their food safety relevance. A total of 189 *S. hyicus* strains from abattoirs A (n=36) and B (n=153) 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%) strains. Six strains harboured the *exh*A and 25 strains the *exh*D gene. *S. aureus* Enterotoxin (SE) genes were detected 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 slaughter process stages in abattoir B, which could indicate a recontamination problem with persisting strains.

Keywords: *Staphylococcus hyicus*, pig carcasses, phenotyping, toxin genes, PFGE

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 of healthy animals at slaughter (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 *S. hyicus.* The coagulase-variable species *S. hyicus* is mainly involved in exudative epidermitis in pigs (Wegener, Andresen, & Bille-Hansen, 1993). Moreover, *S. hyicus* has also been occasionally isolated from subclinical mastitis in cows (Roberson, Fox, Hancock, Gay, & Besser, 1996; Capurro, Concha, Nilsson, & Ostensson, 1999; Waage, Mark, Roros, Aasland, Hunshamar, & Odegaard, 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, Gomze-Lucia, Piriz, Goyache, Orden, & Vadillo, 1990) have also found *S. hyicus* strains producing staphylococcal enterotoxins (SE), which are normally found in *S. aureus* and are responsible for foodborne intoxications. In contrast to *S. hyicus* strains isolated from clinical cases, very little genotypic characterization data are available in the literature for strains isolated from healthy pigs. Consequently, it is of importance to further characterize *S. hyicus* isolated from pig carcasses in order to evaluate a potential food safety risk involved.

The aim of the present study was therefore (i) to identify *S. hyicus* strains among coagulase positive staphylococci (CPS) collected from pig carcasses at two abattoirs during the slaughter process, (ii) to characterize the isolated *S. hyicus* strains by phenotypic and

genotypic traits, iii) to assess these results in view 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, Stephan, & Zweifel, 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 *S. aureus* and were further characterized (Nitzsche, Zweifel, & Stephan, 2007). The remaining 195 CPS isolates were used in the present study.

According to the frequency of CPS on the carcasses at the different process 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. S. hyicus *identification and further biochemical characterization*

For the identification of *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 *sod*A (Table 1). Randomly selected strains were additionally identified by 16S rRNA sequencing.

To evaluate colour and haemolysis of the colonies, the *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 S. hyicus *strains*

Susceptibility to seven antibiotics was determined using the Sensititre NLV 23 system according to the manufacturers' instructions (Trek Diagnostic Systems Ltd., West Sussex, UK). For assessing the MIC-results, breakpoints according to the "Sensititre NLV 23 Veterinary reference card" were used: ampicillin (≥0.5 µg/ml), penicillin (≥0.25 µg/ml), amoxicillin/clavulanic acid (≥8/4 µg/ml), gentamicin (≥16 µg/ml) and kanamycin (≥64 µg/ml). For lincomycin (≥0.5 µg/ml) the breakpoint as stated by De Oliveira et al. (2000) was used. For cloxacillin the breakpoint for oxacillin (≥4 µg/ml) as stated by the Clinical Laboratory Standards Institute was applied.

2.4. Detection of staphylococcal enterotoxins (SE) with the Vidas system

Vidas Staph enterotoxin II (bioMérieux) is an enzyme–linked fluorescent immunoassay (ELFA) used on the automated Vidas instrument for the specific detection of staphylococcal enterotoxins. The Solid Phase Receptacle (SPR) serves as solid phase as well as the pipetting device for the assay. The interior of the SPR is coated with monoclonal anti-staphylococcal enterotoxin antibodies. Reagents for the assay are ready-to-use and pre-dispensed in the sealed reagent strips. The tests were performed on 55 randomly selected isolates according to the manufacturers' instructions.

2.5. Genotypic traits

All PCR assays were performed in a T3 thermocycler (Biometra, Göttingen, Germany). PCR reagents were purchased from PROMEGA (Madison, Wisconsin, USA) and primers

125 (Table 1) were synthesized by MICROSYNTH (Balgach, Switzerland). The 50-µl PCR
126 mixtures consisted of 5 µl of DNA preparation, 38.1 µl of double-distilled water, 5 µl of 10-
127 fold-concentrated polymerase synthesis buffer containing 2.0 mM $MgCl_2$, 1.0 µl dNTP, 0.25
128 µl of each primer (100 µM) and 0.3 µl (5 U/µl) of *Taq* DNA polymerase.
129
130 *2.5.1. Detection of* mecA *gene*
131 Strains were examined for the *mecA* gene with the primers GMCAR 1 and 2 (Mehrotra,
132 Wang, & Johnson, 2000), and the following conditions: initial denaturation at 94°C for 120 s,
133 followed by 5 cycles (30 s at 94°C, 60 s at 45°C, 240 s at 68°C), 10 cycles (10 s at 94°C, 30 s
134 at 55°C, 120 s at 68°C), 10 cycles (10 s at 94°C, 30 s at 55°C, 150 s at 68°C), 10 cycles (10 s
135 at 94°C, 30 s at 55°C, 180 s at 68°C), and a final extension at 68°C for 420 s. The MRSA
136 strain 10272 was used as positive control.
137
138 *2.5.2. Detection of staphylococcal SE genes*
139 To detect the genes of SEA to SED, SEG, SEI, and SEJ, simplex PCR assays were
140 performed (Scherrer, Corti, Muehlherr, Zweifel, & Stephan, 2004). The conditions were (i)
141 for the detection of *sea*, *seb*, *sec*, and *sed* initial denaturation at 94°C for 300 s followed by 35
142 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;
143 (ii) for the detection of *seg,* and *sei,* initial denaturation at 94°C for 240 s followed by 30
144 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;
145 (iii) for the detection of *sej,* initial denaturation at 94°C for 120 s followed by 30 cycles (60 s
146 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
147 strains obtained from a previously performed study were used as positive controls (Scherrer,
148 Corti, Muehlherr, Zweifel, & Stephan, 2004).
149
150 *2.5.4. Detection of the genes encoding exfoliative toxins*

Primers described in the work of Andresen and Ahrnes (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 in order to ensure full extension of the PCR products.

2.6. Macrorestriction analysis by PFGE

S. hyicus isolates were grown aerobically in brain heart infusion broth at 37 °C for 18 h to 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 (50mg/ml) for ten minutes 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 µl 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-Laurylsarkosine, 0,2% Desoxycholic acid) with 20 µl (50 mg/ml) Lysozyme and Lysostaphin and 3.2 µl Achromopeptidase (60 U/ml). Six hours after the start of the incubation 19.5 µl Proteinase K (20 mg/ml) was added. The overnight incubation was followed by a second incubation at 53°C for two hours. 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 minutes. The plugs were then stored in TE buffer at 4 °C.

Plugs were digested with 40 U *Sma*I (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 M Tris, 0.01 M EDTA, 1 M 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 PFG Marker (New England Bio Labs) was used as a molecular size marker. DNA restriction bands were analyzed by using GelCompar II (Applied Maths, Sint-Martens-Latem, Belgium). Similarity coefficients were calculated and dendrograms were constructed using the Dice coefficient and the un-weighted pair group method with arithmetic averages (UPGMA), respectively, with an optimization 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 stain 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.

203 Among strains showing resistances, one strain displayed a double resistance to
204 ampicillin/penicillin, and for one strain each a resistance to lincomycin, cefoperazone and
205 cloxacillin was detected. One strain harboured the *mec*A gene, but showed no other
206 resistances against antimicrobials that have been tested. The PCR product of this strain was
207 sequenced and showed a 100% homology to the *mec*A gene reference sequence (accession
208 number GI 156978331).
209
210 *3.3. Detection of ET genes*
211 ET genes (*exh*A*, exh*B*, exh*C*, exh*D) were detected in 31 (16.4%) strains, isolated in both
212 slaughterhouses. Six strains harboured the *exh*A and 25 strains the *exh*D gene. One strain was
213 positive for the combination of *exh*A and *exh*D genes. None of the strains harboured genes
214 for *exh*B or *exh*C.
215
216 *3.4. Detection of S. aureus Enterotoxin*
217 SE genes (*sea* to *sed*, *seg*, *sei*, *sej*) were not detected in any of the 189 strains tested.
218 Neither were SEs detected by the Vidas Staph enterotoxin II (bioMérieux), which was
219 performed on 55 randomly selected strains.
220
221 *3.5. Macrorestriction analysis*
222 PFGE of the 189 *S. hyicus* DNAs digested with *Sma*I detected 3 to 10 fragments and
223 formed 60 individual pulsotypes (PT) distinguished by at least one band difference. The PT
224 profiles formed fourteen clusters at a similarity level of 80 % and 24 subgroups at 90%
225 similarity. Clusters II and IX each contained 12 PT among 2 subgroups; cluster I contained 8
226 PT among 4 subgroups; cluster X contained 5 PT among 3 subgroups; clusters III and XII
227 each contained 4 PT in 2 subgroups, while V also contained 4 PT. Clusters VI, VIII, XI and
228 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 is given in Figure 1, indistinguishable pulsotypes share 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 (Tanabe et al., 1996; Andresen, 2005; Futagawa-Saito, Ba-Thein, Higuchi, Sakurai, & Fukuyasu, 2007).

In this study, a minority (2.6%) of *S. hyicus* isolated from pig carcasses were resistant to the antibiotics tested. One strain harboured the *mec*A gene. To our knowledge, this is the first study that describes a *mec*A 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, Zweifel, & Stephan, 2007).

Exfoliative toxin genes were detected in 31 (16.4%) strains in this study. Six strains harboured the *exh*A and 25 strains the *exh*D 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, *exh*D-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, Ba-Thein, Higuchi, Sakurai, & Fukuyasu, 2007). Moreover, these authors have not found any 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 already been described by Tanabe et al. (1996).

SE genes were not detected in any of the 189 strains tested. This result correlates with the fact, that to date staphylococcal foodborne intoxications have never been linked to *S. hyicus*. Nevertheless, two studies (Hoover, Tatini, & Maltais, 1983; Valle, Gomze-Lucia, Piriz, Goyache, Orden, & Vadillo, 1990) reported *S. hyicus* strains producing staphylococcal enterotoxins (SE). However, it has to be mentioned that 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 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 are 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 sampling 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, Stephan, & Zweifel, 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 slaughter processes.

The fact that similar PFGE patterns of *S. hyicus* can 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, Zweifel, & Stephan, 2007). Moreover, the genotyping results revealed a remarkable homogeneity in *S. hyicus* strains isolated after the dehairing/singeing from different slaughter process 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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Figure 1. PFGE rendered tree of *S. hyicus* isolates from the two abattoirs based on a Dice coefficient with 1.00% optimisation and a branch similarity cut-off 90%

Legend: A: abattoir A; B: abattoir B; 1: after bleeding; 2: after scalding; 3: after dehairing/singeing; 4: after polishing; 5: after trimming; 6: after washing; 7: scalding water. Roman numerals represent cluster subgroups (90% similarity) and terminal nodes represent indistinguishable pulsotypes.