Assessment of Methicillin Resistance and Toxin Associated Genes in Staphylococcus Species Isolated 
(Case Study of Two Selected Pig Farm in Ogun State)

Ogunsanmi, O.O.¹, Ezeamagu, L.² & Balogun, O.B.³

¹Ogunsanmi, O.O., ²Ezeamagu, L. & ³Balogun, O.B.
Department of Bioscience and Biotechnology, Babcock University Ilishan Remo University
Department of Biological Sciences, Joseph Ayo Babalola University
Nigeria

Abstract: Swine especially pigs have been reported to harbor methicillin-resistant Staphylococcus species and have become a source of a novel and rapidly emerging infection in humans. This study was therefore, designed to investigate methicillin resistance status, susceptibility and exfoliative toxin-encoded genes in Staphylococcus species isolated from pigs. Hundred and fifty (150) samples consisting of 50 anal, nostril and environmental swabs were collected at Ode Remo and Sapade in Ogun state after obtaining ethical clearance. These were transferred into transport medium and transported to Microbiology laboratory of Babcock University. The samples were processed and organisms isolated following Microbiological procedures. The phenotypic detection of methicillin resistance and susceptibility of the isolates to selected antibiotic classes were evaluated by agar diffusion and interpreted according to CLSI, 20011. The data were analyzed by descriptive statistics (frequency). Fifty (50) staphylococcal strains were isolated from anus (28), nostril (17) and environment (5) of which Staphylococcus sciuri (23), Staphylococcus cohnii (11), Staphylococcus piscifermentas (7), Staphylococcus carnosus (1), Staphylococcus condiment (3), Staphylococcus xylosus (2), Staphylococcus Kloosii (1), Staphylococcus pasteuri (1) and Staphylococcus succinus (1). Methicillin resistance was detected in 12 strains S. xylosus (1), S. kloosii (1), S. picifermentas (2) and S. sciuri (8) with phenotypic method while none of the strains were positive by molecular counterpart. Susceptibility to other antibiotics indicated that all the strains were resistant to cefazidimeS. sciuri (23), S. cohnii (11), S. piscifermentas (7), S. carnosus (1), S. condimenti (3), S. xylosus (2), S. kloosii (1), S. pasteuri (1), and S. succinus (1). All the strains were declared negative for exfoliative toxin encoding genes after several trails in PCR. Methicillin resistance is absent among the strains studied and the resistance patterns observed indicated that the pattern of resistance predominantly found in clinical isolates are also emerging in the animal husbandry. Hence, setting up antibiotic surveillance system is necessary to minimize this trend.

Keywords: Staphylococci, Exfoliative Toxin & Methicillin.
1. Introduction

*Staphylococcus aureus* is a Gram-positive spherical bacterium approximately 1 μm in diameter. Its cells form grape-like clusters, since cell division takes place in more than one plane (Aarestrup et al., 1995). It is often found as a commensal associated with skin, skin glands, and mucous membranes, particularly in the nose of healthy individuals (Crossley and Archer, 1997). *Staphylococcus* sp. is catalase-positive, a feature differentiating them from *Streptococcus* sp., and they are oxidase-negative and require complex nutrients. *Staphylococci* are facultative anaerobes capable of generating energy by aerobic respiration and by fermentation which yields mainly lactic acid. *S. aureus* has the ability to adapt to different environments and it may colonize the human skin, nails, nares and mucus membranes and may thereby disseminate among recipient host populations via physical contact and aerosols (Abbott, 2010). Colonization with *S. aureus* is an important risk factor for subsequent *S. aureus* infection (Chait and Kent 1992; von Chavakis et al., 2007). The cell wall of *S. aureus* is a tough protective coat, which is relatively amorphous in appearance, about 20-40 nm thick (Adesiyun, 1991). Underneath the cell wall is the cytoplasm that is enclosed by the cytoplasmic membrane. Peptidoglycan is the basic component of the cell wall, and makes up 50% of the cell wall mass (Nemati et al., 2013).

Livestock-associated methicillin resistance *Staphylococcus aureus* (LA-MRSA) has become a public health concern since its emergence in both animals and the general population. Since its first appearance in Netherlands in 2004, it has been reported in Canada, United States, North America, France, (Novick et al., 2001). Animal associated methicillin resistance is not only limited to *Staphylococcus aureus*, but also to other coagulase negative staphylococci. In 2005, methicillin-resistance prevalence in *S. intermedius* and *S. schleiferi* isolates in 2005 were 15.6 % and 46.6 %, respectively (Nema et al., 2007). Similarly, there has been documentation of methicillin resistance in *S. pseudintermedius* (MRSP) and methicillin-resistant *S. intermedius* group (MRSIG) from different European countries and North America with *S. pseudintermedius* ST71 becoming the most prevalent in Europe (Saroglou, 1980). *Staphylococcus aureus* is considered to be a major pathogen that colonises and infects both hospitalised patients with decreased immunity, and healthy immuno-competent people in the community (O’Riordan and Lee, 2004). This bacterium is found naturally on the skin and in the nasopharynx of the body. It can cause local infections of the skin, nose, urethra, vagina and gastrointestinal tract, most of which are minor and not life-threatening (Ryffel, 1992). In pyogenic infections the pathogenesis results from the combined action of a variety of factors. Infection is initiated when a breach of the skin or mucosal barrier allows *Staphylococci* access to adjacent tissues or to the bloodstream. Whether an infection is contained or spreads depends on a complex interplay between *S. aureus* virulence determinants and host defense mechanisms. The skin and mucous membrane are excellent barriers against local tissue invasion by *S. aureus*. However, if either of these is breached due to trauma or surgery, *S. aureus* can enter the underlying tissue, creating its characteristic local abscess lesion and if it reaches the lymphatic channels or blood can cause septicaemia (Chambers and Deleo, 2009).

Methicillin resistance *Staphylococcus aureus* in animal has been reported in general population without risk factors. Among all livestock, swine seem to be the major reservoir for MRSA (Coimbra et al., 2011). In 2005, a high prevalence of a new livestock-associated MRSA, ST398, was reported in pigs at Dutch slaughterhouses (Choi, et al., 2000). Information from EU on MRSA in swine holdings in 26 countries indicated that 14% of breeding and 27% of production herds were positive for MRSA (Doyle et al., 2011). Highest prevalence of MRSA was recorded in Spain, Germany, Belgium, and Italy. LAMRSA (ST398) accounted for 92.5% of isolates tested (Foster, 2005). Since pigs constitute a separate MRSA reservoir and have become a source of a novel and rapidly emerging type of MRSA in humans, the detection of methicillin resistance in *S. aureus* and other coagulase negative *staphylococci* will help in public surveillance and therefore form baseline information for epidemiological purposes (Fuda, et al 2006). The basic objective is to screen for methicillin resistance in the isolates by both phenotypic and genotypic methods and to determine the antibiogram against selected antibiotic classes and could provide information on the prevalence of methicillin resistance in *Staphylococci* isolates from pig and its environment in Ogun State.
2. Material and Methods

2.1 Sample Collection

Samples were collected using sterile swab sticks from nostrils and anus of pigs and the environment pigs at Ode Remo and Sapade in Ogun state.

2.2 Preparation of Media

Mannitol Salt agar which is a selective medium were used in the isolation of the *Staphylococcus aureus* from the samples. The media were prepared according to the manufacturer’s specifications. The media were homogenized and autoclaved at 121°C for 15 minutes to sterilize media. After sterilization the media were allowed to cool before dispensing into petri dishes. The petri dishes were kept in refrigerator until when needed. Isolates were sub-cultured onto Msa and incubated at 37°C. Discrete colonies of each isolate were stored in eppendorf tubes. The eppendorf tubes contained tryptone soya broth for preservation of the isolates.

2.3 Isolation procedure

All specimens and samples on swab sticks were streaked on prepared agar plates and incubated at 37°C for 18-24 hrs. Colonies showing yellow were then be picked and sub-cultured on Mannitol sat agar and incubated at 37°C. Morphologically distinct colonies were sub-cultured on fresh plates. Pure strains obtained were stored on nutrient agar slants at 4°C for subsequent study.

2.4 Preliminary Identification of Isolate

Identification of isolates was by standard microbiological procedures.

2.5 Gram Staining

A drop or a loop full of water were placed on a well labeled clean grease-free glass slide using a sterile inoculating loop; a colony of a culture of the bacterial isolates was emulsified with the water to make a thin smear. The smear were then air dried and then heat fixed. The slides were flooded with crystal violet stain which is the primary stain for 60 seconds after which it was rinsed with water. The smear were flooded with Lugol’s iodine which is the mordant to fix the primary stain after 60 seconds the iodine was rinsed off. The slide was then flooded with acetone which is the decolourizer and then rinsed off after 5 seconds. Safranin red which is the counter stain was added and rinsed off after 60 seconds. The stained were air-dried, and then observed under the microscope using X100 oil immersion objective lens of the microscope. A cluster of purple colonies was indicative of staphylococci was viewed (Kloos and bannerman, 1994).

2.6 Methicillin resistance

Phenotypic detection of methicillin resistance indicated that only 12 strains of three species { (S. xylosus (1), S. kloosii (1) S. piscifermentas (2) and S. sciuri (8)) were resistant to cefoxitin (Table 1). However, molecular identification revealed that none of the strains studied were methicillin resistant. In this study, there was variability in the performance of disc diffusion using the three acceptable phenotypic test disc (cefoxitin) when compared to molecular method. The variability in the performance of disc diffusion is attributable to the heterogeneous nature of phenotypic expression of resistance in isolates. In a number of studies, sensitivity and specificity of disc diffusion method have been reported between 61.3 - 100 % and 50 - 99.1 % respectively (Kobayashi et al., 1998, Kadlec., et al., 2009). In addition to above factor, other attributable factors like growth conditions (Kahl et al., 1998; Schad et al., 1995), salt concentrations (Milne et al., 1993) inoculums size (Sinsimer, et al., 2005), medium compositions (Ogawa et al., 1981) might have had considerable effects on the susceptibility test. In this study, incessant power supply during the course of susceptibility testing might have also contributed significantly to the variability of the diffusion test. This is because steady incubation temperature was hardly maintained and this could have caused the organisms to grow below its optimum temperature; thus affecting its metabolism. Recent
work has also shown that the susceptibility to antibiotics is highly dependent on the bacterial metabolism and that global metabolic regulators can modulate this phenotype (Morein et al., 2011). This modulation includes situations in which bacteria can be more resistant or more susceptible to antibiotics. Understanding these processes will thus help in establishing novel therapeutic approaches based on the actual susceptibility shown by bacteria during infection, which might differ from that determined in the laboratory.

2.7 Susceptibility

Antibiograms of isolates revealed various patterns to different antibiotics examined. All of the strains were resistant to ceftazidime (Staphylococcus sciuri (23), Staphylococcus cohnii (11), Staphylococcus piscifermentas (7), Staphylococcus carnosus (1), Staphylococcus condimenti (3), Staphylococcus xylosus (2), Staphylococcus kloosii (1), Staphylococcus pasteuri (1), and Staphylococcus succinus (1). Staphylococcus pasteuri (19) and Staphylococcus cohnii (7) were resistant to cefuroxime while Staphylococcus sciuri (23), Staphylococcus cohnii (10) and Staphylococcus piscifermentas (7) were resistant to gentamicin. Also, Staphylococcus sciuri (22), Staphylococcus cohnii (10), Staphylococcus piscifermentas (5), were resistant toceftiraxone. Staphylococcus sciuri (18), Staphylococcus cohnii (10) and Staphylococcus piscifermentas (7) were resistant to erythromycin. Staphylococcus sciuri (17), Staphylococcus cohnii (7), and Staphylococcus piscifermentas (5) were resistant to ofloxacin while Staphylococcus sciuri (16), Staphylococcus cohnii (9), and Staphylococcus piscifermentas (5) showed resistance to augmentin. Staphylococcus sciuri (2), Staphylococcus carnosus (1) and Staphylococcus piscifermentas (1) were resistant to Vancomycin but Staphylococcus condimenti, Staphylococcus xylosus, Staphylococcus succinus, Staphylococcus cohnii, Staphylococcus pasteuri all susceptible. Staphylococcus sciuri (8) and Staphylococcus piscifermentas (2) showed resistance to vancomycin (Table 1).

2.8 Detection of toxin genes

All the staphylococcal isolates were screened for only eta and etb genes by multiplex PCR. However, none of the strains were positive for these genes. The tsst gene was not assayed because S. aureus was not isolated in this study.

2.9 Status of virulent genes in Staphylococcus strains

Identification of the pathogens causing diseases and understanding its resistance pattern have been helpful in the selection of empirical antimicrobial therapy and in infection control measures in health institutions. Identification of staphylococci in many clinical and community settings is often limited to S. aureus, while non- S. aureus isolates are simply reported as coagulase negative staphylococci (CNS). However, a large number of CNS strains recovered from clinical, animal and community based samples have become a serious problem as some of them have been reported to express methicillin resistant gene, which involves all lactam antibiotics and leads to significant limitation of therapeutic options (Loeffler et al., 2007; Kadlec et al., 2010). Particular species of CNS are also associated with distinct types of infections and patterns of antimicrobial susceptibility (Štetina et al., 2005). Therefore, species identification of CNS is increasingly of clinical and epidemiological interest to clinicians. Of the nine species identified, four (S. sciuri, S. xylosus, S. kloosii and S. cohnii) of these have been implicated as pathogens in human infection (Song et al., 1987). The presence of non-motile, non-spore forming Staphylococcus piscifermentans is rather unique as this strain is newly recognized species. This coagulase negative species is phylogenetically and biochemically most closely related to Staphylococcus carnosus subsp. carnosus, Staphylococcus carnosus subsp. uteis and Staphylococcus condiment (Schleifer and Fischer, 1982; Stegger et al., 2012). This strain was also found in healthy dog feces, which was believed to have originated from fermented food residue presented in dog feed (Štetina et al., 2005). Nevertheless, there has been very little report on this species in pigs.

In many clinical laboratories, CNS are not identified to species level and, in most cases, identification of clinically significant CNS is carried out through conventional methods and commercial
identification kits. However, correct identification of all clinical isolates of CNS is not easy, because the biochemical traits of the species are similar and many clinical isolates show intermediate traits (Sundstrom et al., 2006). MALDI-TOF mass spectrometry is a recognized method for both typing and identification of various bacterial species including the staphylococci and has been applied successfully to the identification of CNS to the species level (Kluytmans-Vandenbergh and Kluytmans, 2006; Foster, 2005). This simple, rapid and cost effective assay might provide a potential tool, especially in hospital laboratories, for the full and accurate identification of CNS and thus help to understand better the epidemiology of CNS. Recent reports of misidentification of CNS species by API STAPH (Foster and Hook 1998; Choi, et al., 2006) underlines the importance of molecular methods in the accurate identification and characterization of CNS.

Table 4.1: Resistance patterns of *Staphylococcus* species to different antibiotic classes

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>S. piscifermentas</th>
<th>S. carnosus</th>
<th>S. sciuri</th>
<th>S. condimenti</th>
<th>S. xylosus</th>
<th>S. kloosii</th>
<th>S. succinus</th>
<th>S. cohnii</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEFTAZIDIME 30µg</td>
<td>7</td>
<td>1</td>
<td>23</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td>CEFUROXOME 30µg</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>GENTAMICIN 30µg</td>
<td>7</td>
<td>1</td>
<td>23</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>CEFTRIAXINE 30µg</td>
<td>5</td>
<td>1</td>
<td>22</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>ERYTEROMYCIN 30µg</td>
<td>7</td>
<td>1</td>
<td>18</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>COXACILLIN 5µg</td>
<td>6</td>
<td>1</td>
<td>13</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>OFLOXACIN 5µg</td>
<td>5</td>
<td>1</td>
<td>17</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>AUGMENTIN 30µg</td>
<td>5</td>
<td>1</td>
<td>16</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>VANCOMYCIN 30µg</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 4.2: The summary of preliminary and genotypic tests performed on the *Staphylococcus*

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Gram reaction</th>
<th>Catalase</th>
<th>16SrRNA</th>
<th>Coagulase</th>
<th>eta</th>
<th>etb</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus sciuri</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Staphylococcus cohnii</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Staphylococcus piscifermentas</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Staphylococcus carnosus</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Staphylococcus condimenti</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Staphylococcus xylosus</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Staphylococcus kloosii</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Staphylococcus pasteuri</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Staphylococcus succinus</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

3. Conclusion

Methicillin resistance is absent among the strains studied and the resistance patterns observed indicated that the pattern of resistance predominantly found in clinical isolates are also emerging in the animal husbandry. Hence, setting up antibiotic surveillance system is necessary to minimize this trend. Swine especially pigs have been reported to harbor methicillin-resistant *Staphylococcus* species and have become a source of a novel and rapidly emerging infection in humans. *Staphylococcus* species are Gram-
positive organisms that in the laboratory may be observed as single cells, in pairs or as grape-like irregular clusters, they are catalase positive, non-motile, non-spore-forming and as facultative anaerobes.

Hundred and fifty (150) samples consisting of 50 anal, nostril and environmental swabs were collected at Ode Remo and Sapade in Ogun state. These were transferred into transport medium and transported to Microbiology laboratory of Babcock University. The samples were processed and organisms isolated following Microbiological procedures.

References


