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Research Paper

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

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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 β-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*, *dfr*G, cat_{pC221}, and cat_{pC223}. 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 β-lactam antibiotics, are commonly resistant to other classes of antimicrobial agents

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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 MRS 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. Stocked 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 subcultured on brain heart infusion (BHI) agar (Oxoid). Cultures on BHI agar were tested for coagulase and DNAse activity. Oxacillin/methicillin-resistant mannitol-positive isolates 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), trimethoprim/sulphamethoxazole (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 Societé 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'-TTAAATGAACAAGGTATGAC-3') and gyrA_539-fw (5'-GCCATACCTACCGCGATACC-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-E-CC_{ind}-S-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 cat_{p221} or cat_{p223} genes were detected in the chloramphenicol-resistant strains. Of the 12 sulphamethoxazole/trimethoprim-resistant strains, 9 were positive for *dfr*G 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 *dfr*K-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.

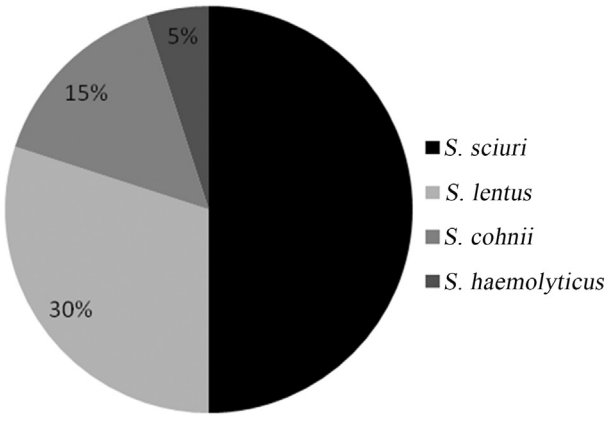

Figure 1 - Percentage distribution of mannitol-positive MRCoNS species from pigs in Nsukka agricultural zone, Nigeria.

Table 1 - Antimicrobial resistance profile of mannitol-positive MRCoNS from pigs in Nsukka Agricultural Zone, Nigeria (n = 20).

Antimicrobial agent (potency)	No. (%) of resistant isolates
Oxacillin (1 μg)	20 (100)
Cefoxitin (30 μg)	20 (100)
Penicillin (10 units)	20 (100)
Fusidic acid (10 μg)	17 (85)
Tetracycline (30 μg)	15 (75)
Streptomycin (10 μg)	13 (65)
Ciprofloxacin (5 μg)	13 (65)
Trimethoprim/Sulphamethoxazole (25 μg)	12 (60)
Clindamycin (2 μg)	10 (50)
Erythromycin (15 μg)	10 (50)
Gentamicin (10 μg)	9 (45)
Kanamycin (30 μg)	9 (45)
Tobramycin (10 μg)	4 (20)
Chloramphenicol (30 μg)	3 (12.5)
Mupirocin (200 μg)	0 (0.0)
Linezolid (30 μg)	0 (0.0)

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

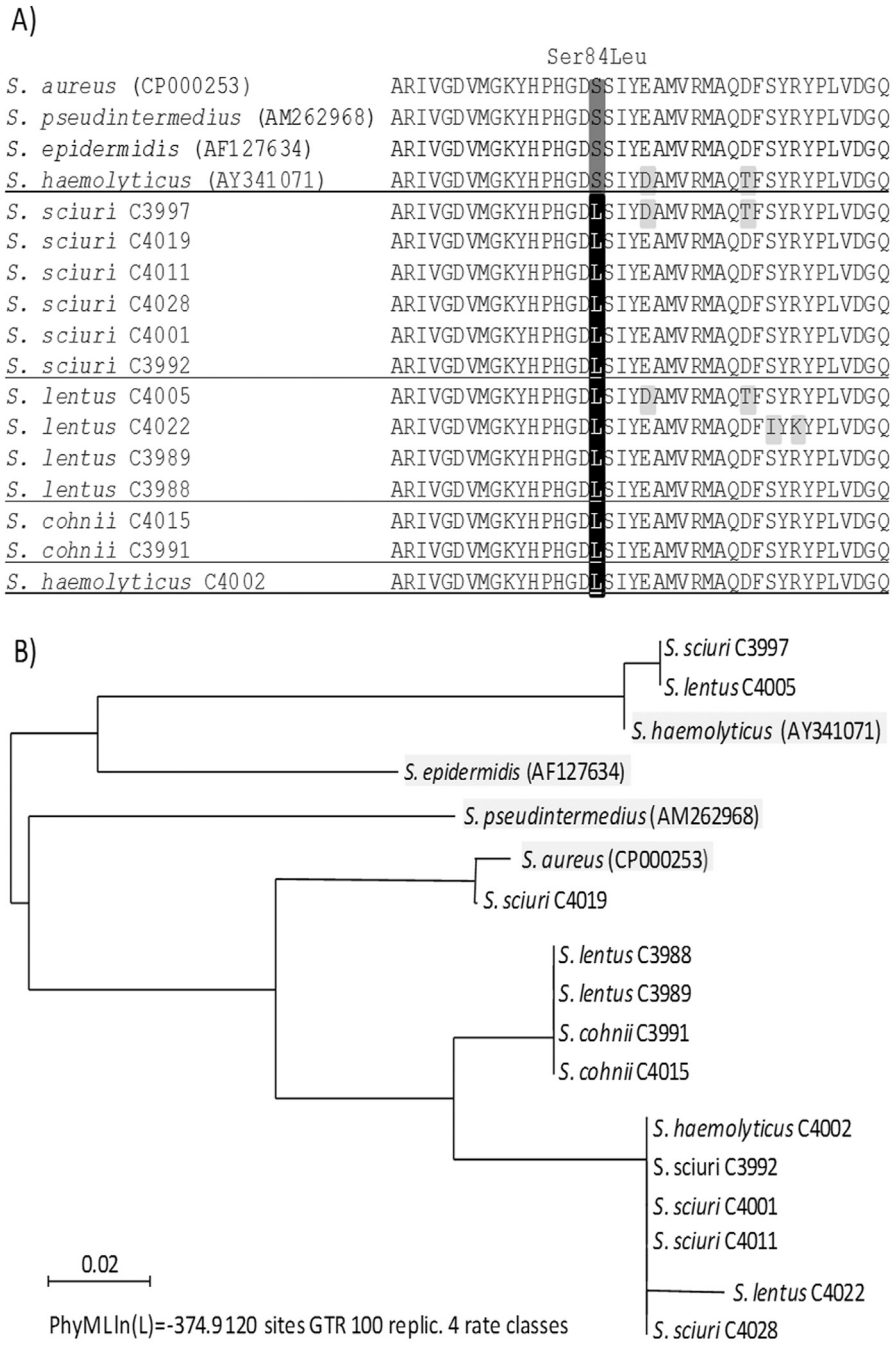

Figure 2 - Molecular evaluation of the quinolone resistance determining region (QRDR) of 13 quinolone- and methicillin-resistant staphylococci isolated from healthy swine in Nsukka, Nigeria, in addition to the QRDR of representative quinolone-susceptible *S. aureus* (Genbank accession no. CP000253), *S. pseudintermedius* (AM262968), *S. epidermidis* (AF127634) and *S. haemolyticus* (AY341071). A) Amino acid sequence comparison of the QRDR of GyrA protein (aa 68-107). B) Phylogenetic relationships among the QRDR nucleotide sequences in *gyrA* gene (nt 202-321).

Table 2 - Species and antimicrobial resistant pheno- and genotypes of mannitol-positive methicillin-resistant coagulase-negative staphylococci (MRCoNS) isolated from pigs in Nsukka, Nigeria.

Strain No.	*Staphylococcus* species	Resistance phenotype[a]	Resistance genes detected
C4019	*S. sciuri*	P-OX-FOX-E-CC_{ind}[b]-S-T-SXT-CIP-FUS	mecA, *erm*(C), *str*, *tet*(M), *dfr*G
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), cat_{pC221}
C3985	*S. sciuri*	P-OX-FOX-E-CC-GM-K-S-T-SXT-FUS	mecA, *erm*(C), *aacA/aphD*, *str*, *tet*(K), *tet*(M) *dfr*G
C3986	*S. lentus*	P-OX-FOX-S-T-SXT-FUS	mecA, *str*, *dfr*G
C4011	*S. sciuri*	P-OX-FOX-E-CC_{ind}-S-T-SXT-CIP-FUS	mecA, *erm*(C), *str*, *tet*(M), *dfr*G
C4012	*S. cohnii*	P-OX-FOX-E-CC_{ind}-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*][c]
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*][c]
C3991	*S. cohnii*	P-OX-FOX-CC_{ind}-CIP-FUS	mecA
C3992	*S. sciuri*	P-OX-FOX-GM-NN-K-CIP-FUS	mecA, *blaZ, aacA/aphD*
C4015	*S. cohnii*	P-OX-FOX-E-CC-GM-K-S-T-SXT-CIP-FUS	mecA, *erm*(B), *aacA/aphD*, *str*, *tet*(K), *tet*(M), [*tet*(L)-*dfrK*][c]
C4024	*S. sciuri*	P-OX-FOX-T-FUS	mecA, *tet*(K)
C4001	*S. sciuri*	P-OX-FOX-E-CC_{ind}-S-T-SXT-CIP-FUS	mecA, *erm*(C), *str*, *tet*(M), *dfr*G
C4002	*S. haemolyticus*	P-OX-FOX-E-CC_{ind}-S-T-SXT-CIP-FUS	mecA, *erm*(C), *str*, *tet*(M), *dfr*G
C4005	*S. lentus*	P-OX-FOX-GM-K-C-CIP-SXT	mecA, *blaZ*, *aacA/aphD, aphA3, dfr*G, cat_{pC223}
C3997	*S. sciuri*	P-OX-FOX-GM-K-C-CIP	mecA, blaZ, aacA/aphD, aphA3, cat_{pC223}
C4022	*S. lentus*	P-OX-FOX-S-T-CIP-FUS	mecA, *str*, *tet*(K), *tet*(M)
C3998	*S. sciuri*	P-OX-FOX-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)

[a]OX, oxacillin; FOX, cefoxitin; P, penicillin; FUS, fusidic acid; T, tetracycline; S, streptomycin; CC, clindamycin; CIP, ciprofloxacin; SXT, sulphamethoxazole-trimethoprin; E, erythromycin; K, kanamycin; GM, gentamycin; NN, tobramycin; C, chloramphenicol; VAN, vancomycin; LIN, linezolid; MUP, mupirocin.
[b]CC_{ind}, Inducible clindamycin resistance.
[c]Genes proved to be physically link.

mannitol-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; Lin *et al.*, 2011; Larsen *et al.*, 2012; Oppliger *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.*, 2007). This species has also been associated with endocarditis (Wallet *et al.*, 2000), urinary tract infections (Stepanovic *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-

sistance to other classes of antimicrobial agents, particularly 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 antimicrobial resistance profiles was observed in this study, for the 20 MRCoNS strains exhibited 16 resistance patterns. This finding suggests the involvement of antimicrobial selective 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 degree of importance of MDR MRCoNS in veterinary medicine is not clearly understood, they may represent a reservoir 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 pathogens such as *S. aureus*. The presence of these MDR bacteria in pigs may be due to the indiscriminate use of antimicrobial 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 capable 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 description of the trimethoprim resistance gene *dfrK* located next to the tetracycline resistance gene *tet*(L) in plasmid pKKS2187 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 representative quinolone-susceptible *S. aureus*, *S. pseudintermedius* and *S. epidermidis* strains, while the representative *S. haemolyticus* strain exhibited two additional substitutions. Interestingly, analysis of the QRDR nucleotide sequences revealed relatively high intra-species divergence 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 predominant species. This is the first report on MRS in pigs in Nigeria. The study highlighted the fact that the CoNS strains were MDR and, therefore, could serve as a pool of resistance genes for pathogenic bacteria. Methicillin-resistant *S. aureus* which is frequently reported in pigs in other countries 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 resistance.

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