Igbinosa & Beshiru. *Afr. J. Clin. Exper. Microbiol.* 2019; 20 (4): 289-298 https://www.afrjcem.org

African Journal of Clinical and Experimental Microbiology ISSN 1595-689X Oct 2019 Vol.20 No.4
AJCEM/1924: https://www.ajol.info/index.php/ajcem

Original Article **Open Access**

Characterization of antibiotic resistance and species diversity of staphylococci isolated from apparently healthy farm animals

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Abstract:

Background: *Staphylococcus* species are adaptable commensals usually involved in a diverse multiplicity of ailments in animals and humans. This study surveyed the occurrence, antibiotic-resistance profile and putative resistant genetic elements of staphylococci isolates from apparently healthy farm animals
Methodology: Nasal and rectal samples were collected from a total of 400 cows and pigs in Benin City between May and December 2017. Staphylococci were isolated following aerobic cultures of samples using standard microbiological methods. Susceptibility profiles of the isolates to eighteen selected antimicrobials were determined using the Kirby-Bauer disk diffusion test. Species of staphylococci were established and antibiotic resistance genes detected by the polymerase chain reaction using species-specific and antibiotic-resistant primers respectively
Result: A total of 139 staphylococci isolates were phenotypically and genotypically identified from the food-producing animals; 87 (62.6%) from pigs and 52 (37.4%) from cows. The most frequent *Staphylococcus* species were *Staphylococcus haemolyticus* 38 (27.3%), *Staphylococcus aureus* 27 (19.4%) and *Staphylococcus capitis* 21 (15.1%). Antibiotic resistance profile showed 120 (86.3%) isolates to be resistant to penicillin G, 100 (71.9%) to nalidixic acid and 99 (71.2%) to minocycline. The prevalence of antibiotic resistance genes assessed were *mec*A 78 (56.1%), *mph*C 23 (16.6%), and *erm*A 20 (14.4%).
Conclusion: Our finding indicates that food animals are potential reservoirs of antibiotic resistant staphylococci which pose a significant threat to food security and public health.

Keywords: food animals; antibiotic-resistant; foodborne pathogen; staphylococci; resistance elements

Received March 21, 2019; Revised May 22, 2019; Accepted May 27, 2019

Caractérisation de la résistance aux antibiotiques et de la diversité des espèces de staphylocoques isolés d'animaux de ferme apparemment en bonne santé

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Abstrait:

Contexte: Les espèces de *Staphylococcus* sont des agents commensaux adaptables généralement impliqués dans une grande diversité de maladies chez les animaux et les humains. Cette étude a examiné l'occurrence, le profil de résistance aux antibiotiques et les éléments génétiques potentiellement résistants d'isolats de staphylocoques provenant d'animaux d'élevage apparemment en bonne santé.
Méthodologie: Des échantillons nasaux et rectaux ont été prélevés chez 400 vaches et porcs au total dans la ville de Benin City entre mai et décembre 2017. Les staphylocoques ont été isolé suite à des cultures aérobies d'échantillons à l'aide de méthodes microbiologiques standard. Les profils de sensibilité des isolats à dix-huit antimicrobiens sélectionnés ont été déterminés à l'aide du test de diffusion sur disque Kirby-Bauer. Les espèces

de staphylocoques ont été établies et les gènes de résistance aux antibiotiques ont été détectés par réaction en chaîne de la polymérase en utilisant respectivement des amorces spécifiques à l'espèce et des bactéries résistantes aux

Résultat: Un total de 139 isolats de staphylocoques ont été identifiés phénotypiquement et génotypiquement à partir des animaux producteurs d'aliments. 87 (62,6%) de porcs et 52 (37,4%) de vaches. Les espèces de *Staphylococcus* les plus fréquentes étaient *Staphylococcus haemolyticus* 38 (27,3%), *Staphylococcus aureus* 27 (19,4%) et *Staphylococcus capitis* 21 (15,1%). Le profil de résistance aux antibiotiques a montré que 120 (86,3%) des isolats étaient résistants à la pénicilline G, 100 (71,9%) à l'acide nalidixique et 99 (71,2%) à la minocycline. La prévalence des gènes de résistance aux antibiotiques évalués était *mec*A 78 (56,1%), *mph*C23 (16,6%) et *erm*A 20 (14,4%).

Conclusion: nos résultats indiquent que les animaux destinés à l'alimentation sont des réservoirs potentiels de staphylocoques résistants aux antibiotiques qui constituent une menace importante pour la sécurité alimentaire et la santé publique

Mots-clés: animaux d'élevage; résistant aux antibiotiques; agent pathogène d'origine alimentaire; staphylocoques, éléments de résistance

Introduction:

Staphylococcus species are adaptable commensals usually involved in a diverse multiplicity of ailments in animals and humans with their pathogenicity associated with invasive capacity, antibiotic resistance, and toxin-mediated virulence (1, 2). In livestock, *Staphylococcus aureus* has been described as a significant cause of skin and soft tissue infections, mastitis and systemic infections (3) and is considered a key foodborne pathogen (4).

The demand for animal proteins is increasing globally at a relatively high rate for human consumption. Concern about the threat of antibiotic-resistant strains of *Staphylococcus* species has increased in recent years (5). The emergence of antibiotic resistance has been recognized to be the result of extensive prophylactic and therapeutic use of antimicrobials as growth promoters in food-producing animals (6, 7). Such antimicrobials are frequently used in human medicine for therapy of infections and prophylaxis during medical procedures such as surgeries, chemotherapy and organ transplantation (8). The widespread use of antimicrobials in food animals contribute to the development of antimicrobial-resistant bacteria (ARB) by means of natural selection and thus constitute a significant risk to public health.

Antibiotic resistance from animals can be disseminated to humans through food products (9), environment (10) and by direct contact to agricultural workers (11). Although it is difficult to establish a direct connection due to the organic character of antibiotic selection pressure, reports have shown a close relationship between the occurrence of livestock-associated antibiotic-resistant bacteria in humans and animals (12). Similarly, the rate of antimicrobial use in food-producing animals and the prevalence of antibiotic-resistant bacteria in humans (13) and animals (14) have been documented. *Staphylococcus* species from food-producing animals frequently harbour resistance elements. *S. aureus* are now generally resistant to methicillin and most other β-lactam antimicrobials. Methicillin resistance in staphylococci is mediated usually by *mecA* gene carried on staphylococcal chromosomal cassette (SCC*mec*) (15) which codes for altered penicillin-binding protein 2a or 2' (PBP2a or 2') with low binding affinity to beta-lactamase resistant penicillins such as oxacillin and methicillin, and other beta-lactam antimicrobials (16).

The genotypic characterization of *Staphylococcus* species is essential to assess the risk of dissemination of resistant staphylococcal isolates between humans, environment and animals. There are enormous concern regarding the public health implication of methicillin-resistant *S. aureus* (MRSA) connected with livestock since MRSA and their resistance genes can spread from humans to animals via the food chain or through direct contact (17). Diversity of MRSA strains have been recovered from small ruminants or cow milk as well as different dairy products in different countries (18, 19).

In 2009, the European Food Safety Authority (EFSA) expressed growing concerns for public health orchestrated by the occurrence of MRSA in food animal production. The authority therefore suggested that additional studies be conducted on sampling, identification and characterization of MRSA carriage in animals and humans, and the environment coupled with food contamination (20). The current study aimed to characterize antibiotic resistant *Staphylococcus* species from food animals in Benin City, Nigeria.

Materials and methods:

Sample collection

A total of 400 samples (200 nasal and 200 rectal) samples were collected from cows and pigs in Benin City between May and

December 2017. Samples were collected with sterile swabs by first moistening in sterile normal saline and gently swabbing the nasal and rectal cavities of the food-producing animals. Informed consent was obtained from the farm owners prior to sampling. Samples were immediately transported on ice packs to the Applied Microbial Processes and Environmental Health Research Group Laboratory, Department of Microbiology, University of Benin, Nigeria for analysis within 24 hours of collection.

Culture isolation and biochemical identification of staphylococci

Swab samples were immediately agitated on 5 mL tryptone soy broth (Lab M, Lancashire, United Kingdom) and incubated aerobically for 18-24 hours at 37°C. After 18 hours, an aliquot of 100 µL was inoculated on mannitol salt agar (Lab M, Lancashire, United Kingdom) and further incubated aerobically for 18-24 hours at 37°C. After incubation, 'golden yellow' and other related colonies were Gram stained and identified by biochemical tests such as coagulase, DNAse, slide agglutination (BBL™ Staphyloslide™), and mannitol and sugar fermentation tests (21, 22). All tests were performed in triplicates with *S. aureus* ATCC 12600 used as control strain in each test procedure. The staphylococci isolates were confirmed with analytical profile index (API) Staph (BioMerieux, France). Identified staphylococci were colony purified on nutrient agar (Lab M, Lancashire, United Kingdom) and stored on nutrient agar slants at 4°C until further use.

Susceptibility profile of staphylococci isolates

Susceptibility profile of the *Staphylococcus* species to antimicrobials was carried out using Kirby-Bauer/CLSI disk diffusion method (23). Briefly, the purified isolates were inoculated into 5.0 mL Mueller-Hinton broth (MHB) (Lab M, Lancashire, United Kingdom) and incubated overnight. The optical density (OD) of the turbidity of the broth was adjusted to OD of 0.5 McFarland standards which gives equivalence of 1 x 10^8 CFU/mL. Using a sterile swab, broth cultures were aseptically swabbed on Mueller Hinton agar (Lab M, Lancashire, United Kingdom). Antibiotic disks were aseptically placed on the agar plates with sterile forcep. Plates were incubated at 37°C for 24 hours and diameter of zone of inhibition for each isolate was measured with a ruler. Susceptibility or resistance of each isolate was determined by comparing the diameter of zone of inhibition with the interpretative chart of the Clinical and Laboratory Standards Institute (23).

The antibiotic disks (Mast Diagnostics, Merseyside, United Kingdom) used were; meropenem (10 µg), penicillin G (10 units), cefoxitin (30 µg, surrogate for testing *S. aureus* against oxacillin), ceftazidime (30 µg), cefotaxime (30 µg), tetracycline (30 µg), doxycycline (30 µg), minocycline (30 µg), clindamycin (2 µg), erythromycin (10 µg), ofloxacin (5 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), nalidixic acid (30 µg), sulfamethoxazole-trimethoprim (23.75 µg/1.25 µg), chloramphenicol (30 µg), kanamycin (30 µg), and gentamicin (10 µg).

Multiple antibiotic resistance index of isolates

The multiple antibiotic resistance index (MARI) for each isolate was calculated as number of antibiotics to which resistance occurred divided by the total number of antibiotics to which the isolate was tested (24). Multidrug resistance was defined as resistance to three or more antimicrobial classes (25).

Genomic DNA extraction

Genomic DNA from *Staphylococcus* isolates was extracted using the boiling method. Briefly, the *Staphylococcus* isolates were re-inoculated in 5.0 mL of tryptone soy broth and incubated at 37 ºC for 18-24 hours. Thereafter, 150 µL of the cell suspension was dispensed into 2.0 mL Eppendorf tube, and the mixture was heated in a dry bath (MK200-2, Shanghai, China) for 15 minutes at 100 °C for cell lysis. The lysed cell mixture was centrifuged with the aid of a mini centrifuge (Mini 14 k, Zhuhai, Guangdong, China) at 14, 500 r/minute, for 5 minutes. The supernatant was carefully separated from the cell residues and stored at -20°C as template target gDNA.

PCR identification of *Staphylococcus* species

PCR was performed for all staphylococcal isolates using genus-specific and species-specific primers (Table 1). For genus specific amplification, the simplex PCR conditions used included denaturation at 96 °C for 3 minutes, followed by 40 cycles at 95 °C for 30 s, annealing at 55 °C for 60 s, extension at 72 °C for 30 s, with a final extension at 72 °C for 3 minutes (26) using a Peltier-based Thermal Cycler (MG96þ/Y, Hangzhou, Zhejiang China). *S. aureus* ATCC 12600 served as positive control and nuclease-free water as negative control. The PCR products were electrophoresed on 1.5 % agarose gel which was stained with ethidium bromide and visualized under the UV transilluminator (Vilber Lourmat, EBOX VX5, France).

Species-specific identification was carried out using multiplex PCR primers targeting *S. epidermidis*, *S. saprophyticus*,

S. aureus and *S. xylosus* (at respective base-pair size in Table 1) and the PCR conditions included denaturation at 94 °C for 3 minutes followed by 40 cycles at 95 °C for 1 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s, with a final extension at 72 °C for 3 minutes (27). The PCR products were electrophoresed using 1.5 % agarose gel (CLSAG100, Warwickshire, United Kingdom).

For other species, the multiplex PCR program conditions were denaturation at 94 °C for 10 minutes followed by 35 cycles at 94 °C for 15 s, 30 s at respective annealing temperature regimen for *S. warneri* (60 °C), *S. haemolyticus* (50 °C) and *S. capitis* (59 °C) respectively, and extension at 72 °C for 30 s (28).

PCR detection of antibiotic resistance genes

PCR detection of macrolide-resistant genes (*erm*A, *erm*B, *erm*C, *mph*C) was done in accordance with multiplex PCR procedure of Sauer et al., (29) using primers presented in Table 2. PCR program conditions included an initial denaturation step for 5 minutes at 94 °C followed by 30 cycles of denaturation for 60 s at 94 °C, with the following respective annealing temperature regimen; *erm*A (51 °C), *erm*B (51 °C), *erm*C (51 °C), *mph*C (55 °C) for 60 s, and extension for 60 s at 72 °C with a final extension for 5 minutes at 72 °C (30, 31, 32).

PCR conditions for *van*A and *van*B genes included an initial denaturation for 5 minutes at 94 °C, followed by 10 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 64 °C, and extension for 45 s at 72 °C (33, 34). PCR condition for *mec*A included an initial denaturation for 5 minutes at 94 °C, followed by 25 cycles, denaturation for 30 s at 94 °C, annealing for 45 s at 50 °C, and extension for 2 minutes at 72 °C, with a final extension for 10 minutes at 72 °C (35).

Table 1: Primers used for staphylococci identification

Microorganisms	Primers	Primer sequence (5′-3′)	Size (bp)	References
Staphylococcus genus	TStaG422	GGCCGTGTTGAACGTGGTCAAATCA	370	Martineau et al. (26)
	TStag765	TIACCATTTCAGTACCTTCTGGTA		
S. haemolyticus	ShaeF	GTTGAGGGAACAGAT	85	Iwase et al. (28)
	ShaeR	CAGCTGTTTGAATATCTT		
S. capitis	ScapF	GCTAATTTAGATAGCGTACCTTCA	208	Iwase et al. (28)
	ScapR	CAGATCCAAAGCGTGCA		
S. xylosus	XylF	AACGCGCAACGTGATAAAATTAATG	539	Morot-Bizot et al. (55)
	XylR	AACGCGCAACAGCAATTACG		
S. warneri	SwarF	TGTAGCTAACTTAGATAGTGTTCCTTCT	63	Iwase et al. (28)
	SwarR	CCGCCACCGTTATTTCTT		
S. aureus	Sa442-1	AATCTTTGTCGGTACACGATATTCTTCACG	1108	Morot-Bizot et al. (55)
	Sa442-2	CGTAATGAGATTTCAGTAGATAATACAACA		
S. saprophyticus	Sap1	TCAAAAAGTTTTCTAAAAAATTTAC	221	Morot-Bizot et al. (55)
	Sap2	ACGGGCGTCCACAAAATCAATAGGA		
S. epidermidis	Se705-1	ATCAAAAAGTTGGCGAACCTTTTCA	1,124	Morot-Bizot et al. (55)
	Se705-2	AAAAGAGCGTGGAGAAAAGTATCA		

Table 2: Primers used for amplification of antibiotic resistance genes in staphylococci isolates

Genes	Primers	Primer sequence (5′-3′)	Size (bp)	References
*mec*A	mecA1	GTAG AAAT GACT GAAC GTCC GATAA	310	Geha et al. (35)
	mecA2	CCAA TTCC ACAT TGTT TCGG TCTAA		
*van*A	van A1	GGGAAAACGACAATTGC	732	Dutka-Malen et al. (33)
	van A2	GTACAATGCGGCCGTTA		
*van*B	van B1	GTGC TGCG AGAT ACCA CAGA	1145	Ramos-Trujillo et al. (34)
	van B2	CGAACACCATGCAACATTTC		
*mph*C	mph (C)-1	GAGA CTAC CAAG AAGA CCTGACG	722	Lüthje and Schwarz (30)
	mph (C)-2	CATA CGCC GATT CTCC TGAT		
*erm*A	erm(A)-1	GCGGTAAACCCCTCTGAG	434	Werckenthin and Schwarz (31)
	erm(A)-2	GCCTGTCGGAATTGG		
*erm*B	erm(B)-1	CATT TAAC GACG AAAC TGGC	425	Jensen et al. (32)
	erm(B)-2	GGAA CATC TGTG GTAT GGCG		
*erm*C	erm(C)-1	ATCT TTGA AATC GGCT CAGG	295	Jensen et al. (32)
	erm(C)-2	CAAA CCCG TATT CCAC GATT		

Results:

Frequency of staphylococci isolation from mannitol salt agar

The frequency of *Staphylococcus* isolates recovered from the food producing animals in Table 3 shows an overall isolation rate from mannitol salt agar of 64.3% (257 of 400), with 98 (24.5%) from cows (47 from nasal and 51 from rectal samples), and 159 (39.8%) from pigs (83 from nasal and 76 from rectal samples).

Table 3: The frequency of isolation of *Staphylococcus* from culture of samples on mannitol salt agar

Number and source of samples examined		Number of samples positive for staphylococci
Cow	nasal sample (n=100)	47 (47)
	rectal sample (n=100)	51 (51)
Pig	nasal sample (n=100)	83 (83)
	rectal sample (n=100)	76 (76)
	Total (n=400)	247 (64.3)

Distribution of the *Staphylococcus* species in cows and pigs

The frequency distribution of *Staphylococcus* species identified by both phenotypic and genotypic methods from cow and pigs is presented in Table 4. A total of 139 *Staphylococcus* species were identified from the 400 samples, giving a 34.8% recovery from these food animals, with 87 (62.6%) from pigs (51 from nasal and 36 from rectal samples) and 52 (37.4%) from cows (33 from nasal and 19 from rectal samples). The frequency distribution of the *Staphylococcus* species in descending order are; *S. haemolyticus* (27.3%), *S. aureus* (19.4%), *S. capitis* (15.1%), *S. epidermidis* (9.4%), *S. saprophyticus* (7.2%), *S. xylosus* (5.0%) and *S. warneri* (2.9%). Other staphylococci species constituted 13.7%.

Table 4: Frequency distribution of *Staphylococcus* species identified by phenotypic and genotypic methods from nasal and rectal samples of cow and pigs

Staphylococcus species	Cow			Pig			Total (%)
	nasal	rectal	Subtotal (%)	nasal	rectal	Subtotal (%)	
S. aureus	11	3	14 (26.9)	8	5	13 (14.9)	27 (19.4)
S. epidermidis	3	3	6 (11.5)	4	3	7 (8.0)	13 (9.4)
S. capitis	5	-	5 (9.6)	13	3	16 (18.4)	21 (15.1)
S. xylosus	3	-	3 (5.8)	3	1	4 (4.6)	7 (5.0)
S. haemolyticus	5	5	10 (19.2)	10	18	28 (32.2)	38 (27.3)
S. saprophyticus	-	3	3 (5.8)	7	-	7 (8.0)	10 (7.2)
S. warneri	2	2	4 (7.7)	-	-	-	4 (2.9)
Other S. species	4	3	7 (13.5)	6	6	12 (13.8)	19 (13.7)
Total	33	19	52 (37.4)	51	36	87 (62.6)	139 (100)

Table 5: Antimicrobial susceptibility profile of the *Staphylococcus* species

Antimicrobial class	Antibiotics	*Staphylococcus* species (*n*=139)		
		Resistant	Intermediate	Sensitive
Carbapenems	Meropenem	18 (12.9)	0 (0)	121 (87.1)
Penicillin	Penicillin G	120 (86.3)	0 (0)	19 (13.7)
Cephalosporins	Cefoxitin	78 (56.1)	0 (0)	61 (43.9)
	Ceftazidime	72 (51.8)	43 (30.9)	24 (17.3)
	Cefotaxime	36 (25.9)	11 (7.9)	92 (66.2)
Tetracyclines	Tetracycline	102 (73.4)	0 (0)	37 (26.6)
	Doxycycline	91 (65.5)	12 (8.6)	36 (25.9)
	Minocycline	99 (71.2)	17 (12.2)	23 (16.5)
Lincosamides	Clindamycin	46 (33.1)	24 (17.3)	69 (49.6)
Macrolides	Erythromycin	36 (25.9)	16 (11.5)	87 (62.6)
Quinolones	Ofloxacin	14 (10.1)	18 (12.9)	107 (76.9)
	Ciprofloxacin	9 (6.5)	13 (9.4)	117 (84.2)
	Levofloxacin	3 (2.2)	0 (0)	136 (97.8)
	Nalidixic Acid	100 (71.9)	0 (0)	0 (0)
Folate inhibitors	Sulfamethoxazole-trimethoprim	97 (69.8)	23 (16.5)	19 (13.7)
Phenicols	Chloramphenicol	0 (0)	7 (5.0)	132 (94.9)
Aminoglycosides	Kanamycin	0 (0)	6 (4.3)	133 (95.8)
	Gentamycin	0 (0)	11 (7.9)	128 (92.1)

Antimicrobial susceptibility profile of the staphylococci isolates

The resistant profile of the *Staphylococcus* species is presented in Table 5 which shows resistance rate to penicillin G of 86.3% (120 of 139), 71.9% to nalidixic acid, 71.2% to minocycline, 69.8% to trimethoprim-sulfamethoxazole, 65.5% to doxycycline, 56.1% to cefoxitin (oxacillin), 51.8% to ceftazidime, and 33.1% to clindamycin. The *Staphylococcus* species were sensitive to levofloxacin 97.8% (136 of 139), 95.8% to kanamycin, 94.9% to chloramphenicol, 92.1% to gentamycin, 87.1% to meropenem, 84.2% to ciprofloxacin, 76.9% to ofloxacin, 66.2% to cefotaxime and 62.6% to erythromycin.

Multidrug-resistance and multiple antibiotic-resistant index of *Staphylococcus* species

The multidrug resistance profile of the *Staphylococcus* species as presented in Table 6 shows 100 (71.9%) isolates resistant to three different antibiotic classes (NAL^R, TET^R and PEN^R), 67 (48.2%) isolates resistant to eight antibiotics in five different classes (TMP^R, NAL^R, MIN^R, DOX^R, TET^R, CAZ^R, OXA^R and PEN^R), and three (2.2%) isolates resistant to fifteen antibiotics in eight different classes (TMP^R, NAL^R, LEV^R, CIP^R, ERY^R, CLI^R, MIN^R, DOX^R, TET^R, CTX^R, CAZ^R, OXA^R, PEN^R and MEM^R). The multiple antibiotic resistant index (MARI) ranged from 0.17 to 0.83 (Table 6).

Distribution of antibiotic-resistant genes in staphylococci isolates

The distribution of antibiotic-resistant genes shows that all 78 staphylococci isolates resistant to cefoxitin (i.e. phenotypic MRSA) carried the *mec*A gene. Of the 36 isolates resistant to the erythromycin (macrolide), 23 (63.8%) harboured the *mph*C gene, 20 (55.6%) had the *erm*A gene, 4 (11.1%) had the *erm*B gene, and 11 (30.6%) had the *erm*C gene (Table 7). However, 12 multidrug resistant (MDR) isolates harboured *van*A gene but none contained *vanB* gene.

Table 6: Multidrug-resistant profile of the *Staphylococcus* species

Number of antimicrobial class	Number of antibiotics	Resistance phenotype	Number of isolates (*n*=139)	MARI
3	3	NAL^R, TET^R, PEN^R	100 (71.9)	0.17
4	5	TMP^R, NAL^R, MIN^R, TET^R, PEN^R	96 (69)	0.27
4	6	TMP^R, NAL^R, MIN^R, DOX^R, TET^R, PEN^R	90 (64.7)	0.33
5	7	TMP^R, NAL^R, MIN^R, DOX^R, TET^R, CAZ^R, PEN^R	70 (50.4)	0.35
5	8	TMP^R, NAL^R, MIN^R, DOX^R, TET^R, CAZ^R, OXA^R, PEN^R	67 (48.2)	0.39
6	9	TMP^R, NAL^R, CLI^R, MIN^R, DOX^R, TET^R, CAZ^R, OXA^R, PEN^R	45 (32.4)	0.50
7	11	TMP^R, NAL^R, ERY^R, CLI^R, MIN^R, DOX^R, TET^R, CTX^R, CAZ^R, OXA^R, PEN^R	35 (25.2)	0.61
8	12	TMP^R, NAL^R, ERY^R, CLI^R, MIN^R, DOX^R, TET^R, CTX^R, CAZ^R, OXA^R, PEN^R, MEM^R	16 (11.5)	0.67
8	13	TMP^R, NAL^R, OFX^R, ERY^R, CLI^R, MIN^R, DOX^R, TET^R, CTX^R, CAZ^R, OXA^R, PEN^R, MEM^R	13 (9.4)	0.72
8	14	TMP^R, NAL^R, CIP^R, ERY^R, CLI^R, MIN^R, DOX^R, TET^R, CTX^R, CAZ^R, OXA^R, PEN^R, MEM^R	9 (6.5)	0.78
8	15	TMP^R, NAL^R, LEV^R, CIP^R, ERY^R, CLI^R, MIN^R, DOX^R, TET^R, CTX^R, CAZ^R, OXA^R, PEN^R, MEM^R	3 (2.2)	0.83

MEM: Meropenem, PEN: Penicillin G, OXA: Oxacillin, CAZ: Ceftazidime, CTX: Cefotaxime, TET: Tetracycline, DOX: Doxycycline, MIN: Minocycline, CLI: Clindamycin, ERY: Erythromycin, OFX: Ofloxacin, CIP: Ciprofloxacin, LEV: Levofloxacin, NAL: Nalidixic Acid, TMP: Trimethoprim-sulfamethoxazole, CHL: Chloramphenicol, KAN: Kanamycin, GEN: Gentamycin, Values in parenthesis denote percentage. MARI: multiple antibiotic resistance index

Table 7: Distribution of antibiotic-resistant genes

Antibiotic-resistant genes	No of phenotypically resistant isolates to the antibiotics used	Frequency of resistance genes screened (%)
*mec*A	78	78 (100)
*van*A	ND	12
*van*B	ND	0
*mph*C	36	23 (63.8)
*erm*A	36	20 (55.6)
*erm*B	36	4 (11.1)
*erm*C	36	11 (30.6)

ND = Not Determined

Discussion:

This study characterized staphylococci isolates from two food animals, cow and pigs, which are common source of animal proteins consumed in our environment. The most frequently identified staphylococci in descending order from the food animals in our study are *S. haemolyticus* (27.3%, n=38), *S. aureus* (19.4%, n=27)), *S. capitis* (15.1%, n=21), *S. epidermidis* (9.4%, n=13), *S. saprophyticus* (7.2%, n=10)*, S. xylosus* (5.0%, n=7) and *S. warneri* (2.9%, n=4). This is different from the pattern in a similar study by Chajecka-Wierzchowska et al., (36), where the most frequently identified staphylococci were *S. xylosus* (n=29, 50%), *S. epidermidis* (n=16, 27.6%), *S. lentus* (n=7, 12.1%), *S. saprophyticus* (n=4, 6.9%), *S. hyicus* (n=1 1.7%) and *S. simulans* (n=1 1.7%). Although *S. xylosus, S. epidermidis* and *S. saprophyticus* were identified in both studies, *S. haemolyticus, S. aureus* and *S. capitis*, the three most frequently isolated staphylococci in our study were absent in Chajecka-Wierzchowska et al., study while *S. hyicus, S. simulans* and *S. lentus* isolated in their study were completely absent in our study.

In the study by Taponen et al., (37) on bovine mastitic milk, the most common coagulase negative staphylococci species identified were *S. simulans*, *S. epidermidis*, *S. chromogenes* and *S. haemolyticus* which are similar to the ones from our study on food animals with respect to *S. epidermidis* and *S. haemolyticus*. Also, in the study by Beyene et al., (38) on 193 samples collected from abattoir and dairy farms, 92 (47.7%) were positive for *Staphylococcus* species with *S. aureus* (n=31; 16.1%), *S. intermedius* (n=21; 10.9%), *S. hyicus* (n=16; 8.3%), and other coagulase negative staphylococci (n=24; 12.4%). The differences in the species of staphylococci identified in different studies may be related to geographical distribution and methods employed in identification of the species from the animals.

There have been reports of alarming high levels of *S. aureus* resistance to commonly used antimicrobials such as tetracycline and penicillins (including amoxicillin) in cows (39, 40). The high resistance of staphylococci isolates in our study to penicillin (86%), tetracycline (73%), sulfamethoxazole-trimethoprim (72%), cefoxitin (surrogate for oxacillin, 56%), and ceftazidime (52%) agrees with reports from earlier studies (39, 40), which suggest that antimicrobial resistance must have developed in the staphylococci isolates occasioned by indiscriminate and prolonged use of antimicrobials. Chajecka-Wierzchowska et al., (36) reported that most of the staphylococci isolates from ready-to-eat food of animal origin in their study were resistant to cefoxitin (41.3%), clindamycin (36.2%), tigecycline (24.1%), rifampicin (17.2%) and erythromycin (13.8%). Majority of the staphylococci isolates from Beyene et al., (38) study also demonstrated resistance to cefoxitin (55.8%), vancomycin (65.1%), cloxacillin (79.1%), nalidixic acid (88.4%) and penicillin G (95.3%). These largely agree with some of the findings in our study.

The staphylococci isolates in Beyene et al., (38) study were multidrug resistant, exhibiting resistance to more than three antibiotic classes, which agrees with findings of the present study, with about 72% of the staphylococci isolates showing resistance to three or more classes of antibiotics. The multidrug resistance rate in our study is however higher that the 32.2% reported by Chajecka-Wierzchowska et al., (36). The predominant multidrug resistance phenotype reported from 46 isolates reported by Li et al., (41) was penicillin-ampicillin-kanamycin-gentamicin-tetracycline but this differs from the commonest phenotype, penicillin-tetracycline-nalidixic acid, reported in the current study.

Globally, livestock farming has improved food production at a reduced cost per unit produced with several pitfalls from increased antimicrobial resistance. This present study has further strengthened the fact that food animals can act as reservoir for antimicrobial resistant *Staphylococcus* species. Linking antimicrobial ingestion in food animals to drug-resistant infections of humans is intrinsically complex due to the environmental nature of the selection pressure for antibiotic-resistant pathogens as well as the occurrence of non-specific routes of transmission throughout the environment. An increasing body of evidence has emerged to strengthen the fact that repeated usage of antimicrobials in intensive livestock farming systems lead to antimicrobial resistance, which is of clinical importance in human medicine (42, 43).

The resistance of *S. aureus* and other staphylococci isolates to beta-lactams such as penicillin G and oxacillin is very evident. Resistance to Penicillin G is a significant concern since this antibiotic is the major antibiotic group that is recommended for staphylococcal mastitic infection. The frequent therapeutic usage of antibiotics in cows may lead to selection and dissemination of resistant strains even as Jaims et al., (44) reported that the development of antimicrobial resistance occurs from repeated therapeutic and/or indiscriminate use of antimicrobials. Resistance to antibiotics is

usually expressed on mobile genetic elements such as plasmids and transposons that can be disseminated from one staphylococcal species to another (45). *S. aureus* resistance to penicillin G is due to the production of beta lactamase enzyme carried on transmissible plasmids, which inactivates penicillin and other beta-lactam anti-microbials. This study also demonstrated the occurrence of macrolide resistance both phenotypically and genotypically. Resistance to macrolide and lincosamide has previously been reported in coagulase negative staphylococci (CoNS) including *S. epidermidis* recovered from cows with mastitis (46).

In this study, all phenotypically methicillin resistant staphylococci (cefoxitin resistance) carried the *mec*A gene while 64%, 56%, 11% and 31% of the isolates that were phenotypically resistant to erythromycin respectively carried the macrolide resistance genes; *mph*C, *erm*A, *erm*B and *erm*C. This is similar to the findings of Chajecka-Wierzchowska et al., (36) where all the MRSA strains in their study also harboured *mec*A gene but the erythromycin resistant isolates carried only the *erm*C gene. However, 84% of *mec*A-positive strains reported by Vyletelova et al., (47) expressed resistance to cefoxitin in the disk diffusion test. In the study by Couto et al., (48) conducted on animals over a 16 year period, the *mec*A gene was identified in 11.6% of the staphylococcal isolates which included MRSA (40.7%), methicillin resistant *Staphylococcus pseudintermedius* (8.7%) and methicillin-resistant coagulase negative staphylococci (26.7%). The prevalence of *mec*A gene in their study was low compared to our study and this difference could be related to differences in the food animals studied. Saputra et al., (49) also reported an overall low frequency of *mec*A gene among *S. pseudintermedius* and *S. aureus* as 11.8% and 12.8% respectively from animals but Ruzauskas et al., (50) reported 20 of 21 *mec*A gene in methicillin resistant staphylococci obtained from 395 clinical samples of diseased animals while the remaining one (1) isolate was positive for *mec*C gene.

The *mec*A gene encodes abnormal penicillin-binding protein 2a or 2' (PBP2a or PBB2') which has a low binding affinity for β-lactam antibiotics. Therefore, this group of antibiotics is not effective against bacteria expressing *mec*A gene. Expression of *mec*A gene however depends on a number of factors such as media type, pH, incubation temperature and presence of beta-lactam agents in the medium (51). The gene may therefore remain silent and unexpressed if these optimum conditions are not met. Other possibility includes mutations in the promoter or coding region of the gene. In addition, staphylococcal isolate may carry another *mec* gene types such as *mec*B, *mec*C or *mec*D, which may also express abnormal PBPs that can cause methicillin resistance (50).

Vancomycin has often be regarded as the last line of antibiotic for staphylococci infections as most isolates have been reported to be sensitive to the antibiotic (52). However, findings from our study revealed that some staphylococci carried *van*A, the gene that has been reported to be responsible for high level resistance to vancomycin in *S. aureus* (53). We could not test our isolates against vancomycin with the CLSI recommended broth dilution or E-test method (23) because this was not available in our centre at the time of this study. This resistant strain (vancomycin resistant *S. aureus*, VRSA) could constitute another important challenge to public health in the near future.

Conclusion:

Antibiotic resistance in pathogens is usually associated with mobile genetic elements such as plasmids, conjugative transposons and integrons (54). Selection and proliferation of antibiotic-resistant strains can occur, and these can be spread to the environment through animal wastes leading to increase in the resistance reservoir pool in the environmental microbiome (55). Findings from our study revealed a high prevalence of antibiotic-resistant *Staphylococcus* species in food-producing animals in Benin City, Nigeria, which could have resulted from overuse of antibiotics which acts as selection pressure and from poor hygiene practices of the animal handlers which is responsible for spread of the resistant pathogens. Improving hygienic measures in handling of food-producing animals and stopping the routine use of antibiotics as prophylactic, therapeutic or growth promoters in animal feeds or water are crucial public health measures.

Conflicts of Interest:

Authors declare no conflict of interest

Acknowledgements:

Authors are grateful to 2013/2014 Tertiary Education Trust fund (TETFUND) Nigeria, Research Project Intervention [Year 2015 TETFUND Research Project Fund 8th Batch] for financial support

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