Molecular Identification and Diversity of Staphylococcus Species Isolated
(Case from Two Selected Pig Farms in Ogun State)

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Abstract: Staphylococcus aureus is a major pathogen of increasing importance due to the rise in antibiotic resistance. This study was therefore, designed to investigate molecular identification of Staphylococcus species and their diversity 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. Microbiological analysis was carried out and the isolates were identified to species level by Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry. 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. piscifermentas (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 ceftazidime S. 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, Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry, Polymerase Chain Reaction.
1. Introduction

*Staphylococcus aureus* belongs to the family Micrococccaceae and is part of the genus *Staphylococcus*, which contains more than 30 species such as *S. epidermidis, S. saprophyticus* and *S. haemolyticus*. Among the staphylococcal species, *S. aureus* is by far the most virulent and pathogenic for humans. *Staphylococcus aureus* is a 1 μm, Gram-positive cell that in the laboratory may be observed as single cells, in pairs or as grape-like irregular clusters (Yamasaki *et al*., 2005). It is characterized as coagulase, positive and oxidase-negative, non-motile, non-spore-forming and as facultative anaerobic chemoorganotrophic coccis with a respiratory and fermentative metabolism at an optimal temperature of 37°C. These organisms are resistant to adverse environmental conditions and can be recovered from non-physiological environments even months after inoculation (Balaban, and Rasooly, 2000).

*Staphylococcus aureus* grows in yellow colonies on nutrient rich media and is referred to as the yellow staphylococci (Winn, 2006). Most species have a relative complex nutritional requirement; however, in general they require an organic source of nitrogen, supplied by 5 to 12 essential amino acids (Antonsson *et al*., 2007; Wilkinson, 1997). Members of this genus are catalase-positive, distinguishing them from the genus *streptococci*, which are catalase-negative, and have a different cell wall composition to *staphylococci* (Wilkinson, 1997). *Staphylococci* are tolerant to high concentrations of salt (Wilkinson, 1997) and show resistance to heat (Kloos and Lambe, 1991). Pathogenic *staphylococci* are commonly identified by their ability to produce coagulase, and thus clot blood (Kloos and Musselwhite, 1975).

*Staphylococcus aureus* is a major pathogen of increasing importance due to the rise in antibiotic resistance (Al-Daccak *et al*., 1998). It is a highly versatile and adaptable pathogen, causing a range of infections of varying severity affecting the skin, soft tissue, respiratory system, bone, joints and endovascular tissues (Basaglia *et al*., 2003). The organism also exists as a commensal, colonizing the anterior nares of about one third of the healthy human population. (Yamasaki *et al*., 2005). *Staphylococcus aureus* causes a wide range of infections from a variety of skin, wound and deep tissue infections to more life-threatening conditions such as pneumonia, endocarditis, septic arthritis and septicemia (Antonsson *et al*., 2007). This bacterium is also one of the most common species in nosocomial infections (Becker *et al*., 2004). However, *S. aureus* may also cause food poisoning, scalded-skin syndrome and toxic shock syndrome, through production of different toxins (Winn, 2006). *Staphylococcus aureus* strains that are capable of causing diseases which express different virulence factors, such as exotoxins, which are molecules on the cell surface associated with adherence and with resistance to various antimicrobials, in addition to enterotoxins, which are extracellular proteins with superantigenic activity (Basaglia *et al*., 2003; Zacconi and Scali. 2013). All these virulence factors contribute to the pathogenicity of such microorganisms (Balaban and Rasooly, 2000). Toxins are poisonous substances produced within living cells or organisms, synthetic toxicants created by artificial processes are thus excluded (Ubukata *et al*., 1989; Voss. and Doebbeling 1995). The term was first used by organic chemist Ludwig Brieger. Toxins can be small molecules, peptides, or proteins that are capable of causing disease on contact with or absorption by body tissues interacting with biological macromolecules such as enzymes or cellular receptors (Kloos. *et al*., 1997; Van Loo, 2007). Toxins vary greatly in their severity, ranging from usually minor to almost immediately deadly. They are non-infectious, non-contagious, and non-curable by antibiotics or chemotherapeutic agents. However, individuals can be protected by vaccination (Udoet *et al*., 2000). The multifactorial nature of virulence of toxin and toxin producers, produces comparative and cooperative pathogenesis, and this makes studies all the more difficult. Antibody raised against all components helps in this pursuit (Van Duijkeren *et al*., 2004).

The study is to isolate *Staphylococcus* species from pigs and identify to species level using Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDITOF-MAS) technique and also to assess the presence of toxin-producing genes (*ETA, ETB and TSST*) in these isolates using simplex and multiple PCR.
2. Materials 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 Msaand 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 (Vosset et al., 2005).

2.6 Maldi-tof mass spectrometry

Direct colony method was used. The bacteria was applied as a thin film onto a 24-spot steel plate and allow to dry at room temperature. Subsequently, 2µl of MALDI matrix (a saturated solution of -cyano-4-hydroxycinnamic acid) in 50% acetonitrile and 2.5% trifluoroacetic acid) were applied onto the colony and allow to dry before testing. For each plate, a bacterial test standard were included to calibrate the instrument and validate the run. MALDI-TOF MS was performed with the Microflex LT mass spectrometer according to the manufacturer’s instructions. Spectra was analyzed by using MALDI Biotyper automation control and the BrukerBiotyper 2.0 software and library (version 2.0, 3,740 entries). Identification score criteria was used as recommended by the manufacturer: a score of ≥2.000 indicates species-level identification, a score of 1.700 to 1.999 indicates identification to the genus level, and a score of ≤1.700 was interpret as no identification. Isolates that failed to produce a score of 1.700 with direct colony was retested (Fitzgerald, et al., 1993).

2.7 Extraction of DNA by Boiling Method

DNA from sample was prepared by boiling. The samples was centrifuged at 15,000 × g for 15 min. The supernatant was eliminated, and the pellet was resuspended in sterilized distilled water and centrifuged
at 15,000 × g for 10 min. The supernatant was discarded, and the pellet was resuspended in 40 µl of sterilized distilled water, subjected to boiling at 100°C in a heating block for 10 min, and centrifuged at 15,000 × g for 10 s. The supernatant was then transferred into a fresh eppendorf tubes before it was stored at -20°C. Aliquots of 2 µl of template DNA were used for PCR (Zell et al., 2008).

2.7 Purity Determination

The purity of the DNA was checked using an automated Nanodrop spectrophotometer (3300) connected to a computer. This procedure involved blanking the machine first with sterile distilled water which has optical density of zero. Subsequently, 20µl of the extracted DNA samples was transferred into the optical sensitive aperture of the machine (loading chamber). The machine arithmetically gave the required purity as well as the quantities of each DNA per micro liter. Samples with optical density in the range of 1.40- 2.0 were used for PCR reactions. However, DNA samples of all isolates below this range of optical density was repeated (Zell et al., 2008).

2.8 Preparation of Oligonucleotide Primer Solutions

All primers used were synthesized by Zigma-Aldrich, Germany. In the laminar flow hood, solutions of the oligonucleotide primers (µM) was prepared by multiplying the concentration of the dried oligonucleotide de primers in nanomole of each forward and reverse primer (given by manufacturer in the primer data sheet) by ten. The products gave an equivalent of micro-litre of sterilized double distilled (molecular biology grade water) required to 100µl solution. After mixing the equivalent quantity of water, the solution was mixed on a vortex and centrifuged at 12000rpm for 5minutes at 4°C. This were the stock solution which was incubated 30minutes on ice. To avoid repeated thawing, 50µl (working solution) was taken from the stocked for PCR reactions while the stocked were kept in freezer at -20°C (Garcia et al., 2008).

2.9 PCR amplification of gene of Staphylococcus sp

An aliquot of 2.0µl of DNA suspension was added to make up 25µl of PCR mixture consisting of 5µl standard reaction buffer (20mMTris-HCl, 1.8mM MgCl₂, 22mM NH₄Cl, 22mM KC1, 0.06% IGEPL CA-630, 0.05% Tween 20, pH 8.9), a 0.5µl (10mM) concentration of each of the four deoxyribonucleoside triphosphates (dATP, dCTP, dGTP, and dTTP), 0.25 µl of TsaG422 as forward primer and Tsag765 as the reverse primer, 16.875 of water and 0.125 U of TaqDNA polymerase. DNA amplification was carried out on PCR system 9700 thermocycler with the following thermal cycling profile: Initial denaturation at 94°C for 1minutes, denaturation 94°C for 30 seconds, annealing 58°C for 30 seconds, extention 68°C for 1 minute and final extension 68°C for 5minutes with a programmable period of 30 cycles. After PCR amplification, 10µl of PCR product was removed and subjected to agarose gel electrophoresis (Garcia et al., 2008).

3. Data Analysis

3.1 Staphylococcal identification

Fifty (50) staphylococcal strains; anus (28), nostril (17) and environment (5) were isolated from 150 pig samples and its environment. The Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDITOF-MAS) technique classified the 50 staphylococcal strains into nine species: Staphylococcus sciuri (23), Staphylococcus cohnni (11), Staphylococcus piscifermentas (7), Staphylococcus carnosus (1), Staphylococcus condimenti (3), Staphylococcus xylosus (2), Staphylococcus kloosii (1), Staphylococcus pasteuri (1) and Staphylococcus succinus (1).
3.2 Staphylococcal identification

Fifty (50) staphylococcal strains; anus (28), nostril (17) and environment (5) were isolated from 150 pig samples and its environment. The plate shows electro photogram showing positive amplification with 16SrRNA using staphylococcal specific primers.

Figure 4.1: The frequency of staphylococcal isolates from different sources
The anus shows the major source at which the staphylococcal isolates were isolated from because of the twenty three (23) isolates were found and the least is environment which has only five (5) staphylococcal isolates.

![Figure 4.2: Frequency of Staphylococcus species identified using MALDIOF-MS](image)

**Figure 4.2: Frequency of Staphylococcus species identified using MALDIOF-MS**

The Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDITOF-MAS) technique classified the 50 staphylococcal strains into nine species: *Staphylococcus sciuri* had the highest occurrence, it has (23) twenty three species, then is *Staphylococcus cohnii* which had (11) eleven species *Staphylococcus piscifermentas* had seven (7) species, *Staphylococcus condiment* had (3) three species, *Staphylococcus xylosus* had two (2) species, The lowest occurrence was *Staphylococcus carnosus Staphylococcus kloosii Staphylococcus pasteuri* and *Staphylococcus succinus* which occur just once (1).

4. Discussion

Identification of the pathogens causing diseases and 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 lead to diseases. Particular species of CNS are also associated with distinct types of infections and patterns of antimicrobial susceptibility Therefore, species identification of CNS is increasingly of clinical and epidemiological interest to clinicians (Voss et al., 2005). Out 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 (Balaban and Rasooly. (2000). The presence of non-motile, non-sporo 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. *utilis* and *Staphylococcus condiment* (Garcia et al., 1998). This strain was also found in healthy dog feces, which was believed to have originated from fermented food residue presented in dog feed (Kloos 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 (Udo et al., 2000). 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. 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 (Van Loo 2007) underlines the importance of molecular methods in the accurate identification and characterization of CNS.

5. Conclusion
Swine especially pigs have been reported to harbor 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. Flight Mass Spectrometry which is a recognized method for both typing and identification of various bacterial species including the staphylococci spp and have been applied successfully to the identification of CNS to the species level.

6. Recommendations
We recommend increased awareness among healthcare professionals that animals are a possible source of MRSA and CNS infections and that the potential for person-to-person spread exists and also Government should provide equipment and expertise to both public and private universities such as MALDI-TOF mass spectrometry to identify organisms to species level instead of traditional method used because no financial support. Grant should be made available for both public and private universities from government and should be monitored.

7. References


