Short-Form paper

Clonality and antimicrobial susceptibility of *Staphylococcus aureus* and methicillin-resistant *S. aureus* from food and other animals

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Running title: Clonality of *S. aureus* populations in animals

Keywords: spa typing, multilocus sequence typing; methicillin-resistant *Staphylococcus aureus*

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ABSTRACT

Of 3081 animals studied, 24.9% pigs, 4.7% chickens, 6.3% dogs, 10.5% cats and 7.1% rodents were *S. aureus* positive. Prevalence of methicillin-resistant *S. aureus* (MRSA) was high in pigs (21.3% animals, 46.5% batches), with all MRSA and most methicillin-sensitive *S. aureus* belonging to clonal complex 9 and being multidrug-resistant. Predominant *S. aureus* CCs among dog and cats isolates were similar. Among rodent isolates, CC398 predominated with spa t034 as the most frequent spa-type detected.

(75 words)
Staphylococcus aureus is a major pathogen in human and veterinary medicine (3,4,14). Since 2005, there has been a marked increase in methicillin-resistant S. aureus (MRSA) among farm animals, especially in pigs (14). These MRSA isolates belong to specific clones (ST9 and ST398) and were often resistant to other non-β-lactam antibiotics (14). However, limited studies have been performed to compare the clonal structure of MSSA and MRSA populations in food and other animals. Here, we investigated the prevalence of MRSA and MSSA among various animals and the strains were characterized by molecular methods. In September 2008-August 2011, nasal or tracheal swabs were obtained from animals in a central slaughterhouse (cattle and pigs), wet markets (chicken) and urban areas (stray dogs, stray cats, wild rodents) (6). ChromID MRSA (bioMerieux, France) and mannitol salt agar plates were used for recovery of S. aureus following an overnight broth enrichment step (5). The bacteria were identified as S. aureus by PCR methods based on a S. aureus chromosomal fragment (sau) (11). The PCR assay could distinguish S. aureus from other closely related species (11). Isolates from cats and dogs were further tested by a multiplex PCR method based on the thermonuclease (nuc) gene, allowing separation from the newly described S. pseudointermedius (16). The disc diffusion method was used for susceptibility testing according to the CLSI (2). The isolates were characterized by spa typing, multilocus sequencing (MLST) and SCCmec typing, as previously described (5,8). PCR assays were used to detect the macrolides, lincosamides and streptogramin B (MLS) resistance determinants (ermA, ermA, ermC, mefA/E) and two virulence genes (pvl and arcA) (5,7,8).

In total, 3081 animals including 609 cats, 660 chickens, 589 dogs, 310 cattle, 305 pigs and 608 rodents (281 Rattus norvegicus, 22 R. rattus, 151 R. andamanensis, 100 Niviventer fulvescens and 54 unidentified species) were cultured (Table 1). Overall, 24.9 % pigs, 4.7% chickens, 6.3% dogs, 10.5% cats and 7.1% rodents were S. aureus positive. A total of 254 S. aureus, including 188 MSSA and 66 MRSA were recovered from 252 animals (Table 2). All
but one of the isolates from chicken and pigs were resistant to three or more non-β-lactam drugs. In contrast, most of the isolates from dogs, cats and rodents were fully susceptible.

Overall, 20.3% (22/108) and 73.1% (79/108) erythromycin-resistant isolates were positive for the \textit{erm}B and \textit{erm}C genes, respectively (Table 2). No isolates were found to have the \textit{erm}A or \textit{mef}A/E genes.

Seventy-two unique spa types were identified including 68 and five spa types among the MSSA and MRSA isolates, respectively. Fig. 1 showed that 78.9% (194/246) of the isolates were clustered into seven spa clonal cluster (spa-CC): spa-CC899 (65 MRSA and 6 MSSA isolates), spa-CC034 (35 MSSA isolates), spa-CC189 (27 MSSA isolates), spa-CC002 (22 MSSA isolates), spa-CC091 (15 MSSA isolates), spa-CC701 (15 MSSA isolates) and spa-CC084 (9 MSSA isolates). All porcine and the only chicken MRSA isolates clustered into spa-CC899 (Fig. 1). Representative isolates for each spa type were tested by MLST. The results showed that all spa types within spa-CC899 belong to sequence type (ST) 9, which is within the clonal complex (CC) 9. The porcine MSSA isolates with spa t899 were also found to belong to ST9. Among chicken isolates, the two predominating spa types were t002 (ST5/CC5) and t034 (ST398/CC398 and ST2169/CC398). Isolates from dogs and cats shared similar spa type distribution with the major spa types (MLST) being t189 (ST188/CC188), t701 (ST6/CC6), t091 (ST7/CC7 and ST943/CC7) and t084 (ST15/CC15). Overall, 54.1% (20/37) and 68.8% (44/64) of the isolates from dogs and cats belonged to the four spa-CCs including spa-CC189, spa-CC701, spa-CC091 and spa-CC084. The 43 rodent isolates were found to belong to 21 unique spa types of which 24 (55.8%) isolates were of spa-CC034.

Multiplex PCR showed that MRSA isolates from pigs had either SCC\textit{mec} type IVb (n=62) or type V (n=3). The only MRSA from chickens had SCC\textit{mec} type IV. Presence of the \textit{pvl} and \textit{arc}A genes were sought in 96 isolates (66 MRSA and 30 MSSA, which were chosen to represent the major spa types) and all were PCR negative.
This study demonstrated that ST9-MRSA is the major livestock-associated MRSA (LA-MRSA) in pigs. In China, the predominant spa type in this lineage was reported to be t899 while those identified in Thailand, Malaysia and The Netherlands were t337, t4358 and t1430, respectively (9,12,13). Our data showed that the predominant MSSA carried by pigs was also spa type t899 and they share a similar multidrug resistance profiles as their MRSA counterparts. The findings indicate that this LA-MRSA might have emerged through the introduction of a meca-carrying element into the ST9/t899 MSSA lineage. Despite the prevalence of MRSA-ST9 among pigs in Asia, infections caused by this clone in animals and humans could not be found in the literature. As LA-MRSA has the potential to colonize human and its virulence may change over time (14), ongoing surveillance is needed to detect changes in epidemiology. In pigs and chickens, the S. aureus populations are far more clonally conserved than those in the other animal species; and were often multidrug-resistant. We did not collect any information about antibiotic usage in the farms. Nonetheless, the observation might be the result of farm over-crowding and antibiotic selection, facilitating the spread of multidrug-resistant clones (1). Our data on the S. aureus population structure corroborates previous observations, based primarily on animal-associated MRSA that there are host specific subsets of S. aureus typically associated with different animal species (10,17). Remarkably, the t034/CC398 clone was dominating among isolates from rodents (mainly R. norvegicus) and revealed that urban rodents could be an important reservoir of CC398 isolates. Dogs are not natural hosts of S. aureus (15), therefore, the S. aureus detected in this study may be accidental hosts of S. aureus clones circulating in the community. In conclusion, this study revealed distinctive distribution of major spa and MLST types among S. aureus populations and that only a limited numbers were associated with multidrug resistance phenotypes.
Acknowledgement

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TABLE 1. Prevalence of Staphylococcus aureus by animal groups, September 2008-August 2011

<table>
<thead>
<tr>
<th></th>
<th>dogs</th>
<th>cats</th>
<th>rodents</th>
<th>chickens</th>
<th>pigs</th>
<th>cattle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animals with S. aureus</td>
<td>6.3 (37/589)</td>
<td>10.5 (64/609)</td>
<td>7.1 (43/608)</td>
<td>4.7 (31/660)</td>
<td>24.9 (76/305)</td>
<td>0.3 (1/310)</td>
</tr>
<tr>
<td>Animal with MRSA</td>
<td>0 (0/589)</td>
<td>0 (0/609)</td>
<td>0 (0/608)</td>
<td>0.2 (1/660)</td>
<td>21.3 (65/305)</td>
<td>0 (0/310)</td>
</tr>
<tr>
<td>Batches with S. aureus</td>
<td>24.1 (32/133)</td>
<td>33.8 (47/139)</td>
<td>57.8 (26/45)</td>
<td>36.4 (12/33)</td>
<td>53.5 (46/86)</td>
<td>3.2 (1/31)</td>
</tr>
<tr>
<td>Batches with MRSA</td>
<td>0 (0/133)</td>
<td>0 (0/139)</td>
<td>0 (0/45)</td>
<td>3.0 (1/33)</td>
<td>46.5 (40/86)</td>
<td>0 (0/31)</td>
</tr>
</tbody>
</table>

The animals were randomly sampled in batches: chicken (20 animals per batch), cattle (10 animals per batch), pigs (2 to 7 animals per batch), stray cats (1-10 animals batch), stray dogs (1-10 animals per batch) and urban rodents (2 to 23 animals per batch). For cats, dogs and rodents, the batches referred to animals held in the same area in a holding facility. Of the 43 S. aureus from rodents, 40 were recovered from R. norvegicus and one each were recovered from R. andamanensis and N. fulvescens. The species source for one rodent S. aureus was unknown.
### TABLE 2. Occurrence of resistance among *Staphylococcus aureus* in different animal species

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>% nonsusceptible</th>
<th>MLS phenotype (genotype)***</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CHL</td>
<td>CIP</td>
<td>SXT</td>
</tr>
<tr>
<td>Dogs, MSSA</td>
<td>37</td>
<td>2.7</td>
<td>2.7</td>
</tr>
<tr>
<td>Cats, MSSA</td>
<td>64</td>
<td>0</td>
<td>4.7</td>
</tr>
<tr>
<td>Rodents, MSSA</td>
<td>43</td>
<td>0</td>
<td>2.3</td>
</tr>
<tr>
<td>Chicken, MSSA</td>
<td>30</td>
<td>20</td>
<td>96.7</td>
</tr>
<tr>
<td>Pigs, MSSA</td>
<td>13</td>
<td>84.6</td>
<td>53.8</td>
</tr>
<tr>
<td>Pigs, MRSA</td>
<td>65</td>
<td>89.2</td>
<td>100</td>
</tr>
</tbody>
</table>

**CHL, chloramphenicol; CIP, ciprofloxacin; SXT, cotrimoxazole; ERY, erythromycin; CLI, clindamycin; GEN, gentamicin; TET, tetracycline; MLS, macrolides, lincosamides and streptogramin B antibiotics; M phenotype, only ERY-resistant; L phenotype, only CLI-resistant; and cMLS, constitutive CLI resistance.**

***PCR results for 108 ERY-resistant isolates with indicated phenotype. Values show number of isolates. One MSSA from cattle and one MRSA from chicken were not included in the table. The cattle MSSA isolate was susceptible to all the antibiotics. The chicken MRSA was susceptible to SXT but resistant to CHL, CIP, GEN, TET, ERY and CLI (cMLS and *ermC* positive).**
FIG 1 Minimum-spanning tree based on spa sequence data. A total of 246 *Staphylococcus aureus* isolates (65 methicillin-resistant and 181 methicillin-sensitive) with 65 unique spa types were clustered by the BioNumerics software (version 6.6) using the default parameters. Sources of the isolates were indicated by colors. The numbers of isolates from each animal source were: dogs (n=36), cats (n=63), rodents (n=42), chicken (n=29) and pigs (n=76). Seven isolates with spa types of fewer than 5 repeats, including t2922 (MRSA, n=1), t111 (MSSA, n=2), t4070 (MSSA, n=1), t605 (MSSA, n=1), t7295 (MSSA, n=1) and t8615 (MSSA, n=1), and one spa PCR negative isolate were excluded. The size of the nodes (i.e. circles) was drawn in proportions to the number of isolates for each spa type. The values between the nodes were the distance matrix.
References


