Characterization of mannitol-fermenting methicillin-resistant staphylococci isolated from pigs in Nigeria

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Submitted: July 29, 2014; Approved: February 2, 2015.

Abstract

This study was conducted to determine the species distribution, antimicrobial resistance pheno- and genotypes and virulence traits of mannitol-positive methicillin-resistant staphylococci (MRS) isolated from pigs in Nsukka agricultural zone, Nigeria. Twenty mannitol-positive methicillin-resistant coagulase-negative staphylococcal (MRCoNS) strains harboring the mecA gene were detected among the 64 Staphylococcus isolates from 291 pigs. A total of 4 species were identified among the MRCoNS isolates, namely, Staphylococcus sciuri (10 strains), Staphylococcus lentus (6 strains), Staphylococcus cohnii (3 strains) and Staphylococcus haemolyticus (one strain). All MRCoNS isolates were multidrug-resistant. In addition to ß-lactams, the strains were resistant to fusidic acid (85%), tetracycline (75%), streptomycin (65%), ciprofloxacin (65%), and trimethoprim/sulphamethoxazole (60%). In addition to the mecA and blaZ genes, other antimicrobial resistance genes detected were tet(K), tet(M), tet(L), erm(B), erm(C), aacA-aphD, aphA3, str, dfrK, dfrG, catpC221, and catpC223. Thirteen isolates were found to be ciprofloxacin-resistant, and all harbored a Ser84Leu mutation within the QRDR of the GyrA protein, with 3 isolates showing 2 extra substitutions, Ser98Ile and Arg100Lys (one strain) and Glu88Asp and Asp96Thr (2 strains). A phylogenetic tree of the QRDR nucleotide sequences in the gyrA gene revealed a high nucleotide diversity, with several major clusters not associated with the bacterial species. Our study highlights the possibility of transfer of mecA and other antimicrobial resistance genes from MRCoNS to pathogenic bacteria, which is a serious public health and veterinary concern.

Key words: methicillin-resistant, mannitol-fermenting, staphylococci, quinolone resistance, pigs.

Introduction

Staphylococci are Gram-positive bacteria, and they are classified into two groups, coagulase-positive (CoPS) and coagulase-negative (CoNS), based on their ability to produce the enzyme coagulase (Bergeron et al., 2011). Staphylococcus aureus and Staphylococcus pseudintermedius are the most important species in the CoPS group as they are major pathogens for both humans and animals, especially S. aureus. Although CoNS are saprophytic and rarely pathogenic (Kloos and Bannerman, 1994), multidrug-resistant (MDR) strains have been associated with severe cases of difficult to treat infections, especially in immunocompromised individuals (Zell et al., 2008).

Methicillin-resistant staphylococci (MRS) are among the most important bacteria in both human and veterinary medicine and of major clinical, public health and economic concern (Kolar et al., 2010). The problem is aggravated by the fact that MRS, in addition to ß-lactam antibiotics, are commonly resistant to other classes of antimicrobial agents.
including aminoglycosides, macrolides, phenicols, tetracyclines and fluoroquinolones (Lee, 2003; Khanna et al., 2008). Methicillin resistance is conferred by the mecA gene which encodes an altered penicillin-binding protein (PBP2a or PBP2′) with a low affinity for β-lactam antimicrobials (Weese, 2010).

Methicillin-resistant S. aureus (MRSA) colonization in pigs was first reported in the Netherlands in 2005 (Voss et al., 2005). Since then, MRSA, particularly strains of lineage ST398, have been isolated from livestock, especially pigs, in several countries in Europe, America and Asia (Khanna et al., 2008; Denis et al., 2009; Wagenaar et al., 2009; Baba et al., 2010; Golding et al., 2010; Gomez-Sanz et al., 2010; Graveland et al., 2011; Lin et al., 2011; Larsen et al., 2012; Oppliger et al., 2012). Recently, Chah et al. (2014) characterized methicillin-resistant CoNS from dogs in Nsukka, Nigeria. There are, however, no documented reports on the characterization of methicillin-resistant staphylococci from pigs in Nigeria. Thus, this study was conducted to gain insight into the species distribution, antimicrobial resistance pheno- and genotypes and virulence traits of mannitol-positive MRSA from intensively reared pigs in the Nsukka agricultural zone, Nigeria.

Materials and Methods

Bacterial strains

Sixty-four staphylococcal strains isolated from nasal and ear swabs of 291 pigs from 16 farms in the Nsukka agricultural zone, Enugu State, Nigeria, were used in the study. Informed consent of each pig owner was obtained prior to sample collection. Stacked cultures of the isolates were sub-cultured on nutrient agar and confirmed as staphylococci based on their microscopic and biochemical characteristics (Gram-positive cocci in bunches and catalase-positive). Each confirmed Staphylococcus isolate was cultured on oxacillin resistance screening agar base (ORSAB) (Oxoid) supplemented with 2 μg/mL oxacillin (Oxoid) and incubated at 37 °C for 24-48 hours. Blue colonies (mannitol-positive isolates) were picked from ORSAB and sub-cultured on brain heart infusion (BHI) agar (Oxoid). Cultures on BHI agar were tested for coagulase and DNAses activity. Oxacillin/methicillin-resistant staphylococci were identified to the species level by PCR amplification and sequencing of the sodA and 16S rRNA genes (Mellmann et al., 2006). Detection of the mecA gene was carried out by PCR using specific primers (Al-Talib et al., 2009).

Pheno- and genotype of the mannitol-positive methicillin-resistant staphylococci

Mannitol-positive methicillin-resistant isolates were tested for susceptibility to 16 antimicrobial agents using the disk diffusion method, and the interpretive criteria were as specified by the Clinical and Laboratory Standards Institute (CLSI, 2014) guidelines. The antimicrobial agents tested were penicillin (P), oxacillin (OX), cefoxitin (FOX), erythromycin (E), clindamycin (CC), gentamicin (GM), kanamycin (K), streptomycin (S), tobramycin (NN), tetracycline (T), trimethoprimsulphamethoxazole (SXT), chloramphenicol (C), ciprofloxacin (CIP), mupirocin (MUP), fusidic acid (FUS), and linezolid (LIN). The breakpoints for fusidic acid/mupirocin and streptomycin were as recommended by EUCAST (http://www.eucast.org) and the Société Francaise de Microbiologie (www.sfm.asso.fr), respectively. A double-disk diffusion test (D-test) was performed on the isolates to detect inducible clindamycin resistance. Detection of antimicrobial resistance genes (with the exception of that for fusidic acid) was performed by specific PCRs (Gomez-Sanz et al., 2010). Positive and negative controls from the collection of the University of La Rioja were used in each reaction.

Molecular basis for quinolone resistance

Ciprofloxacin-resistant isolates were evaluated by amplification and sequencing of the quinolone resistance-determining region (QRDR) of the DNA gyrase A subunit gene gyrA between positions equivalent to Ala-68 and Gln-107 (Takahata et al., 1997), using primers gyrA_157-fw (5’-TTAAATGAAACAGTGATGAC-3’) and gyrA_539-fw (5’-GCCCATACCTACCGCGTACC-3’) (Takahata et al., 1997). The phylogenetic relationships between the QRDR nucleotide sequences (nt 202-321 of gyrA) of all ciprofloxacin-resistant isolates were investigated by constructing a maximum likelihood phylogenetic tree using the SeaView program version 4.4.0. The QRDR nucleotide sequences of representative strains of quinolone-susceptible S. aureus (Genbank accession no. CP000253), S. pseudintermedius (Genbank accession no. AM262968), Staphylococcus epidermidis (Genbank accession no. AF127634), and Staphylococcus haemolyticus (Genbank accession no. AY341071) were included in the analysis for comparison purposes. The sequences were aligned using MUSCLE, and the trees were constructed with PhyML using the general time reversible (GTR) model.

Detection of virulence genes

The presence of the eta, etb, etc, and etd genes encoding for exfoliative toxins was assessed by PCR (Chen et al., 2007). Genes involved in biofilm formation, icaA, icaB, icaC, icaD, icaR and IS256, as well as bap, were also investigated by specific PCRs as previously described (Ziebuhr et al., 1999; Cucarella et al., 2001).
Results

Methicillin-resistant Staphylococcus species

Out of the 64 Staphylococcus strains, 20 were able to grow on ORSAB supplemented with oxacillin and formed blue colonies, thus indicating mannitol fermentation. All 20 strains were negative in the coagulase and DNAse tests and harbored the mecA gene. Thus, the strains were all methicillin-resistant coagulase-negative staphylococci (MRCoNS). These mannitol-positive MRCoNS belonged to 4 species, namely, S. sciuri (10 strains), S. lentus (6 strains), S. cohnii (3 strains) and S. haemolyticus (one strain) (Figure 1).

Antimicrobial resistance phenotypes of the MRCoNS strains

Seventeen (85%) of the 20 MRCoNS strains were resistant to fusidic acid, while resistance to tetracycline, streptomycin and ciprofloxacin was shown by 15 (75%), 13 (65%) and 13 (65%) strains, respectively (Table 1). Five (25%) of the isolates expressed inducible clindamycin resistance as revealed by the typical D-shaped halo around a clindamycin disc, while the rest exhibited constitutive resistance to clindamycin. None of the isolates was resistant to mupirocin or linezolid. Sixteen different resistance patterns were found among the 20 MRCoNS strains, with P-OX-FOX-CCox-T-SXT-CIP-FUS being the predominant pattern (4/20). All the isolates were MDR as they displayed resistance to at least 3 classes of antimicrobial agents (Table 2).

Antimicrobial resistance and virulence genes detected

The antimicrobial resistance genes detected in the MRCoNS strains are presented in Table 2. The mecA and blaZ genes mediating β-lactam resistance were detected in all 20 and 4 MRCoNS strains, respectively. Tetracycline resistance was found to be mediated mainly by tet(K) alone (3 strains) or in combination with tet(M), or tet(M) and tet(L) (3 strains each). Two tetracycline-resistant strains lacked any of the genes encoding tetracycline resistance. Various combinations of the aacA-aphD, aphA3 and str genes were detected among the aminoglycoside-resistant MRCoNS strains. The catp223 or catp223 genes were detected in the chloramphenicol-resistant strains. Of the 12 sulphamethoxazole/trimethoprim-resistant strains, 9 were positive for dfrG while the remaining 3 harbored the dfrK gene. These latter strains (2 strains of S. lentus and one strain of S. cohnii) also carried the tet(L) gene linked to dfrK, as determined by specific PCR using primers tet(L)-fw (5’-CATTTGGTCTTATTGGATCG-3’) and dfrK-rv (5’-CAAGAAGCTTTTCGCTCATAAA-3’) and by sequencing of a 1,732-bp fragment. All the MRCoNS strains were negative for the genes mediating exfoliative toxin and biofilm formation.

Analysis of the QRDR of quinolone-resistant strains

All 13 ciprofloxacin-resistant strains (6 strains of S. sciuri, 4 strains of S. lentus, 2 strains of S. cohnii and one strain of S. haemolyticus) harbored a Ser84Leu mutation within the QRDR of the GyrA protein at the position identical to that involved in quinolone resistance in S. aureus, S. pseudintermedius, S. epidermidis and S. haemolyticus (Sreedharan et al., 1991; Yonezawa et al., 1996; Gómez-Sanz et al., 2013) (Figure 2A). In addition to this, 3 isolates
showed two extra substitutions, Ser98Ile and Arg100Lys in *S. lentus* strain C4022 and Glu88Asp and Asp96Thr in *S. sciuri* C3997 and *S. lentus* C4005, with the latter also present in the representative quinolone-susceptible *S. haemolyticus* strain (Figure 2A). The phylogenetic tree of the QRDR nucleotide sequences in the *gyrA* gene revealed a high nucleotide diversity, with several major clusters not associated with the bacterial species. The quinolone-susceptible *S. pseudintermedius* representative strain showed the most divergent QRDR (Figure 2B).

**Discussion**

In this study, the species distribution, antimicrobial resistance pheno- and genotypes and virulence traits of...
mannotol-fermenting MRS isolated from intensively reared pigs in the Nsukka agricultural zone, Nigeria, were investigated. The study provides the first report on MRS in pigs in Nigeria. MRCoPS, particularly MRSA, have been reported from pigs in several European, American and Asian countries (Khanna et al., 2008; Denis et al., 2009; Wagenaar et al., 2009; Baba et al., 2010; Golding et al., 2010; Gomez-Sanz et al., 2010; Graveland et al., 2011; Larsen et al., 2012; Oppliker et al., 2012). Surprisingly, none of the 20 strains in the present study was MRSA, despite the fact that they formed blue colonies on ORSAB. Oxacillin resistance screening agar base is a modification of mannitol-salt agar supplemented with 2 µg/mL of oxacillin, on which mannitol-fermenting isolates turn intensely blue due to the presence of aniline. Although ORSAB is intended for detection of MRSA (Simor et al., 2001), the results of the present study as well as those of previous work (Becker et al., 2002; Dzen et al., 2007) have shown that colonies of mannitol-positive CoNS on this medium are similar to those of MRSA. These findings, therefore, emphasize the need for accurate identification of mannitol-fermenting methicillin-resistant staphylococci.

Three of the four Staphylococcus species reported in the present study have recently been reported in dogs in the same study area (Chah et al., 2014). S. sciuri was the predominant MRCoNS species found among the pig Staphylococcus isolates in this study. In Switzerland, S. sciuri has been identified as the major MRCoNS species isolated from various animal sources, including pigs (Huber et al., 2011). S. sciuri was also reported as the major MRCoNS species in the bovine population in Belgium (Vanderhaeghen et al., 2013). Although the degree of importance of CoNS in veterinary medicine is not clearly understood, they are increasingly found to be implicated in cases of bovine mastitis (Inegol and Turkyilmaz, 2012; Kaynarca and Turkyilmaz, 2010) and canine pyoderma (Lima et al., 2012). S. sciuri has been reported to cause fatal exudative epidermitis in piglets in China (Chen et al., 2003) and wound infections (Shiyuu et al., 2004; Coimbra et al., 2011) in humans.

In addition to resistance to β-lactam antibiotics, the MRCoNS in this study also demonstrated high rates of re-

| Strain No. | Staphylococcus species | Resistance phenotypea | Resistance genes detected |
|------------|------------------------|-----------------------|---------------------------|
| C4019      | S. sciuri              | P-OX-FOX-E-CCind-GMT-SXT-CIP-FUS | mecA, erm(C), str, tet(M), dfrG |
| C4017      | S. lentus              | P-OX-FOX-S-T-SXT      | mecA, str, dfrG            |
| C4020      | S. sciuri              | P-OX-FOX-SXT-Cci-C-FUS | mecA, dfr(G), catE223     |
| C3985      | S. sciuri              | P-OX-FOX-E-CC-GM-K-S-T-SXT-FUS | mecA, erm(C), aacA/aphD, str, tet(K), tet(M) dfrG |
| C3986      | S. lentus              | P-OX-FOX-S-T-SXT-FUS  | mecA, str, dfrG            |
| C4011      | S. sciuri              | P-OX-FOX-E-CCind-S-T-SXT-CIP-FUS | mecA, erm(C), str, tet(M), dfrG |
| C4012      | S. cohnii              | P-OX-FOX-E-CCind-GM-K-S-T-FUS | mecA, blaZ, erm(C), aacA/aphD, aphA3, str, tet(K) |
| C3988      | S. lentus              | P-OX-FOX-E-CC-GM-NN-K-S-T-SXT-CIP-FUS | mecA, erm(B), aacA/aphD, str, tet(K), tet(M), [tet(L)-dfrK] |
| C3989      | S. lentus              | P-OX-FOX-E-CC-GM-NN-K-S-T-SXT-CIP-FUS | mecA, erm(B), aacA/aphD, str, tet(K), tet(M), [tet(L)-dfrK] |
| C3991      | S. cohnii              | P-OX-FOX-CCind-CIP-FUS | mecA               |
| C3992      | S. sciuri              | P-OX-FOX-FOX-NN-K-CIP-FUS | mecA, blaZ, aacA/aphD   |
| C4015      | S. cohnii              | P-OX-FOX-FOX-CCind-GM-K-S-T-SXT-CIP-FUS | mecA, erm(C), str, tet(M), dfrG |
| C4024      | S. sciuri              | P-OX-FOX-T-FUS        | mecA, str(T)             |
| C4001      | S. sciuri              | P-OX-FOX-FOX-CCind-S-T-SXT-CIP-FUS | mecA, erm(C), str, tet(M), dfrG |
| C4002      | S. haemolyticus        | P-OX-FOX-E-CCind-GM-K-S-T-SXT-CIP-FUS | mecA, erm(C), str, tet(M), dfrG |
| C4005      | S. lentus              | P-OX-FOX-FOX-FOX-NN-K-C-CIP-SXT | mecA, blaZ, aacA/aphD, aphA3, dfrG, catE223 |
| C3997      | S. sciuri              | P-OX-FOX-FOX-FOX-NN-K-C-CIP-SXT | mecA, blaZ, aacA/aphD, aphA3, catE223 |
| C4022      | S. lentus              | P-OX-FOX-S-T-CIP-FUS  | mecA, str, tet(K), tet(M) |
| C3998      | S. sciuri              | P-OX-FOX-S-T-FUS      | mecA, tet(K)             |
| C4028      | S. sciuri              | P-OX-FOX-E-CC-GM-NN-K-S-T-CIP-FUS | mecA, erm(B), aacA/aphD, aphA3, str, tet(K), tet(M) |

aOX, oxacillin; FOX, cefoxitin; P, penicillin; FUS, fusidic acid; T, tetracycline; S, streptomycin; CC, clindamycin; CIP, ciprofloxacin; SXT, sulphamethoxazole-trimethoprim; E, erythromycin; K, kanamycin; GM, gentamycin; NN, tobramycin; C, chloramphenicol; VAN, vancomycin; LIN, linezolid; MUP, mupirocin.

bCCind. Inducible clindamycin resistance.

†Genes proved to be physically linked.
sistance to other classes of antimicrobial agents, particu-
larly tetracycline, aminoglycosides, fluoroquinolones and
macrolides. Similar findings (except fluoroquinolones)
have been reported for CoNS from dogs in Nsukka (Chah
et al., 2014). As has been pointed out by Aslantas et al.
(2013), MRCoNS could pose a major therapeutic challenge
for veterinarians due to a limited choice of antimicrobial
agents in the case of infection. A high diversity of anti-
microbial resistance profiles was observed in this study, for
the 20 MRCoNS strains exhibited 16 resistance patterns.
This finding suggests the involvement of antimicrobial se-
lective pressure in the pig farms.

Antimicrobial resistance in these MDR MRCoNS
was mediated by multiple antimicrobial resistance genes.
This observation is similar to that reported for MRCoNS
isolated from dogs in Nsukka, Nigeria (Chah et al., 2014)
and Hatay, Turkey (Aslantas et al., 2013). Although the de-
gree of importance of MDR MRCoNS in veterinary medi-
cine is not clearly understood, they may represent a reser-
voir of resistance genes which can be spread to pathogenic
bacteria within and across species and genera. These MDR
staphylococci, therefore, represent a public health risk as
they may transfer their resistance genes to human patho-
gens such as S. aureus. The presence of these MDR bacteria
in pigs may be due to the indiscriminate use of antimicro-
bial agents in pig production in Nigeria. The fact that the
MRS strains harbor several resistance genes against the
same antimicrobial agent indicates that the strains are capa-
bles of acquiring and maintaining multiple resistance genes
at the same time. A possible adaptive advantage of carrying
these redundant genes is unknown yet.

Detection of the resistance gene cluster tet(L)-dfrK in
S. lentus and S. cohnii is a novel observation. A first de-
scription of the trimethoprim resistance gene dfrK located
next to the tetracycline resistance gene tet(L) in plasmid
pKK52187 from a MRSA ST398 isolate of pig origin was
reported in Germany (Kadlec and Schwarz, 2009). The
presence of this gene cluster in MRCoNS isolates from
swine in Nigeria indicates a broad geographic distribution
of both resistance determinants. The use of tetracyclines
and trimethoprim in pig farming in Nigeria may favor the
spread and maintenance of their resistance genes.

All 13 quinolone-resistant MRCoNS strains harbored
the Ser84Leu substitution in the GyrA protein. To our
knowledge, this is first report of this mutation in S. sciuri, S.
lentus and S. cohnii, suggesting that quinolone resistance
in these bacterial species is commonly associated with this
mutation. Until now, this type of mutation has been most
frequently found in S. aureus, S. pseudintermedius, S.
epidermidis and S. haemolyticus (Sreedharan et al., 1991;
Yonezawa et al., 1996; Takahata et al., 1997; Gómez-Sanz
et al., 2013). The majority of the strains carried a single
amino acid substitution in the same region in the represen-
tative quinolone-susceptible S. aureus, S. pseudintermedius and S. epidermidis strains, while the repre-
sentative S. haemolyticus strain exhibited two additional
substitutions. Interestingly, analysis of the QRDR nucleo-
tide sequences revealed relatively high intra-species diver-
gence in this region, with S. pseudintermedius clustered
farthest away from the others. These results broaden the
current knowledge on the quinolone resistance mechanisms
among different CoNS species.

This study demonstrated the presence of mannitol-
positive MRCoNS in intensively reared pigs in the Nsukka
agricultural zone, Nigeria, with S. sciuri being the predomi-
nant species. This is the first report on MRS in pigs in Nige-
ria. The study highlighted the fact that the CoNS strains
were MDR and, therefore, could serve as a pool of resis-
tance genes for pathogenic bacteria. Methicillin-resistant S.
aureus which is frequently reported in pigs in other coun-
tries was not detected in this study. Our study provides a
new insight into bacterial resistance mechanisms in
MRCoNS from pigs, with special attention to quinolone re-
sistance.

Acknowledgments

The molecular analysis was financially supported by the
grant awarded to Ugwu, Chidozie Clifford by Universi-
dad de La Rioja, Logroño, Spain. This work was par-
tially supported by Project SAF2012-35474 from the
Ministerio de Economía y Competitividad of Spain and the
Fondo Europeo de Desarrollo Regional (FEDER).

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*Associate Editor: Nilton Erbet Lincopan Huenuman*

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