Novel Apramycin Resistance Gene apmA in Bovine and Porcine Methicillin-resistant Staphylococcus aureus ST398

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Short title: Apramycin resistance gene apmA in MRSA ST398

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A novel apramycin resistance gene, \textit{apmA}, was detected on the ca. 40-kb resistance plasmid pAFS11 from bovine MRSA ST398. The \textit{apmA} gene coded for a protein of 274 amino acids which was only distantly related to acetyltransferases involved in chloramphenicol or streptogramin A resistance. Nsil deletion of \textit{apmA} resulted in a 16- to 32-fold decrease in the apramycin MICs. An \textit{apmA}-specific PCR identified this gene in one additional bovine and four porcine MRSA ST398 isolates.

Methicillin-resistant \textit{Staphylococcus aureus} (MRSA) of sequence type ST398 has been identified mainly as colonizer of the skin and the mucosal surfaces of swine (6, 26, 28). Although more rarely, such isolates were also found to be involved in infections of swine (9, 17, 18, 24). Moreover, MRSA ST398 was also detected in other animals such as cattle (8, 23), horses (25, 27), poultry (15), dogs (16) and rats (22), and in humans with exposure to MRSA ST398-colonized animals (7, 20, 28, 30). As a colonizer, MRSA ST398 is subject to selective pressure by antimicrobial agents, which are not primarily used to control staphylococcal infections, and as a consequence may acquire novel or uncommon resistance genes. One such example was the observation that the chloramphenicol-resistant MRSA ST398 isolates from swine (9) and cattle (8) did not carry any of the usually found staphylococcal \textit{cat} genes for chloramphenicol resistance (19), but harbored the phenicol exporter gene \textit{fexA} that also confers resistance to florfenicol. Florfenicol is a fluorinated chloramphenicol derivative which is widely used for the control of respiratory tract infections in cattle and swine. Another example is apramycin resistance.
Apramycin is an aminocyclitol antibiotic which is exclusively used in veterinary medicine for the treatment of *Escherichia coli* infections in swine, cattle, sheep, poultry or rabbits. Studies of apramycin-resistant Enterobacteriaceae identified the gene *aac(3)-IV*, which was mostly located on plasmids and conferred resistance to apramycin and gentamicin (2, 3, 5, 21, 29). In contrast to the wealth of data available for apramycin resistance in Enterobacteriaceae (1, 31, 32), no information exists about apramycin resistance in staphylococci.

During two survey studies on MRSA ST398 from diseased swine and dairy cattle, 4/54 porcine and 2/16 bovine isolates revealed high apramycin MIC values of ≥ 32 µg/ml (8, 9). These isolates were tentatively classified as resistant although no clinical breakpoints for apramycin approved by the Clinical and Laboratory Institute (CLSI) are currently available (4). One of these isolates, the bovine MRSA isolate 11, was chosen for further analysis of the genetic basis of apramycin resistance. The bovine MRSA isolate 11 carried a SCC*mec* element of type V and displayed the MLST type ST398, the *spa* type t2576, and the *dru* type dt11a (8). Plasmid analysis identified the ca. 40-kb plasmid pAFS11 which upon transformation into *S. aureus* RN4220 mediated a multi-resistance phenotype (Table 1). The corresponding resistance genes were detected by specific PCR assays (8, 9, 14). In addition to kanamycin/neomycin resistance via *aadD*, macrolide/lincosamide/streptogramin B resistance via *erm*(B), tetracycline resistance via *tet*(L) and trimethoprim resistance via *dfr*K, plasmid pAFS11 also conferred a high apramycin MIC of ≥ 128 µg/ml. The *S. aureus* RN4220 transformant carrying pAFS11, however, was classified as intermediate to gentamicin (MIC 8 µg/ml) (Table 1).

An 11,312-bp EcoRI fragment of pAFS11 was cloned into pBluescript II SK+ (Stratagene). Recombinant plasmids were transformed into *E. coli* strain JM101 and transformants were selected on apramycin-supplemented Luria-Bertani agar (15 µg/ml). Sequence analysis was conducted by primer walking starting with M13 universal and reverse primers. The schematic presentation of the seven reading frames found on this
EcoRI fragment is shown in Fig. 1. This segment comprised part of a Tn917 transposon with one terminal repeat and the entire \textit{erm}(B) gene. A reading frame for a 315-amino acids (aa) protein with 30.9 or 31.4\% identity to distinctly larger chromosome replication initiation/membrane attachment proteins of \textit{Staphylococcus hominis} (ZP_04059882) or \textit{Staphylococcus warneri} (ZP_04678490), respectively, was detected. Further downstream, the reading frame for a 263-aa ParA protein which corresponded closely (96.2 and 95.1\% identity) to the chromosome partitioning ATPases of \textit{Staphylococcus capitis} (ZP_03614545) and \textit{S. aureus} (ACY12632), respectively. A complete IS257 element was identified which, however, did not exhibit 8-bp direct repeat sequences in the up- and downstream part. The lack of these direct repeats suggested that recombination events via this insertion sequence have occurred. A complete reading frame for a 347-aa protein and the 3\' end of a reading frame (190 aa) showed 48.4\% and 54.5\% identity to IcaC (YP_189846) and IcaB (YP_189845), respectively, both from the chromosome of a \textit{S. epidermidis} isolate.

To confirm the role of the seventh reading frame, designated \textit{apmA}, in apramycin resistance, the EcoRI fragment was digested with Nsil which cuts once within the \textit{apmA} reading frame, once within the IS257 sequence and once within the \textit{icaB}-like gene. Deletion clones in \textit{E. coli} JM101 were tested for their apramycin MICs by broth microdilution according to the CLSI document M31-A3 (4). In comparison to clones carrying the original EcoRI fragment, all three deletion clones showed a 16- to 32-fold decrease in the apramycin MICs and also an 8-fold decrease in the gentamicin MICs.

The \textit{apmA} gene codes for a 274-aa protein which shows limited similarity to other proteins deposited in the databases. The best matches were 38.1\% identity to a VatB-like xenobiotic acetyltransferase protein from \textit{Pasteurella multocida} (NP_246134) and 33.3\% identity to a putative chloramphenicol acetyltransferase from \textit{Escherichia fergusonii} (YP_002383245). Based on the \textit{apmA} sequence, a PCR assay using the primers \textit{apmA-fw} (5'-CGTTTGCTTCGTGCATTAAA-3') and \textit{apmA-rev} (5'-
TTGACACGAAGGAGGGTTTC-3') [annealing temperature: 52°C; amplicon size: 656 bp] was developed and applied to MRSA ST398 isolates. While the remaining bovine and the four porcine apramycin-resistant isolates were positive for *apmA*, the isolates with MICs of ≤16 µg/ml were negative. All five additional isolates harbored a SCCmec type V element and showed the *spa* type t011 and the *dru* type dt11a (8, 9). Transfer and hybridization experiments identified *ampA* in all five cases on plasmids of ca. 40 kb which were indistinguishable or closely related to pAFS11 in their EcoRI, HindIII, BglII and PvuI restriction patterns. All these plasmids also harbored *tet*(L), *dfrK*, *aadD* and *erm*(B) in addition to *apmA*.

Recent studies on antimicrobial resistance genes in MRSA ST398 led to the identification of a number of novel or unusual resistance genes, such as *dfrK* (10), *vga*(C) (11), *erm*(T) (12) and *cfr* (13). All these genes were located on plasmids. Analysis of these plasmids suggested that recombination and co-integrate formation played a major role in the acquisition of novel resistance genes by MRSA ST398. In most of the described plasmids, insertion sequences, such as IS257 or the ISSau10 (10, 12), seemed to be involved in recombination processes. This is to the best of our knowledge the first description of an apramycin resistance gene in Gram-positive cocci. The presence of *apmA* on the multiresistance plasmid pAFS11 enables its persistence and co-selection under the selective pressure imposed by the use of kanamycin, neomycin, tetracyclines, macrolides, lincosamides or trimethoprim.

**Nucleotide sequence accession number.** The sequence of the 11,321-bp EcoRI fragment of plasmid pAFS11 has been deposited in the EMBL database under accession number FN806789.
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References


Fig. 1:

\[ \text{MIC (µg/ml)} \]

- 1 0.25
- 2 0.25
- 32 2

\text{apramycin}
\text{gentamicin}

\text{erm(B) aprmA rep parA IS257 icaC-like icaB-like}

\text{N N N}

\text{apmAerm(B) IS257}

\text{MIC (µg/ml)}

- 1 0.25
- 2 0.25
- 32 2
Table 1: Comparative analysis of the bovine MRSA ST398 isolate 11, *S. aureus* RN4220 and the *S. aureus* RN4220 transformant carrying the plasmid pAFS11.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Resistance genes</th>
<th>MICs (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>APR</td>
</tr>
<tr>
<td>MRSA ST398 isolate 11</td>
<td><em>apmA, erm(B), tet(L), tet(M), tet(K), dfrK, aadD, mecA, blaZ</em></td>
<td>≥ 128</td>
</tr>
<tr>
<td><em>S. aureus</em> RN 4220</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td><em>S. aureus</em> RN 4220 (pAFS11)</td>
<td><em>apmA, erm(B), tet(L), dfrK, aadD</em></td>
<td>≥ 128</td>
</tr>
</tbody>
</table>

Antimicrobial agents are abbreviated as follows: APR (apramycin), GEN (gentamicin), ERY (erythromycin), CLI (clindamycin), TET (tetracycline), TMP (trimethoprim), KAN (kanamycin), NEO (neomycin), OXA (oxacillin).