Detection and new genetic environment of the pleuromutilin–lincosamide–streptogramin A resistance gene lsa(E) in methicillin-resistant Staphylococcus aureus of swine origin

Beibei Li1†, Sarah Wendlandt2†, Jiannan Yao1, Yiqiu Liu3, Qing Zhang4, Zixue Shi1, Jianchao Wei1, Donghua Shao1, Stefan Schwarz2, Shaohui Wang1 and Zhiyong Ma1*

1Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Shanghai 200241, China; 2Institute of Farm Animal Genetics, Friedrich-Loeffler-Institut (FLI), 31535 Neustadt-Mariensee, Germany; 3China Institute of Veterinary Drug Control, Beijing 100081, China; 4The Fifth People’s Hospital of Shanghai, Fudan University, Shanghai 200240, China

*Corresponding author. Tel: +86-2134293139; Fax: +86-2154081818; E-mail: zhiyongma@shvri.ac.cn
†These authors contributed equally to this study.

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Objectives: To investigate the genetic basis of pleuromutilin resistance in porcine methicillin-resistant Staphylococcus aureus (MRSA) and to map the genetic environment of the identified plasmid-borne resistance gene.

Methods: Seventy porcine MRSA isolates, which exhibited high MICs of tiamulin, valnemulin and retapamulin, were investigated for pleuromutilin resistance genes and mutations. They were characterized by staphylococcal cassette chromosome mec (SCCmec) typing, spa typing and multilocus sequence typing (MLST). Plasmid DNA was extracted from the lsa(E)-positive strains and transferred to S. aureus RN4220 for selection of resistance plasmids. The plasmid-borne lsa(E) gene region was sequenced and 10 overlapping PCR assays for the analysis of the genetic environment of lsa(E) were developed.

Results: All 70 MRSA isolates were ST9 (MLST)-t899 (spa)-IVa (SCCmec). Sixteen isolates carried the lsa(E) gene; all others were negative for known pleuromutilin resistance mechanisms. An lsa(E)-carrying plasmid of ~41 kb was detected in a single isolate. Sequence analysis revealed that the lsa(E) gene was located in a multiresistance gene cluster, which showed partial homology to clusters identified in MRSA, methicillin-susceptible S. aureus (MSSA) and Enterococcus faecalis. PCR analysis of the remaining isolates revealed a partly deleted multiresistance gene cluster in 6/15 isolates and solely the lsa(E) gene without the known flanking regions in 9/15 isolates.

Conclusions: We identified the pleuromutilin–lincosamide–streptogramin A resistance gene lsa(E) in porcine MRSA isolates. The multiresistance gene cluster in which lsa(E) was located differed from the previously described ones found in human MRSA/MSSA or in E. faecalis. The location of lsa(E) on a multiresistance plasmid facilitates its persistence and dissemination.

Keywords: multidrug resistance, ABC transporters, MRSA, plasmids

Introduction

Pleuromutilin antibiotics interfere with protein synthesis by binding to the peptidyl transferase centre of the 50S ribosomal subunit and inhibiting peptide bond formation.1 Pleuromutilins, such as tiamulin and valnemulin, have been used exclusively in veterinary medicine to treat infections in pigs and poultry. Recently, the novel pleuromutilin retapamulin has been approved for topical treatment of skin infections in humans caused by Staphylococcus aureus or Streptococcus pyogenes.2 Pleuromutilin resistance may be due to alterations of the drug target site by mutations or methylation and the expression of specific ATP-binding cassette (ABC) transporters. Mutations in ribosomal protein L3 or in domain V of 23S rRNA at the peptidyl transferase centre are known to mediate pleuromutilin resistance in various bacteria.3–7 The methyltransferase Cfr, which methylates 23S rRNA at nucleotide A2503, also confers resistance to pleuromutilins and to other classes of antibiotics, including phenicols, lincosamides, oxazolidinones, streptogramin A and the 16-membered macrolides spiramycin and josamycin.8,9 In addition, the
ABC transporter genes vga(A), vga(C), vga(E), lsa(C) and lsa(E) can mediate combined resistance to pleuromutilins, lincosamides and streptogramin A antibiotics in Gram-positive bacteria. While lsa(C) was identified in Streptococcus agalactiae, all other resistance genes were found on plasmids or transposons in staphylococci. However, the gene lsa(E) has been so far exclusively detected in methicillin-resistant S. aureus (MRSA) sequence type (ST) 398 and methicillin-susceptible S. aureus (MSSA) ST9 of human origin from Spain.

In 2011, a surveillance study on MRSA of swine origin in Jiangsu and Zhejiang provinces, China, identified 70 MRSA isolates from the nasal cavity of pigs, all of which showed high MICs of tiamulin, valnemulin and retapamulin. The purpose of the present study was to investigate the genetic basis of pleuromutilin resistance in these porcine MRSA isolates and to map the genetic environment of the identified resistance gene.

Materials and methods

Bacterial strains, molecular typing and antimicrobial susceptibility testing

The 70 porcine MRSA isolates, which originated from five unrelated pig farms in Jiangsu and Zhejiang provinces, were characterized by staphylococcal chromosomal cassette mec (SCCmec) typing, spa typing and multilocus sequence typing (MLST), as described previously. Antimicrobial susceptibility testing by broth microdilution followed the recommendations given in CLSI documents M31-A3 and M100-S21. S. aureus ATCC 29213 served as the quality control strain for antimicrobial susceptibility testing. These isolates were investigated for the pleuromutilin resistance genes cfr, vga(A), vga(C), vga(E), lsa(C) and lsa(E) by PCR (Table S1, available as Supplementary data at JAC Online) and for mutations in ribosomal protein L3 and domain V of the 23S rRNA.

Isolation of plasmid DNA and transformation

Plasmid DNA of the 16 lsa(E)-positive MRSA isolates was extracted using the Qiagen plasmid extraction mid kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions with the following modification: after resuspending the bacterial pellet in buffer P1, lysisostaphin was added to a final concentration of 50 mg/L and the mixture was incubated for 2 h at 37°C before adding buffer P2. The purified plasmid DNA was transferred into the S. aureus recipient strain RN4220 by electrottransformation. Transformants were selected on brain heart infusion (BHI) agar containing 10 mg/L valnemulin. The identified resistance plasmid, designated pV7037, was digested with several restriction enzymes (XbaI, BamHI, HindIII, XhoI and EcoRI) to estimate the plasmid size and to choose a suitable enzyme for subsequent cloning (data not shown).

Cloning and sequencing of the genetic environment of lsa(E)

Plasmid pV7037 extracted from the valnemulin-resistant transformant was digested with XbaI and the restriction fragments were cloned into the Escherichia coli–S. aureus shuttle vector pL150. The resulting recombinant plasmids were separately transformed into the recipient strain S. aureus RN4220 and plated on BHI agar supplemented with 10 mg/L valnemulin. Growth on the selection plates suggested that the individual colonies carried the pleuromutilin resistance determinant and the presence of the lsa(E) gene was confirmed by PCR using primers lsa(E)-fw and lsa(E)-rv (Table S1). Using the corresponding recombinant plasmid as a template and starting with primers complementary to the vector pL150 sequence, the lsa(E)-containing XbaI fragment was sequenced by primer walking. Sequence analysis was performed with the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) and with the ORF Finder program (http://www.ncbi.nlm.nih.gov/orf/). The 17577 bp sequence of the resistance plasmid pV7037 has been deposited in the GenBank database under accession number JX560992.

To analyse the 15 isolates, for which an lsa(E)-carrying plasmid could not be isolated, for the presence of the pV7037-associated genetic environment of lsa(E), 10 overlapping PCR assays were developed and applied. The primers, amplicon sizes and annealing temperatures are listed in Table S2 (available as Supplementary data at JAC Online).

Results and discussion

Characterization of lsa(E)-positive MRSA isolates

Although the 70 porcine MRSA isolates were from five different farms in two different provinces, they all belonged to the same genotype: ST9 (MLST)-t899 (spa)-Iva (SCCmec). All MRSA isolates showed elevated MICs of tiamulin (MIC >128 mg/L), valnemulin (MIC 32–64 mg/L) and retapamulin (MIC 32–64 mg/L). PCR analysis revealed that 16 of the 70 MRSA isolates were positive for the lsa(E) gene, which was confirmed by sequencing of the PCR products. In contrast, none of the remaining 54 isolates harboured any of the other so far known pleuromutilin resistance genes. In addition, point mutations in domain V of 23S rRNA and in the gene for the ribosomal protein L3 were not detected in these isolates. This observation suggested that other pleuromutilin resistance genes may be present in these isolates.

Transfer of resistance and genetic environment of the lsa(E) gene

Only one of the 16 lsa(E)-positive MRSA isolates, namely isolate SA7037, yielded transformants after electrottransformation. Plasmid profiling identified the ~41 kb plasmid pV7037 in the corresponding S. aureus RN4220 transformants. The transformants exhibited high MICs of tiamulin, valnemulin and retapamulin (Table 1) and PCR analysis confirmed the presence of the lsa(E) gene. Restriction analysis of plasmid pV7037 with XbaI generated four fragments of about 17.5, 12.5, 6.5 and 4.5 kb. Four recombinant plasmids, each carrying one of the four XbaI fragments of pV7037, were successfully constructed and separately transformed into the recipient strain S. aureus RN4220. Only the clones carrying the recombinant vector with the 17.5 kb XbaI fragment (designated pL1-175) grew on BHI agar supplemented with 10 mg/L valnemulin and harboured the lsa(E) gene. This 17.5 kb XbaI fragment contained 14 opening reading frames (ORFs) of >100 amino acids. Structural analysis identified a centre region carrying five antibiotic resistance genes bracketed by the two insertion sequences IS1216 and IS257 (Figure 1). The detected resistance genes included the Inu(B) gene for lincosamide resistance, the lsa(E) gene for pleuromutilin–lincosamide–streptogramin A resistance, the aadE gene for streptomyacin resistance, the erm(B) gene for macrolide–lincosamide–streptogramin B (MLSβ) resistance and the aacA-aphD gene for gentamicin–kanamycin–tobramycin resistance. Consistent with the presence of these resistance genes, the original MRSA isolate SA7037 and the transformants S. aureus RN4220/pV7037 and S. aureus RN4220/pL1-175 exhibited high MICs or—if applicable...
A closer look at these resistance genes revealed that only an IS256-deficient relic of transposon Tn4001, which commonly harbours the aacA-aphD gene bracketed by IS256 elements, was present. In the aacA-aphD upstream part, the IS1216 element had integrated into the non-coding spacer region between IS256L and the start codon of aacA-aphD, thereby deleting the IS256L and 141 of the 399 bp in the spacer region. Moreover, the 3’-terminal region of the aacA-aphD gene was truncated by the insertion of a Tn917 relic. Of this Tn917 relic, only the left-hand segment including the 73 bp terminal repeat, the erm(B) gene and 39 bp of the internal 73 bp repeat sequence were present. This insertion deleted the terminal 44 bp of the aacA-aphD gene (including the regular translational stop codon), generated an alternative stop codon and thus extended the aacA-aphD reading frame by 12 codons. Based on the high gentamicin MICs observed (Table 1), these modifications at the C-terminus obviously had no negative impact on the aminoglycoside resistance mediated by this AacA/AphD protein.

Comparative analysis revealed that the (E)-containing multiresistance gene cluster of plasmid pV7037 showed partial homology to genetic structures found in one MRSA ST398 and two MSSA ST9 of human origin from Spain\(^{21}\) (accession number JQ861959) and the one located on plasmid pEF418 from Enterococcus faecalis (accession number AF408195) (Figure 1). The multiresistance gene cluster found in the present study was even more expanded as it included another two resistance genes, aacA-aphD and erm(B), both of which are commonly found in enterococci. The presence of IS257 and IS1216 insertion sequences in the three multiresistance gene clusters suggested that these insertion sequence elements might have been involved in both the formation of the multiresistance gene cluster and its dissemination. Based on the genes present, it is likely that this multiresistance gene cluster has evolved in enterococci. Since enterococcal plasmids often do not replicate in staphylococci, either integration into the chromosomal DNA of the staphylococcal host or co-integrate formation with a plasmid already present in the staphylococcal host are possible. In staphylococci, IS257 has been shown to be involved in such chromosomal integration and plasmid co-integrate formation processes.\(^{22}\) The observation that the resistance genes present in this cluster confer resistance to five classes of antimicrobial agents, including macrolides, lincosamides, streptogramins, pleuromutilins and various aminoglycosides, is alarming. The use of any member of these five classes of antimicrobial agents could provide selection pressure for the maintenance and dissemination of the multiresistance plasmid pV7037. Continued surveillance for the (E)-gene and the multiresistance plasmid pV7037 in MRSA isolates is warranted, both to determine the current prevalence and to monitor its dissemination.

**Genetic environment of (E) in the 15 other MRSA isolates**

To investigate the genetic environment of the (E)-gene in the remaining 15 MRSA isolates we designed 10 different PCRs (PCR1 to PCR10, Figure 1 and Table S2) that covered the entire multiresistance gene cluster located on plasmid pV7037. PCR mapping showed that

<table>
<thead>
<tr>
<th>Bacterial isolate</th>
<th>TIA, valnemulin</th>
<th>VAL, retapamilin</th>
<th>VIR, M1</th>
<th>VIR, viridamycin</th>
<th>M1, C1</th>
<th>lincomycin</th>
<th>ERY, erythromycin</th>
<th>AZM, azithromycin</th>
<th>A2, aztreonam</th>
<th>AZT, aztreomycin</th>
<th>GEN, gentamicin</th>
<th>STR, streptomycin</th>
<th>FFC, florfenicol</th>
<th>LZD, linezolid</th>
<th>V AND, vancomycin</th>
<th>DAP, daptomycin</th>
<th>LVX, levofloxacin</th>
<th>SXT, sulfamethoxazole/trimethoprim</th>
<th>DOX, doxycycline</th>
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Table 1. MICs for the original MRSA SA7037, S. aureus RN4220 and the S. aureus RW-220 transformants carrying plasmids pV7037, pLI-175 and pLI50.
six of the 15 MRSA isolates were positive for PCR4 to PCR10. This observation indicated that these isolates carried a large part of the multiresistance gene cluster of plasmid pV7037, but obviously had the area that comprised the genes aacA-aphD and erm(B) deleted. The remaining nine MRSA isolates carried the gene lsa(E), but were only positive for the PCR10. This observation suggested a novel genetic environment of the lsa(E) gene in these isolates.

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Transparency declarations
None to declare.

Supplementary data
Tables S1 and S2 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References


