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# First Report on the Antibiotic Resistance Profiles and Virulence Genes of Staphylococcus Pseudintermedius Colonizing Shelter Dogs and Dog Owners in Nigeria

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## ABSTRACT

The increase in antibiotic-resistant staphylococci among pets and its transfer to humans threaten veterinary medicine and public health. This study was designed to determine the antibiotic resistance patterns and the prevalence of virulence genes among S. pseudintermedius obtained from dogs and dog owners in Abakaliki, Nigeria. Exactly 112 swab samples (perineum, nares, and mouth) were obtained from shelter dogs while nasal swabs of 97 dog owners and 150 non-dog owners were collected. Swab samples were processed and isolates were identified using standard microbiological procedures. MIC was determined by broth micro-dilution using the sensititre system. Isolates were screened for sec, siet, exi, and lukD genes by PCR. A total of 99 S. pseudintermedius isolates [86 (76.8 %) from dogs and 13 (13.4 %) from dog owners] were obtained, out of which 52 (52.5 %) were identified as methicillin-resistant S. pseudintermedius (MRSP) strains as they harboured mecA genes. No S. pseudintermedius isolate was recovered from non-dog owners. Isolates were highly resistant to penicillin (100 %) and ampicillin (94.2 %). Equal resistance frequency (51.2 %) was each observed for fluoroquinolones, clindamycin, trimethoprim/sulfamethoxazole, and erythromycin. Isolates also exhibited resistance to gentamycin (46.5 %), chloramphenicol (23.1 %), tetracycline (19.8 %), and tigecycline (8.1 %). Isolates harboured sec (73.7 %), exi (2 %), siet (62.6 %), and lukD (55.6 %) virulence genes. S. pseudintermedius isolates, including MRSP strains which harboured mecA genes in this study were multi-drug resistant and notably more resistant than those reported in literature. Sec, exi, siet, and lukD virulence genes were haboured by the isolates. There was phenotypic homogeneity in the antibiogram of isolates from dogs and their owners, thus depicting a possible zoonotic transmission. The ability of S. pseudintermedius to cause human infections highlights its lack of host specificity and the importance of considering inter-species transmission.

## MATERIALS AND METHODS

**Study population:** The study population were shelter dogs and humans (dog owners) living within Abakaliki Metropolis, Ebonyi state, South-East Nigeria. A total of 112 shelter dogs were sampled (one sample per dog) and 97 dog owners volunteered for the study between January, 2017 and February, 2018 from 69 different households. Also included in the study are 150 people who have no contacts with dogs. All samples were collected in triplicate from each dog and human.

**Ethical consideration:** Ethical clearance for the collection of bacterial isolates from dogs and humans (dog owners and non-dog owners) were obtained from the Ministry of Health, Ebonyi state (Reference number: SMOH/ERC/19/061). Informed consent of dog owners and other people (who had no contacts with dogs) were obtained prior to sample collection.

**Sample collection:** Sterile cotton swabs moistened in sterile normal saline were used to collect samples from dogs (nares, perineum and mouth) and humans (nasal swabs). These samples were delivered to the laboratory within few hours on the same day for bacteriological analysis.

**Bacteriological analysis:** Each swab sample was streaked onto already prepared mannitol salt agar (Oxoid, UK) and incubated at 35°C for 24 h. Colonies displaying typical Staphylococcus pseudintermedius characteristics (pink-yellow, yellow colonies) were picked and sub-cultured onto CHROM agar Staph aureus (CHROM agar, Paris, France) and incubated at 35°C for 24 h. After incubation, small blue colonies typical of Staphylococcus pseudintermedius were picked and further sub-cultured onto 5% sheep blood Columbia agar. Suspected S. pseudintermedius colonies on Columbia sheep blood agar (small, creamy grey to white, round colonies with a small margin and double zone of hemolysis) were then purified through successive streaking so as to obtain pure cultures. Purified colonies were then subjected to Gram-staining and catalase test. Presumptive Staphylococcal colonies (Gram-positive cocci in bunches with positive catalase test) were further evaluated for coagulase production (tube coagulase using rabbit plasma), hyaluronidase test, acetoin production, beta-galactosidase production, pyrrolidonylarylamidase (PYR) test, DNAse activity and some other biochemical tests such as maltose utilization and mannitol utilization17,18. Identified S. pseudintermedius colonies were then preserved at -80°C in Trypticase Soy Broth (TSB) with 15% glycerol for further analysis, including molecular characterization.

Antibiotic susceptibility test and Methicillin Resistant Staphylococcus pseudintermedius (MRSP) determination: This was used to determine the antibiotic susceptibility pattern of the Staphylococcus pseudintermedius isolates. Antibiotic susceptibility test was done using the Kirby Bauer disc diffusion method in accordance with Clinical Laboratory Standards Institute (CLSI) guidelines, M100-S2419. Mueller-

Hinton agar (Oxoid, UK) was prepared according to the manufacturer's instructions. The medium was cooled to 45-50°C and poured into plates. Plates were allowed to set on a level surface to a depth of approximately 4 mm. When the agar has solidified, plates were allowed to dry before use. Staphylococcus aureus ATCC 29213 and Enterococcus faecalis ATCC 29212 were used as quality control strains. An 18-24 h old broth culture of the Staphylococcus pseudintermedius isolate was standardized by diluting to 0.5 Mcfarland's standard. A sterile swab stick was inserted into the standardized Staphylococcus pseudintermedius inoculum, drained to remove excess inoculum load and inoculated by spreading on the surface of prepared Mueller-Hinton agar plate. After this, the inoculated Mueller-Hinton agar plate was allowed to dry for a few minutes at room temperature with the lid closed. After the agar surface has dried for few minutes, antibiotic impregnated discs (Oxoid, UK) of known concentrations; amoxicillin-clavulanic acid (30 µg), cefepime (30 µg), ertapenem (10 µg), ofloxacin (5 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), levofloxacin (5 µg), doripenem (10 µg), ceftazidime (30 µg) and oxacillin+2% NaCl (1 µg) were carefully applied on the inoculated Mueller-Hinton agar plates using sterile forceps. The plates were then incubated at 37°C for 18-24 h. After incubation, the diameters of the zones of inhibition were measured with a ruler to the nearest millimeter and recorded. The results were recorded as resistant, intermediate and susceptible according to the guidelines of Clinical Laboratory Standards Institute (CLSI) guidelines, M100-S2419. Oxacillin antibiotic (with 2% NaCl) was used in MRSP determination. Isolates were considered to be Methicillin Resistant Staphylococcus pseudintermedius (MRSP) when they exhibit resistance to oxacillin+2% NaCl (Inhibition zone diameter <17 mm).

Determination of Multiple Antibiotic Resistance Indices (MARI) of the isolates: The MARI of the bacterial isolates was calculated with the technique described by Christopher et al.20 and Subramanian et al.21. This was calculated as the number of antibiotics to which the tested isolates were resistant to, divided by the total number of antibiotics to which the organisms were tested against.

**Molecular characterization of S. pseudintermedius isolates**

**DNA extraction:** DNA was extracted from overnight cultures of S. pseudintermedius grown at 37°C on blood agar plates isolates by boil prep technique using heat block matrix (VWR Digital Heat Block, Henry Troemner, LLC, USA). Sterile Eppendorf tubes were labelled in duplicates according to the isolates' codes and one set of the tubes was filled with 200 µL of sterile water each. The Eppendorf tubes filled with sterile water were used for isolate inoculation while the second set of Eppendorf tubes without sterile water was used for DNA storage. The first set of the tubes was each inoculated with a loopful of a 24 h old culture of the isolates. Next, each of the inoculated tube was vortexed for proper mixing. The vortexed tube was tapped occasionally to allow inoculum droplets drain down the tube. After this, the vortexed Eppendorf tube was fixed into the heat block boil prep machine and heated at 100°C for 10 min. After the boiling time of 10 min elapsed, the tubes were allowed to cool a bit for about 2-3 min before centrifuging them. The Eppendorf tube which now contains a mixture of the lysed bacterial cells and DNA was removed from the heat block matrix and centrifuged at 10,000 rpm for 2 min to isolate the bacterial cell's DNA. After centrifugation, the cell pellets settle at the bottom of the Eppendorf tube while the cell's DNA remain in the supernatant in the tube. The centrifuged Eppendorf tube was then removed from the centrifuge and the supernatant carefully aliquoted into the second set of pre-labelled empty Eppendorf tube. The second set of the Eppendorf tube which now contains the bacterial DNA was then put in small-sized boxes and kept in the refrigerator at -20°C for preservation and further use.

Amplification reaction were carried out in a 25 µL PCR mixture containing 17.55 µL of PCR water, 2.5 µL of 10x buffer, 1.25 µL of 50 mM MgCl2, 0.5 µL of 2 mM dNTP, 1 µL primer 1 (Forward), 1 µL primer 2 (Reverse), 0.2 µL of Taq polymerase and 1 µL of the genomic DNA. The thermocycler (BIO-RAD Finnegan C1000 TouchTM, USA) was programmed for optimum conditions. The PCR mixture was poured into microcentrifuge tubes and vortexed for proper mixing before loading them into the thermocycler. The PCR reaction for siet gene was performed as follows: an initial denaturation at 95°C for 5 min, 40 cycles of denaturation at 94°C for 30 sec, annealing temperature of 58°C for 30 sec, elongation at 72°C for 60 sec and final 10 min extension period at 72°C. The PCR reaction for sec gene was performed as follows: an initial denaturation at 95°C for 5 min, 40 cycles of denaturation at 94°C for 30 sec, annealing temperature of 66°C for 30 sec, elongation at 72°C for 60 sec and final 10 min extension period at 72°C. The PCR reaction for exi gene was performed as follows: an initial denaturation at 95°C for 5 min, 40 cycles of denaturation at 94°C for 30 sec, annealing temperature of 61°C for 30 sec, elongation at 72°C for 60 sec and final 10 min extension period at 72°C. The PCR reaction for lukD gene was performed as follows: an initial denaturation at 95°C for 5 min, 40 cycles of denaturation at 94°C for 30 sec, annealing temperature of 55°C for 30 sec, elongation at 72°C for 60 sec and final 10 min extension period at 72°C.

**Agarose gel electrophoresis:** Gel electrophoresis was used to detect amplified DNA products. DNA fragments were analyzed by electrophoresis in 1x TBE buffer (Fisher Scientific International Inc., USA) on a 1% Ultra Pure agarose gel (Invitrogen, UK) stained with ethidium bromide. A 1% (w/v) agarose gel was used to resolve the amplified Polymerase Chain

Reaction (PCR) products of the S. pseudintermedius isolates. The 1% (w/v) agarose gel was prepared by combining 1 g agarose with 100 mL of 1X TBE ((Tris-borate-EDTA) buffer in a 250 mL conical flask and heated in a microwave for about 2 min until the agarose was completely dissolved. The agarose solution was allowed to cool to about 60°C. After cooling, 1 µL of ethidium bromide (10 mg mL–1) was added to the dissolved agarose solution with swirling to mix. The melted agarose was then poured into a gel electrophoresis mould and the casting combs were inserted. It was allowed to gel for 30 min. The casting comb was then carefully removed after the gel had completely solidified. The solidified agarose gel was then carefully placed in the gel electrophoresis tank. One times concentration (1X) TBE electrophoresis buffer was then added to the Gel electrophoresis tank until the buffer just covered the agarose gel. Exactly 3 µL of amplified PCR products was mixed with 2 µL of gel tracking dye (bromophenol blue) and loaded in the sample wells of the prepared agarose gel (the marker was loaded on lane 1 followed by the controls and later followed by the samples). The electrophoresis tank was then covered and the electrodes were connected to the power pack (BIO-RAD, BIO-RAD Laboratories, USA) in such a way that the negative terminal is at the end where the samples have been loaded. The agarose gel was then subjected to electrophoresis at 90 volts for 30 min. TrackItTM 100 bp DNA ladder (Invitrogen, Thermo Fisher Scientific, USA) was used as the molecular weight marker. At the completion of electrophoresis, electrodes were then disconnected and the power pack was turned-off. After this, the gel was removed from the buffer and the band pattern images of the DNA fragments in the gel were viewed using the Molecular Imager® (Gel Doc™ XR, BIO-RAD Laboratories, inc., USA) documentation system.

**Statistical analysis:** Statistical analysis was performed using SPSS 16.0 version statistical software package. Comparison between categorical variables was calculated using the t-test, ANOVA and Tukey post hoc multiple comparison test. Results were considered statistically significant if the p-value is less than 0.05 ($p<0.05$).

## RESULTS

Prevalence of S. pseudintermedius isolates in dogs and dog owners (humans): Out of the 45 perineum swab samples, 42 (93.3%) were positive for S. pseudintermedius while 25 (71.4%) out of the 35 nares swab samples were positive for S. pseudintermedius.

A total of 19 (59.4%) swab samples were positive for S. pseudintermedius out of the 32 mouth swab samples obtained from dogs. In total, 86 (76.8%) dogs were positive for S. pseudintermedius (Table 2). There was no statistically significant difference in the prevalence of S. pseudintermedius in the perineum, nares and mouth [$p = 0.074$ (at $p<0.05$)]. A total of 97 nasal swab samples of dog owners that volunteered for this study were collected. Out of the 97 volunteers, 13 (13.4%) were positive for S. pseudintermedius. None of the nasal swab samples from the 150 volunteers who have no contacts with dogs was positive for S. pseudintermedius.

Prevalence of Methicillin Resistant S. pseudintermedius (MRSP) isolates among dog and humans (dog owners) isolates: A total of 52 (52.5%) out of all the 99 isolates from both shelter dogs and humans were Methicillin Resistant Staphylococcus pseudintermedius (MRSP) as they were resistant to oxacillin with 2% NaCl. Out of the 52 MRSP isolates, 46 were from dogs while six were from humans (dog owners) (Table 3). There was no statistically significant difference in the prevalence of Methicillin Resistant Staphylococcus pseudintermedius (MRSP) isolates between dogs and humans [$p = 0.417$ (at $p<0.05$) (Table 3)].

Percentage occurrence of responses of the S. pseudintermedius isolates from dogs to antibiotics tested: Antibiotic susceptibility test results revealed that S. pseudintermedius isolates from dogs were highly resistant to ceftazidime and Amoxicillin-clavulanic acid with resistance frequencies of 95.3 and 94.2%, respectively (Table 4). Resistance was also observed to cefepime (80.2%), levofloxacin (51.2%) and ciprofloxacin (50%) (Table 4). Isolates were susceptible to ofloxacin (80.2%), chloramphenicol (76.9%), doripenem (66.3%) and ertapenem (66.3%). There was a statistically significant difference in the mean percentage resistances of the S. pseudintermedius isolates from dogs [$p = 0.000$ (at $p<0.05$)]. Results also showed that 46 (53.5%) of the dog isolates were methicillin-resistant strains as they were resistant to oxacillin with 2% NaCl (Table 4).

Percentage occurrence of responses of the S. pseudintermedius isolates from dog owners (humans) to antibiotics tested: Antibiotic susceptibility test results showed that S. pseudintermedius isolates from dog owners (humans) were highly resistant to ceftazidime, amoxicillin-clavulanic acid and cefepime with resistance frequencies of 100, 92.3 and 69.2%, respectively (Table 5). Isolates were susceptible to chloramphenicol (76.9%), doripenem (76.9%), ofloxacin (69.2%), levofloxacin (53.8%), ertapenem (53.8%) and ciprofloxacin (53.8%). There was a statistically significant difference in the mean percentage resistances of the S. pseudintermedius isolates from humans [$p = 0.000$ ($p<0.05$) (Table 5)]. There was no statistically significant difference between the mean resistances of S. pseudintermedius from dogs when compared to those from humans ($p<0.05$).

Prevalence of virulence genes among the S. pseudintermedius isolates from dogs and humans (dog owners): A total of 73 (73.7%), 2 (2%), 62 (62.6%) and 55 (55.6%) isolates haboured sec, exi, siet and lukD virulence genes respectively (Table 6,

Fig. 1-4). Out of the 73 sec virulence genes, 62 (84.9%) were found in the dog isolates while 11 (15.1%) were found in the human (dog owners) isolates (Table 6, Fig. 1). One exi virulence gene was present in both a dog (D014) and human (D103) isolates (Table 6, Fig. 2). Siet virulence gene was present in 53 (85.5%) isolates while 9 (14.5%) were present in the human isolates.

**DISCUSSION**

Staphylococcus pseudintermedius is an important opportunistic pathogen of dogs. Increasing antimicrobial resistance by S. pseudintermedius, especially MRSP strains in dogs represents a major challenge for veterinarians in terms of antibiotic therapy3. This is the first study in Nigeria which reports S. pseudintermedius colonization in both dogs and dog owners. The S. pseudintermedius isolates from this study, including the MRSP strains were multi-drug resistant and more prevalent in dogs than among dog owners. Isolates were notably more resistant than those reported in literature and harboured virulence genes encoding various pathogenic factors. Interestingly, there was phenotypic homogeneity in the antibiotic resistance profiles of isolates from both dogs and dog owners in each household that was sampled, thus depicting a possible zoonotic transmission event from dogs to their owners. The carriage frequencies of S. pseudintermedius among shelter dogs and dog owners in this study were 76.8 and 13.4%, respectively. All the nasal swab samples from 150 volunteers who had no contact with dogs were negative for S. pseudintermedius. Among the three major dog sites that were sampled (perineum, nares and mouth) for S. pseudintermedius, the perineum was the most colonized site with a recovery frequency of 93.3%. This was closely followed by the nares and the mouth with recovery frequencies of 71.4 and 59.4%, respectively. The S. pseudintermedius isolates exhibited resistance (100-50%) to ceftazidime, amoxicillin-clavulanic acid, cefepime, levofloxacin and ciprofloxacin. Isolates also exhibited multi-drug resistant traits as they were resistant to at least three different classes of antibiotics. There was a statistically significant difference in the mean percentage resistances of the S. pseudintermedius isolates from dogs [$p = 0.000$ (at $p<0.05$)]. However, isolates were highly susceptible (80.2-66.3%) to ofloxacin, chloramphenicol, doripenem and ertapenem. The Multiple Antibiotic Resistance Index (MARI) values of the S. pseudintermedius isolates in this study ranged from 0.2-0.9, thus further depicting multidrug resistance and the gradual phasing out of the tested antibiotics in treating infections caused by S. pseudintermedius. This study revealed that 46 (53.5%) of the S. pseudintermedius isolates from dogs and 6 (46.2%) of those from dog owners were methicillin-resistant strains. Amoxicillin-clavulanic acid+ceftazidime resistance antibiotype (AMCR CAZR) was the most prevalent antibiotype as it was present in virtually all the antibiotypes observed. Interestingly, there was phenotypic homogeneity in the antibiotic resistance profiles of isolates obtained from dogs and their owners in each of the households that were sampled, thus depicting a possible zoonotic transmission event between dogs and their owners. The S. pseudintermedius isolates in this study harboured sec, exi, siet and lukD virulence gene encoding pathogenic factors. Sec gene, mostly implicated in dog pyoderma cases was the most predominant virulence gene detected among the 99 isolates as 73 (73.7%) of the isolates were positive. This was closely followed by siet gene [62 (62.6%] and lukD gene [55 (55.6%], while exi gene [2 (2%)] was the least predominant. The 76.8% S. pseudintermedius carriage frequency among dogs in this study was higher than the 55% carriage frequency reported by Gharsa et al.22 in Tunisia, North Africa, but less than the 90% carriage frequency reported by Rubin and Chirino-Trejo25 in Western Canada. Although few human cases have been described by Stegmann et al.5, Van Hoovels et al.6, Chuang et al.7 and Savini et al.8, the presumptive transmission of S. pseudintermedius from dogs to dog owners has also been reported as 3.9-13% of those humans have been found to harbor S. pseudintermedius3,26-28. Even though the resistance frequencies of the S. pseudintermedius isolates in this study is higher than those reported in other studies, similar S. pseudintermedius resistance to beta-lactams, fluoroquinolones and chloramphenicol have been reported by Rubin et al.18, Gharsa et al.22 and Saab et al.29. The prevalence frequency of MRSP among dogs in this study is far higher than the 0.8-7% prevalence frequency reported by Priyantha et al.30, Ruscher et al.31 and Griffeth et al.32 in North America and Europe9,33,34. This study is in total concord with other studies from Asia where 32-45% MRSP carriage frequency was reported in Thailand, Japan and Hong Kong26,35,36. Gharsa et al.22, Ruzauskas et al.37, Pitchenin et al.38, Garbacz et al.39, Matanovic et al.40 and Ruscher et al.41 reported luk virulence gene in 29-95% of S. pseudintermedius isolates in their studies. In this study, the high prevalence frequency of siet gene (62.6%), responsible for the production of an exfoliative toxin, mostly associated with skin infections in dogsis in agreement with the 69 and 91% prevalence frequency reported by Ruzauskas et al.37 and Pitchenin et al.38 in Lithuania and Brazil, respectively. This study also agrees with other studies on canine S. pseudintermedius in Korea42, Poland39 and Tunisia22. Interestingly, most of the isolates that harboured lukD gene also harboured siet gene. This was also reported by Ruzauskas et al.37 and Borjesson et al.43. Contrastingly, Borjesson et al.43 reported 8% prevalence for sec virulence gene. This frequency value (8%) for sec virulence gene reported by Borjesson et al.43 does not completely agree with the 73.7% prevalence in this study. The occurrence of virulence genes encoding toxins among the S. pseduintermedius isolates in this study is ubiquitous with the exception of exi gene which was present in just 2% of the isolates. The high frequency of isolates harbouring a significant number of these virulence genes suggests that these genes may play an

important role in infection caused by S. pseudintermedius. The epidemiological situation of S. pseudintermedius infections is further exacerbated as the gene driving the drug resistance is highly mobile and can be transferred between different staphylococcal species colonizing humans and zoonotic hosts10. It is therefore pertinent to further investigate the diversity and dissemination of S. pseudintermedius infections in both dogs and humans so as to better understand their pathogenesis, risk factors and transmission characteristics which will greatly help in developing strong mitigation and control strategies and for future epidemiological studies.

## CONCLUSION

Staphylococcus pseudintermedius isolates in this study were notably more resistant than those reported in literature. This study revealed that amoxicillin-clavulanic acid, ceftazidime and cefepime are no longer effective in treating bacterial infections caused by S. pseudintermedius isolates in Ebonyi state as a large number of the isolates obtained in this study were resistant to these antibiotics. This study also showed that ofloxacin, chloramphenicol, doripenem and ertapenem are still highly active against S. pseudintermedius and MRSP isolates from both dogs and humans in Ebonyi state. The S. pseudintermedius isolates from dogs and dog owners in this study harboured sec, siet, exi and lukD virulence genes. High prevalence of isolates positive for a significant number of these toxin genes suggests that these genes may play an important role in infection caused by S. pseudintermedius. The strange phenotypic homogeneity in the antibiotic resistance profiles observed among human and dog isolates linedepicts a likely zoonotic transfer between the dogs and their owners. The ability of S. pseudintermedius to cause human infections highlights its lack of host specificity and the importance of considering inter-species transmission.

## SIGNIFICANCE STATEMENT

This is the first study in Nigeria which reports the antibiogram and prevalence of S. pseudintermedius, including the methicillin-resistant strains among dogs and dog owners. This study is also the first to report the prevalence of various virulence genes; sec, siet, exi and lukD genes encoding different pathogenic factors among S. pseudintermedius isolates obtained from dogs and dog owners. The high prevalence of sec, siet and lukD virulence genes in S. pseduintermedius isolates obtained from dogs and humans in this study indicates that these toxins may play an important role in the pathogenesis of infection and could be very important in active infection. High prevalence of isolates positive for a significant number of toxin genes suggests that these toxins may play an important role in infection caused by S. pseudintermedius. Knowledge about the toxigenic profile of S. pseudintermedius strains will greatly help in understanding the pathogenesis of infection caused by this microbe.