Multi-drug resistance traits of methicillin-resistant *Staphylococcus aureus* and other Staphylococcal species from clinical and environmental sources

Adegboyega O. Oladipo, Oluwatosin G. Oladipo and Cornelius C. Bezuidenhout

ABSTRACT

Multi-drug resistance traits of *Staphylococcus* species especially methicillin-resistant *Staphylococcus aureus* (MRSA) in the clinical settings are well established. Of environmental concern is hospital effluents discharging into wastewaters. This article investigated the prevalence and detection of antibiotic resistance genes in *Staphylococcus* species from clinical and environmental sources in Ile-Ife, Nigeria. Standard culture-based and molecular protocols were used. Seventy-six (27 clinical, 14 hospital effluent and 35 environmental) *Staphylococcus* isolates were recovered: 56.58% were coagulase-negative and 43.42% coagulase-positive (*S. aureus*). For the clinical isolates, 10, 6, 4, 4 and 1 were isolated from urine, skin, wounds, blood and pus, respectively. Isolates were resistant to methicillin and amoxycillin (91.7%), cloxacillin (88.0%), ciprofloxacin (84.0%), ofloxacin (83.3%), azithromycin (78.0%), ceftazidime (76.0%), gentamycin (75.0%), cefuroxime (75.0%) and erythromycin (72.0%). Nearly, all isolates (90.8%) had multiple antibiotic resistance (MAR) index >0.2. Overall MAR indices for *Staphylococcus* species isolated from the clinical, hospital effluent and environmental wastewaters were relatively similar (0.482; 0.500; 0.435). *mecA*, *nuc* and *luk-pvl* genes were detected in *S. aureus*, while *mecA* was detected in *S. arlettae*, *S. sciuri*, *S. cohnii*, *S. epidermidis* and *S. saprophyticus*. This study informs on the potential contamination of environmental waters downstream from hospitals and possible impacts that this could have on human and animal health.

Key words | antibiotic resistance genes, coagulase-negative *Staphylococcus* species (CoNS), hospital effluents, MRSA, receiving wastewaters, *Staphylococcus* species

Adegboyega O. Oladipo
Oluwatosin G. Oladipo
Cornelius C. Bezuidenhout (corresponding author)
Unit for Environmental Sciences and Management: Microbiology,
North-West University,
Private Bag X6001, Potchefstroom 2520,
South Africa
E-mail: *carlos.bezuidenhout@nwu.ac.za*

Adegboyega O. Oladipo
Department of Medical Microbiology and Parasitology,
Obafemi Awolowo University Teaching Hospitals Complex (OAUTHC),
P.M.B 5538, Ile-Ife,
Nigeria

INTRODUCTION

Currently in human medicine, antibiotics are the most effective antimicrobials used for combatting bacterial infections. However, the uncontrolled, excessive and misuse of antibiotics has played a key role in the selection of antibiotic-resistant bacteria (ARB) (Finley *et al.* 2013; Tripathi & Tripathi 2017; Jensen *et al.* 2019).

Hospital effluents have been identified as a major reservoir for pollutants such as pharmaceutical wastes (antibiotics) and pathogenic microorganisms (Huang *et al.* 2019). Studies have reported that the discharge of hospital wastewaters is a highly selective route for the dissemination of ARB into natural environments (receiving water bodies) (Yilmaz *et al.* 2017; Kumar *et al.* 2019). However, the effect of antibiotics as environmental pollutants has been largely overlooked (Moges *et al.* 2014; Sabri *et al.* 2018). According to Finley *et al.* (2013) and Burgmann *et al.* (2018), there are no regulations guiding the release of antibiotic residues into wastewaters from hospital effluents. It is documented

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that only a few countries recommend the pre-treatment of hospital effluents before they are discharged into receiving water bodies (Szekeres *et al.* 2017).

Staphylococcus species are among the most frequently encountered bacteria in hospital settings and have been incriminated for many infections in humans such as skin and soft tissue infections, surgical site/wound infections, pneumonia, septicaemia and bone infections (Nanoukona *et al.* 2017). The detection of *Staphylococcus* species in hospital wastewaters had been reported by various authors (Gómez *et al.* 2017; Tripathi & Tripathi 2017). It has been demonstrated that pathogenic *Staphylococcus* species could be resistant to a wide array of antibiotics (WHO 2017). *Staphylococcus* spp. comprise of two major groups – coagulase-positive Staphylococci (CoPS) and coagulase-negative Staphylococci (CoNS) (Casey *et al.* 2009). The CoPS include *Staphylococcus aureus* as the major pathogen. There is now increasing evidence that some of the CoNS species (*S. cohnii*, *S. epidermidis*, *S. haemolyticus*, *S. warneri* and *S. xylosus*) could also be pathogenic and cause nosocomial infections (Tayyar *et al.* 2015; Hitzenbichler *et al.* 2017).

S. aureus and specifically methicillin-resistant *S. aureus* (MRSA) have been isolated from wastewater effluent (Börjesson *et al.* 2009, 2010; Rosenberg Goldstein *et al.* 2012; Ekwanzala *et al.* 2018). In addition, the presence and multi-drug resistance of CoNS in reclaimed water had also been confirmed (Goldstein *et al.* 2017). Among the antibiotics to which *Staphylococcus* species exhibit resistance are ampicillin and methicillin (Moges *et al.* 2014; Delorme *et al.* 2017). Susceptibility/sensitive to vancomycin and imipenem is still being recorded among large numbers of isolates (Tayyar *et al.* 2015; Hitzenbichler *et al.* 2017; Premanadham *et al.* 2017).

Antibiotic resistance could be the result of mutations or the acquisition of antibiotic resistance genes (ARGs) due to horizontal gene transfer (HGT) (Calero-Caceres *et al.* 2017). HGT, thus, facilitates the inter- and intraspecies transfer of antibiotic resistance and virulence genes (Tripathi & Tripathi 2017; Lerminiaux & Cameron 2019).

The detection of these *Staphylococcus* species in wastewaters is an important environmental risk which may result in the contamination of natural water reservoirs (groundwaters) and may eventually lead to public health challenges. In West Africa and particularly Nigeria, there is a paucity of data on the detection of *Staphylococcus* species in hospital effluents and the interplay with receiving wastewaters.

The aim of this study was therefore to investigate the prevalence of MRSA in clinical and environmental (hospital effluents and surrounding receiving wastewaters) sources in the South-western region of Nigeria and to determine antibiotic resistance profiles and *mecA*, *nuc* and *luk-pvl* genes in *Staphylococcus* species.

MATERIALS AND METHODS

Study location

This study was carried out at a hospital in Ile-Ife (7°16′48″N, 4°20′24″E), Osun State, South-western Nigeria. The medical institution provides health care services to about two million inhabitants in Ile-Ife and Southwest Nigeria.

Sample collection

Isolates from clinical samples

Isolates from various clinical samples were used and included wounds, skin, pus, urine, bones and blood. These were samples from different wards in the hospital submitted to the Microbiology Laboratory Unit for analysis. Clinical isolates were obtained from the Laboratory over a period of 6 months between May and October 2017. The clinical isolates were aseptically cultured on appropriate MacConkay agar (Difco™ MacConkey Agar, New Brunswick, NJ, USA) and blood agar (Blood agar base, Oxoid Ltd, Hampshire, UK) to select for the growth of staphylococci. Purification and growth on Mannitol salt (Biotec Laboratories, Kentford, UK) and MRSA CHROMagar base (CHROMagar™ MRSA-ITK Diagnostics BV, Uithoorn, The Netherlands) agars were to enhance the growth of *S. aureus* and MRSA, respectively. Isolates were incubated on the two media at 33 and 37 °C, respectively, for 24–48 h following standard protocols (Cheesbrough 2006; Igbinosa *et al.* 2016).

Processing of water samples

Environmental samples were collected from wastewater generated within the hospital facility (hospital effluents). Water

samples were also collected downstream at three receiving wastewater bodies within proximity (2 km) of the hospital. Each receiving wastewater body was approximately 750 m from each other. Sampling was done weekly over a period of 6 months for the sole purpose of collecting as many *Staphylococcus* isolates as possible. The water samples were collected in sterile bottles, preserved in cooler boxes and stored at 4 °C until analysed (within 24 h). Once at the laboratory, the water samples were filtered using sterile 0.45 μm membrane filters. These filters were enriched within Bacto tryptic soy broth (soybean-casein digest medium) (Becton Dickinson, USA). They were then placed on Mannitol salt agar (Biotec Laboratories, Kentford, UK) and incubated for 24 h at 33 °C. Resulting yellow colonies were presumptively considered as *S. aureus*. The yellow colonies were further plated on MRSA CHROMagar base (CHROMagar™ MRSA-ITK Diagnostics BV, Uithoorn, The Netherlands) to obtain pure isolates. These were at 37 °C for 48 h.

Isolation of *Staphylococcus* species

Clinical and environmental isolates were grown on Mannitol salt agar and chromogenic agars. Those that provided both the characteristic yellow and purple colours, respectively, were included in further analyses. These were subjected to Gram stain and identified as Gram-positive cocci with grape-like clusters under a microscope. *S. aureus* including MRSA and other *Staphylococcus* species were confirmed with the coagulase and catalase tests using standard protocols (Cheesbrough 2006; Igbinosa *et al.* 2016).

Antimicrobial susceptibility testing

All isolated and identified *Staphylococcus* species were then subjected to antibiotic susceptibility testing using the standard Kirby-Bauer's disk diffusion technique (CLSI 2014).

Partial 16S rRNA gene-based identification of isolates

Extraction of genomic DNA

The Nucleospin® tissue extraction kit (Macherey-Nagel, Düren, Germany) was used to extract genomic DNA from the isolates following the manufacturer's instructions. Briefly, pure isolates were incubated overnight in Nutrient Broth (Biolab Diagnostics, RSA) at 37 °C. About 2 mL of samples from the broth cultures were dispensed into a 2 mL microfuge tube and centrifuged at 8,000 rpm for 5 min at room temperature, the supernatant discarded, and the pellet treated, following the Macherey-Nagel Nucleospin® tissue extraction kit instructions. The quality and integrity of extracted DNA products were verified on 1% agarose gels, and images were captured using a GeneGenius Bioimaging system (Syngene, Cambridge, UK) and GeneSnap software V. 2.2.2 (Syngene, Cambridge, UK). DNA purity and concentration were verified using NanoDrop spectrophotometer (ND-1000, NanoDrop Technologies Inc., Wilmington, DE, USA).

PCR amplification

Polymerase chain reaction (PCR) was performed in a C1000TM thermal cycler (Bio-Rad, Hercules, CA, USA), and amplification of the 16S rRNA gene was done using universal primer sets 27F and 1492R with PCR conditions (Table 1), as previously described (Lane 1991). Each PCR reaction including positive and negative controls contained 12.5 μL of 2x PCR Master mix (Thermos Scientific Technologies, Waltham, MA, USA), 50 ng DNA template, 5 μM each of forward (27F) and reverse (1492R) primers and nuclease-free water to a final volume of 25 μL.

Sequencing of 16S rRNA genes

Sequencing of purified PCR products was carried out using the Big Dye terminator V. 3.1 cycle sequencing kit (Applied Biosystems, Warrington, UK) on a 3130 Genetic analyzer (Applied Biosystems/Hitachi, Tokyo, Japan). Generated sequence electropherograms were then manually edited after inspection using Finch TV 1.4.0 (http://www.geospiza.com/Products/finchtv.shtml). Edited sequences were aligned against other sequences for comparison with the Basic Local Alignment Search Tool (BLAST) program alignment tool of the GenBank on the National Centre for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/). Multiple sequence alignment was performed using MUSCLE (Edgar 2004) integrated into MEGA V. 7.0 (http://www.megasoftware.net/; Kumar *et al.* 2016). Phylogenetic sequence dendrograms with closely related sequences in GenBank

Table 1 | Primers used for the identification of *Staphylococcus* species and the detection of marker genes

Primers	Primer sequence (5′–3′)	PCR conditions	Size (bp)	References
27F 1492R	5′GAGTTTGATCATGGCTCAG3′ 5′GGTTACCTTGTTACGACTT3′	1 cycle of 2 min at 95 °C; 35 cycles of 30 s at 94 °C; 30 s at 53 °C; 1 min at 72 °C; 1 cycle of 10 min at 72 °C	1,500	Lane (1991)
*mec*A-F *mec*A-R	5′AACGATTGTGACACGATAGCC3′ 5′GGGATCATAGCGTCATTATC3′	1 cycle of 5 min at 94 °C; 30 cycles of 30 s at 94 °C; 30 s at 55 °C; 1 min at 72 °C; 1 cycle of 10 min at 72 °C	527	Kumar *et al.* (2016)
nuc-1 *nuc*-2	5′TCAGCAAATGCATCACAAACAG3′ 5′CGTAAATGCACTTGCTTCAGG3′	1 cycle of 5 min at 94 °C; 35 cycles of 30 s at 94 °C; 30 s at 55 °C; 1 min at 72 °C	255	Othman *et al.* (2014)
luk-F *luk*-R	5′ATCATTAGGTAAATGTCTGGCA TGATCC3′ 5′AGCATCAAGTGTATTGGATAGC AAAAGC3′	1 cycle of 4 min at 94 °C; 30 cycles of 45 s at 94 °C; 1 min at 72 °C; 1 cycle of 2 min at 72 °C	433	McClure *et al.* (2006)

were constructed with the neighbor-joining tree method using the substitution model. The partial 16S rRNA sequences from this study are available in the GenBank under the accession numbers: KY290702-KY290711; KY290719-KY290745 and MK208534-MK208572.

PCR amplification of *mecA*, *nuc* and *luk-pvl* genes

For MRSA differentiation, PCR amplification of the *mecA* gene (that encodes for methicillin resistance), the thermonuclease (*nuc*) gene and the *luk-pvl* gene that encodes for virulence * in *S. aureus* species was performed. Each PCR reaction contained 12.5 μL of 2x PCR Master mix (Thermos Scientific Technologies, Waltham, MA, USA), 50 ng DNA template, 5 μM each of forward and reverse primers and nuclease-free water to a final volume of 25 μL. Information on the primers and conditions are detailed in Table 1. DNA amplification was performed in a C1000 thermocycler (Bio-Rad, Hercules, USA). Electrophoresis of the amplicons were performed with 1% w/v agarose gel and visualized with GelRed staining under a UV transilluminator.

Statistical analyses

Antimicrobial resistance data were analysed using WHONET 2017 software V. 5.6 (WHO; http://www.whonet.org/software.html). The multiple antibiotic resistance (MAR) index for individual isolates was calculated and interpreted according to Krumperman (1983) using the formula:

$$\text{MAR index per isolate} = \frac{\text{the number of antibiotics to which the test isolate showed resistance to}}{\text{the total number of antibiotics tested}}$$

MAR index values greater than 0.2 indicate high-risk source of contamination where antibiotics are often used (Krumperman 1983). The MAR index for each of the three compartments (clinical, hospital wastewater and environmental water) was determined using the method of Krumperman (1983) using the formula:

$$\text{MAR index per compartment} = \frac{\text{number of isolates in a specific sample population resistant to antibiotics}}{(\text{number of antibiotics tested}) \times (\text{total number of organisms in sample})}$$

Multiple sequence alignment was performed using MUSCLE (Edgar 2004) integrated into Molecular Evolutionary Genetics Analysis (MEGA) V. 7.0 (Kumar *et al.* 2016).

RESULTS

Prevalence of *Staphylococcus* strains isolated from clinical and environmental samples

Over the sampling period, a total of 360 bacterial isolates were obtained, of which 140 isolates were presumptively

and phenotypically identified as Gram positive. Further biochemical testing confirmed 76 of the total number of samples collected both clinical and environmental isolates were *Staphylococcus* species.

Phenotypic and taxonomic identification of *Staphylococcus* strains

This study further confirmed the identities of the *Staphylococcus* species obtained from the clinical and environmental sources by 16S rRNA gene sequencing analysis. Of *Staphylococcus* species ($n = 76$), 56.58% were coagulase-negative (CoNS) and 43.42% coagulase-positive (*S. aureus*). Twenty-seven of these were from clinical sources, 14 from hospital effluents and 35 isolates originated from environmental (wastewater) sources. Among the clinical isolates, 10 were from urine, 4 from wounds, 6 from skin, 4 from blood and 1 isolate from pus. Environmental samples constituted 40% ($n = 14$) from the hospital generated wastewaters (effluents) and 60% ($n = 21$) from surrounding wastewater bodies at proximity to the hospital.

Distribution of Staphylococcal species

A total of 11 different *Staphylococcus* species were obtained in this study (Figure 1). Forty-three percent of the isolates were identified as *S. aureus*. Other species included *S. sciuri* (17.1%) and *S. cohnii* (11.8%) as well as *S. arlettae*, *S. epidermidis* and *S. saprophyticus* (6.5%). There were single representatives of *S. equorum*, *S. warneri*, *S. xylosus* and *S. kloosii*.

In terms of species distribution, 78.87% of the *Staphylococcus* strains obtained were found in both clinical and environmental sources (Figure 1). These were *S. aureus*, *S. cohnii*, *S. sciuri*, *S. haemolyticus*, *S. arlettae*, *S. saprophyticus* and *S. epidermidis*. On the other hand, *S. sciuri* and *S. haemolyticus* were isolated more frequently from environmental water sources. It was observed that four of the species were found either in the clinical or environmental source but not in both. Among these species were *S. equorum* (found only in clinical sources) and *S. warneri*, *S. kloosii* and *S. xylosus* occurring only in environmental samples. Furthermore, three *Staphylococcus* species exclusively found in environmental sources were isolated from the hospital effluents (Table 2). These observations are not

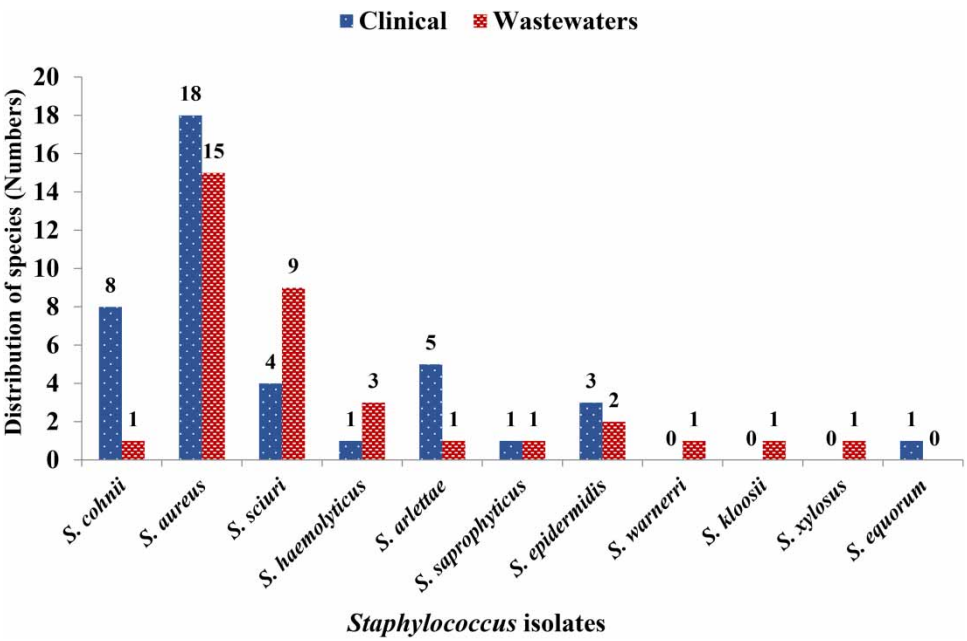

Figure 1 | Distribution of *Staphylococcus* isolates obtained from clinical and wastewater samples.

Table 2 | Identification, source, antibiotic resistance pattern and MAR indices of *Staphylococcus* species isolated from clinical and environmental samples

					Antibiotics														
S No.	Isolates	Accession number	Sources	Sample type	FOX	OFX	ERY	CLO	CRM	GEN	IPM	AZM	CAZ	AMC	AUG	CIP	VAN	R	MAR per isolate
1.	*S. cohnii*	KY290719	Clinical	Wound	R	R	S	R	R	R	S	R	S	R	R	R	S	9	0.7
2.	*S. arlettae*	KY290720	Clinical	Skin	S	R	R	R	R	R	S	S	S	R	R	R	S	8	0.6
3.	*S. equorum*	KY290721	Clinical	Pus	R	R	S	R	S	S	S	R	R	R	R	S	R	8	0.6
4.	*S. aureus*	KY290722	Clinical	Wound	R	R	S	R	S	R	S	R	R	R	S	S	S	7	0.5
5.	*S. aureus*	KY290723	Clinical	Skin	R	S	S	R	S	S	S	R	S	R	S	R	S	5	0.4
6.	*S. cohnii*	KY290724	Clinical	Wound	R	R	R	S	S	R	S	S	S	R	S	R	S	6	0.5
7.	*S. arlettae*	KY290725	Clinical	Urine	S	S	R	R	S	R	S	S	R	S	S	S	S	4	0.3
8.	*S. cohnii*	KY290726	Clinical	Wound	S	S	R	S	R	R	S	S	S	R	S	S	S	4	0.3
9.	*S. epidermidis*	KY290727	Clinical	Skin	S	R	R	R	S	R	S	R	R	R	S	R	S	8	0.6
10.	*S. sciuri*	KY290728	Clinical	Urine	S	S	S	R	R	R	S	R	R	R	S	R	R	8	0.6
11.	*S. saprophyicus*	KY290729	Clinical	Urine	R	S	R	S	R	R	S	S	S	R	S	R	R	6	0.5
12.	*S. aureus*	KY290730	Clinical	Urine	R	R	R	S	R	R	S	S	S	R	S	S	S	6	0.5
13.	*S. cohnii*	KY290731	Clinical	Urine	S	S	R	S	R	S	S	S	S	R	R	R	R	6	0.5
14.	*S. aureus*	KY290732	Clinical	Blood	R	R	S	R	S	S	S	S	S	S	S	S	S	3	0.2
15.	*S. arlettae*	KY290733	Clinical	Urine	R	R	S	S	S	R	R	S	S	R	S	R	R	6	0.5
16.	*S. cohnii*	KY290734	Clinical	Skin	S	R	R	R	R	R	R	R	R	R	R	R	S	11	0.8
17.	*S. aureus*	KY290735	Clinical	Bone	S	S	R	R	S	S	S	S	S	R	R	S	S	3	0.2
18.	*S. cohnii*	KY290736	Clinical	Blood	R	R	S	R	R	S	S	S	S	R	S	S	S	5	0.4
19.	*S. sciuri*	KY290737	Clinical	Urine	R	R	S	S	S	R	S	S	S	R	S	S	S	4	0.3
20.	*S. haemolyticus*	KY290738	Clinical	Skin	R	R	S	R	R	S	R	S	R	S	S	R	S	7	0.5
21.	*S. sciuri*	KY290739	Clinical	Urine	R	R	S	S	S	S	S	S	S	R	S	S	R	4	0.3
22.	*S. sciuri*	KY290740	Clinical	Urine	R	R	S	R	S	S	S	R	R	R	S	S	S	6	0.5
23.	*S. aureus*	KY290741	Clinical	Bone	R	R	S	R	S	R	S	R	R	R	S	R	R	9	0.7
24.	*S. cohnii*	KY290742	Clinical	Urine	R	R	R	R	R	R	R	R	R	R	R	R	S	12	0.9
25.	*S. cohnii*	KY290743	Clinical	Blood	S	S	S	R	R	S	S	R	R	R	S	S	S	5	0.4
26.	*S. epidermidis*	KY290744	Clinical	Skin	R	R	S	S	S	R	S	S	S	R	R	S	S	5	0.4
27.	*S. arlettae*	KY290745	Clinical	Blood	S	S	S	R	S	R	S	S	S	R	S	R	S	4	0.3
MAR index for clinical isolates = 0.482																			
28.	*S. aureus*	KY290702	Hospital wastewater	Effluent	R	R	S	S	R	R	S	S	R	R	S	S	S	6	0.5

(*continued*)

Table 2 | continued

S No.	Isolates	Accession number	Sources	Sample type	Antibiotics														MAR per isolate
					FOX	OFX	ERY	CLO	CRM	GEN	IPM	AZM	CAZ	AMC	AUG	CIP	VAN	R	
29.	*S. sciuri*	KY290703	Hospital wastewater	Effluent	S	S	R	S	S	R	S	R	S	R	R	R	R	7	0.5
30.	*S. haemolyticus*	KY290704	Hospital wastewater	Effluent	S	S	R	S	S	S	S	R	S	R	R	R	R	6	0.5
31.	*S. arlettae*	KY290705	Hospital wastewater	Effluent	R	R	R	S	R	R	S	S	S	S	R	S	S	6	0.5
32.	*S. saprophyticus*	KY290706	Hospital wastewater	Effluent	R	R	S	R	R	S	S	S	S	R	R	S	R	7	0.6
33.	*S. epidermidis*	KY290707	Hospital wastewater	Effluent	S	R	S	R	S	R	S	R	R	R	S	R	S	7	0.6
34.	*S. epidermidis*	KY290708	Hospital wastewater	Effluent	S	S	R	S	S	S	S	R	R	R	R	R	S	6	0.5
35.	*S. warneri*	KY290709	Hospital wastewater	Effluent	S	S	R	R	S	S	S	R	S	R	R	S	S	5	0.4
36.	*S. Kloosi*	KY290710	Hospital wastewater	Effluent	R	R	S	S	S	S	S	S	R	R	S	S	S	4	0.3
37.	*S. xylosus*	KY290711	Hospital wastewater	Effluent	R	S	S	R	S	R	S	R	S	R	R	S	R	7	0.6
38.	*S. aureus*	MK208534	Hospital wastewater	Effluent	R	S	S	S	S	S	R	S	R	R	S	R	R	6	0.5
39.	*S. aureus*	MK208535	Hospital wastewater	Effluent	R	R	S	R	R	R	R	R	S	R	R	R	S	10	0.8
40.	*S. aureus*	MK208536	Hospital wastewater	Effluent	R	S	R	R	R	R	R	R	R	R	R	R	R	12	0.9
41.	*S. aureus*	MK208537	Hospital wastewater	Effluent	R	S	S	S	S	S	S	S	S	S	S	S	R	2	0.1
MAR index for hospital wastewater = 0.500																			
42.	*S. aureus*	MK208538	Environmental	Water	S	R	R	S	S	S	R	R	S	R	R	R	S	7	0.7
43.	*S. aureus*	MK208539	Environmental	Water	S	S	S	S	R	R	S	S	S	S	R	R	S	4	0.3
44.	*S. aureus*	MK208540	Environmental	Water	S	R	R	R	R	R	S	S	R	R	R	R	R	10	0.8
45.	*S. aureus*	MK208568	Environmental	Water	R	S	S	R	R	S	S	R	S	R	S	S	R	6	0.5
46.	*S. aureus*	MK208568	Environmental	Water	R	S	R	R	S	R	S	S	R	R	R	R	S	8	0.6
47.	*S. aureus*	MK208568	Environmental	Water	S	R	R	R	S	S	S	S	R	R	R	R	S	7	0.6
48.	*S. aureus*	MK208568	Environmental	Water	S	S	S	S	S	S	S	S	R	R	R	S	R	4	0.3

(*continued*)

Table 2 | continued

S No.	Isolates	Accession number	Sources	Sample type	Antibiotics														MAR per isolate
					FOX	OFX	ERY	CLO	CRM	GEN	IPM	AZM	CAZ	AMC	AUG	CIP	VAN	R	
49.	*S. aureus*	MK208545	Environmental	Water	S	S	S	S	S	S	S	S	S	R	S	S	S	1	0.1
50.	*S. aureus*	MK208546	Environmental	Water	R	S	S	R	R	R	R	S	R	R	R	R	S	9	0.7
51.	*S. aureus*	MK208547	Environmental	Water	S	R	S	R	S	S	R	S	S	R	R	S	S	5	0.4
52.	*S. aureus*	MK208548	Environmental	Water	R	R	S	S	R	S	R	R	R	R	R	R	S	9	0.7
53.	*S. aureus*	MK208549	Environmental	Water	R	S	R	R	S	R	R	R	S	S	R	S	R	8	0.6
54.	*S. aureus*	MK208550	Environmental	Water	R	R	R	R	S	R	S	R	S	S	R	S	S	7	0.5
55.	*S. aureus*	MK208551	Environmental	Water	S	S	S	S	S	S	S	S	R	R	R	S	S	3	0.2
56.	*S. aureus*	MK208552	Environmental	Water	S	S	S	S	S	S	S	S	S	R	R	S	S	2	0.1
57.	*S. haemolyticus*	MK208553	Environmental	Water	S	S	S	R	S	S	R	S	S	R	R	s	S	4	0.3
58.	*S. aureus*	MK208554	Environmental	Water	S	S	S	S	S	S	R	S	S	R	S	S	S	2	0.1
59.	*S. aureus*	MK208555	Environmental	Water	S	S	S	S	S	S	S	S	S	S	R	S	R	2	0.1
60.	*S. cohnii*	MK208556	Environmental	Water	R	R	R	R	S	R	S	R	R	R	R	S	R	10	0.8
61.	*S. epidermidis*	MK208557	Environmental	Water	S	S	S	R	R	S	S	S	R	R	S	S	R	5	0.4
62.	*S. epidermidis*	MK208558	Environmental	Water	S	R	S	R	R	S	S	S	R	R	R	S	S	6	0.5
63.	*S. aureus*	MK208559	Environmental	Water	S	R	R	S	S	R	R	S	S	R	R	R	S	7	0.5
64.	*S. epidermidis*	MK208560	Environmental	Water	S	S	S	S	S	S	S	S	S	R	S	S	S	1	0.1
65.	*S. epidermidis*	MK208561	Environmental	Water	S	S	R	S	S	S	S	S	S	R	S	S	S	2	0.1
66.	*S. epidermidis*	MK208562	Environmental	Water	S	R	R	S	S	S	S	R	R	S	S	S	S	4	0.3
67.	*S. epidermidis*	MK208563	Environmental	Water	S	S	S	S	S	S	S	R	S	R	R	S	R	4	0.3
68.	*S. aureus*	MK208564	Environmental	Water	R	S	R	R	R	R	R	S	R	R	R	R	R	11	0.8
69.	*S. epidermidis*	MK208565	Environmental	Water	S	R	R	R	R	R	S	S	S	R	R	R	S	8	0.6
70.	*S. haemolyticus*	MK208566	Environmental	Water	R	R	S	R	R	R	S	S	R	R	S	R	S	8	0.6
71.	*S. epidermidis*	MK208567	Environmental	Water	S	R	R	S	S	R	S	R	R	S	S	S	S	5	0.4
72.	*S. aureus*	MK208568	Environmental	Water	S	R	S	S	S	S	S	R	R	R	R	S	R	6	0.5
73.	*S. aureus*	MK208569	Environmental	Water	S	R	S	R	S	S	R	S	S	S	R	S	S	4	0.3
74.	*S. epidermidis*	MK208570	Environmental	Water	S	S	S	S	R	R	S	R	S	R	R	S	S	5	0.4
75.	*S. haemolyticus*	MK208571	Environmental	Water	S	S	S	S	R	R	S	S	R	R	R	S	R	6	0.5
76.	*S. aureus*	MK208572	Environmental	Water	S	S	R	R	R	R	S	S	S	R	R	R	R	8	0.6
MAR index for environmental receiving water = 0.435																			

Notes: R is the number of antibiotics an isolate was resistant to. FOX (Methicillin) (30 μg), OFX (Ofloxacin) (5 μg), ERY (Erythromycin) (15 μg), CLO (Cloxacillin) (5 μg), CRM (Cefuroxime) (30 μg), GEN (Gentamycin) (10 μg), IPM (Imipenem) (10 μg), AZM (Azithromycin) (15 μg), CAZ (Ceftazidime) (30 μg), AMC (Amoxycillin) (2 μg), AUG (Augmentin) (30 μg), CIP (Ciprofloxacin) (5 μg) and VAN (Vancomycin) (30 μg).

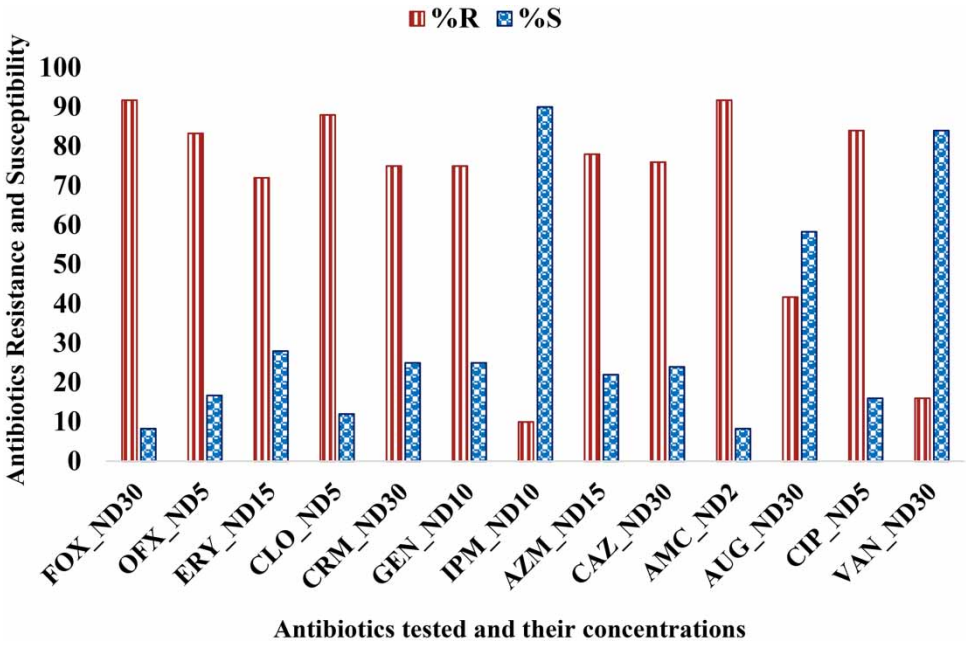

Figure 2 | Antibiotic resistance profile of *Staphylococcus* isolates. **Key**: R indicates 'resistant' and S 'susceptible'. FOX (Methicillin) (30 μg), OFX (Ofloxacin) (5 μg), ERY (Erythromycin) (15 μg), CLO (Cloxacillin) (5 μg), CRM (Cefuroxime) (30 μg), GEN (Gentamycin) (10 μg), IPM (Imipenem) (10 μg), AZM (Azithromycin) (15 μg), CAZ (Ceftazidime) (30 μg), AMC (Amoxycillin) (2 μg), AUG (Augmentin) (30 μg), CIP (Ciprofloxacin) (5 μg) and VAN (Vancomycin) (30 μg).

absolute and could be biased since the isolation process was a culture-based selective process. Nonetheless, it provided some insights into culturable staphylococci associated with hospital effluent, environmental water and a potential link to clinical staphylococci.

Antibiotic resistance patterns of *Staphylococcus* species

Many of *Staphylococcus* species were resistant to several classes of antibiotics (Table 2). The antibiotic(s) to which the highest number of isolates were resistant to included methicillin (the antibiotic of interest) and amoxycillin (91.7%). More than 80% were also resistant to cloxacillin, ciprofloxacin, ofloxacin and more than 70% to azithromycin, ceftazidime, gentamycin, cefuroxime and erythromycin (Figure 2). Forty-two percent were resistant to ceftriaxone. Among all these isolates, 90% and 84% were susceptible to imipenem and vancomycin, respectively. This implies that these antibiotics were still effective against the staphylococci from this region.

About 60% of the isolates were resistant to at least 6 of the 13 antibiotics (Table 2). Over 50% of the *S. aureus* isolates were methicillin-resistant (thus MRSA), while the others were susceptible to methicillin (MSSA).

Multiple antibiotic resistance

In this study, nearly all the isolates (69 of 76; 90.8%) had an MAR index higher than 0.2 while only 7 (9.2%) had an MAR index <0.2. The *Staphylococcus* isolates with an MAR index greater than 0.2 were mainly clinical isolates (Table 2). Furthermore, the mean overall MAR values of clinical, hospital wastewater effluents and environmental water were calculated and compared. The overall MAR index values for *Staphylococcus* species isolated from the clinical, hospital effluent and environmental wastewaters were 0.482, 0.500 and 0.435, respectively (Table 2). These MAR index values were relatively similar.

Detection of resistance and virulence genes in *Staphylococcus* species

The detection of resistance and virulence genes among the *Staphylococcus* species varied. It was observed that of the 11 different species, six (54.5%) tested positive for the

resistance gene – *mecA*. Of all 11 species, *S. aureus* was the only strain that was positive for all three genes. *S. sciuri* and *S. cohnii* (six isolates each), *S. arlettae* and *S. epidermidis* (five isolates each) and a single isolate of *S. saprophyticus* were positive for only the *mecA* gene but neither *nuc* nor *luk-pvl* genes. Overall, approximately 55.3% (42 out of 76) of the isolates analysed in this study were positive for the ARG – *mecA*. Neither resistance nor virulence genes were detected in *S. haemolyticus*, *S. equorum*, *S. warnerii*, *S. kloosii* and *S. xylosus* species.

The *mecA* gene was detected among 83.3% of the *Staphylococcus* species originated from clinical sources, while it was detected in 16.7% environmental samples. These were mainly from the hospital wastewater. Similarly, for the *nuc* gene, 54.5% of the strains that were positive for this gene were isolated from clinical sources, while 45.5% were isolated from wastewater sources. However, with the *luk-pvl* virulence gene, all the isolates were *S. aureus* from clinical sources. Largely, 63% of the isolates which were positive for the resistance and virulence genes originated from clinical samples while 37% were from environmental sources.

DISCUSSION

Studies have focussed on *S. aureus* and CoNS from clinical sources in West Africa (Nanoukona *et al.* 2017) and Nigeria (Shittu *et al.* 2012; Vitali *et al.* 2014). However, there is a paucity of data regarding these species in environmental sources (wastewater effluents and water bodies). In the present study, we found that 43.42% of isolates were coagulase-positive (*S. aureus*) and 56.58% coagulase-negative (CoNS). These isolates were from clinical, wastewater and environmental water samples. The 10 different CoNS species identified included – *S. cohnii*, *S. epidermidis*, *S. saprophyticus*, *S. haemolyticus*, *S. arlettae*, *S. scuiri*, *S. xylosus*, *S. warneri*, *S. equorum* and *S. kloosii*. Similar observations were made in previous studies. A study conducted by Gómez *et al.* (2017) on wastewaters in Spain demonstrated the occurrence of 16.67% *S. aureus* and 83.33% CoNS species. These authors observed 12 different species. Furthermore, Shittu *et al.* (2012) and Tayyar *et al.* (2015) found *S. haemolyticus*, *S. epidermidis*, *S. saprophyticus* and *S. xylosus* in clinical samples. Similarly, Nanoukona *et al.* (2017) identified *S. cohnii*, *S. sciuri*, *S. arlettae*, *S. warneri* and *S. kloosii* also in clinical samples. Gómez *et al.* (2017) found *S. epidermidis*, *S. sciuri*, *S. xylosus*, *S. equorum* and *S. warneri* in wastewater samples.

The present study demonstrated the occurrence of *S. cohnii* and *S. equorum* in clinical samples and *S. xylosus*, *S. warneri* and *S. kloosii* in wastewater sources. These results are corroborated by previous studies. Both Chen *et al.* (2015) and Gómez *et al.* (2016) suggested that the reason few studies have reported *S. cohnii* in wastewater is because they are mainly associated with nosocomial infections. Therefore, finding them in wastewater may suggest that their origin is from a clinical setting (Vitali *et al.* 2014; Hitzenbichler *et al.* 2017; Nanoukona *et al.* 2017). A study by Gómez *et al.* (2017) demonstrated that *S. xylosus* and *S. warneri* to occur in wastewaters. This is consistent with the findings from the present study. These observations confirmed that the *S. warneri* and *S. kloosii* could be present in hospital effluents. This may possibly suggest that they are likely to originate from clinical sources and then could be transported to environmental water bodies where they could be a potential health threat. *Staphylococcus equorum*, a very rare species could be present in wastewaters (Gómez *et al.* 2017). It had earlier been reported to also be associated with clinical scenarios (Nováková *et al.* 2006). *S. kloosii* isolated from wastewaters in this study was also previously isolated from clinical sources (Nanoukona *et al.* 2017). In the present study, various *Staphylococcus* sp. had been isolated from clinical samples, hospital effluent and receiving environmental waters. These findings were supported by previous studies showing similar trends. The prevalence of the nine different species found in these aquatic environmental habitats may be ascribed to their capability to survive under the existing conditions for extended periods (Šolić & Krustulović 1994; Tolba *et al.* 2008; Abulreesh 2011).

With respect to antimicrobial resistance, almost all the *Staphylococcus* species were resistant to several classes of antibiotics. Isolates were defined as multi-resistant based on resistance to methicillin and at least two other classes of antibiotics (Magiorakos *et al.* 2012). Specifically, over 50% of *S. aureus* isolates from both clinical and environmental sources were methicillin-resistant (MRSA), while the others were susceptible to methicillin (MSSA).

S. saprophyticus isolates were all resistant to methicillin. Furthermore, a strain of *S. cohnii* was resistant to 12 of the 13 antibiotics tested. This finding is supported by Delorme *et al.* (2017) and Nanoukona *et al.* (2017) that found *S. chonii* resistant to a large number of antibiotics. In the present study, it was demonstrated that most of the isolates (91.7%) were resistant to methicillin and amoxycillin. This trend had also been confirmed by previous studies (Moges *et al.* 2014; Tayyar *et al.* 2015; Premanadham *et al.* 2017). On the contrary, most of the *Staphylococcus* species were susceptible to vancomycin (84.0%) and imipenem (90.0%) demonstrating that these antibiotics are still useful in clinical settings. A similar trend of antibiotic susceptibility of *Staphylococcus* spp. had been documented in previous studies (Thomas *et al.* 2016; Hitzenbichler *et al.* 2017). The high susceptibility rate recorded for vancomycin and imipenem had been ascribed to the fact that these drugs are usually not commonly prescribed in the hospital settings (Thomas *et al.* 2016). This demonstrates that antibiotic stewardship and regulation may still delay the onset of antibiotic resistance, even in developing regions of the world. Vancomycin is a glycopeptide antibiotic that is seen as a drug of choice and a good therapeutic option for treating severe bacterial infections, especially multi-drug resistance MRSA strains (Hitzenbichler *et al.* 2017). Although the efficacy of vancomycin may be reduced when administered intravenously as a single dose, its combination with other antibacterial agents, such as imipenem, has given better treatment outcomes (Erjavec *et al.* 1994; Courvalin 2006).

Overall, all the species from *Staphylococcus* sp. displayed multiple resistance traits towards antibiotics. The CoNS in this study were resistant to a higher number of antibiotics compared with *S. aureus*. This result confirms the multi-resistance potential of *S. aureus* and CoNS to antibiotics used in clinical settings. Our finding is corroborated by Center *et al.* (2003) who reported that CoNS species generally have decreased susceptibilities to various antibiotics.

It was confirmed that strains of the same *Staphylococcus* species demonstrated similar antibiotic resistance/susceptibility patterns regardless of their isolation sources (clinical or environmental). Of note is the very high number of *Staphylococcus* species that had an MAR index greater than 0.2. Furthermore, the overall MAR values of clinical, hospital wastewater effluents and environmental water numerically relatively quite similar. These similar MAR indices may indirectly suggest that all the isolates were from the sources where antibiotics are often used. This also implies that the strains from the clinical/hospital wastewater effluent and environmental water might have a common origin (Krumperman 1983; Riaz *et al.* 2011). Furthermore, this index demonstrates frequent exposure of the bacteria to antibiotics of various classes (Krumperman 1983; Riaz *et al.* 2011). It could also indicate that ARGs may be well distributed among the bacteria in environmental wastewaters where there is the interphase of the clinical and environmental strains (Rosenberg Goldstein *et al.* 2012; Gómez *et al.* 2017). MAR in bacteria is most commonly associated with the presence of plasmids or other mobile elements which contain one or more resistance genes, each encoding a single antibiotic resistance phenotype (Riaz *et al.* 2011).

The detection of multi-drug resistance phenotype, with *mecA* (54.5%) and *nuc* (100%) resistance and virulence genes, respectively, were confirmed in *S. aureus* isolates. Only the *mecA* gene was detected in *S. arlettae*, *S. scuiri*, *S. cohnii*, *S. epidermidis* and *S. saprophyticus*. The detection of the *mecA* resistance gene in CoNS species had been reported by previous studies (Shittu *et al.* 2012; Vitali *et al.* 2014; Gómez *et al.* 2017). The *mecA* gene allows a bacterium to be resistant to antibiotics such as methicillin and penicillin. The most commonly known carrier of the *mecA* gene is the bacterium known as MRSA (Igbinosa *et al.* 2016). In addition, the detection of the virulence gene, *luk-pvl* was confirmed in the six *S. aureus* representatives isolated from clinical samples only. Panton–Valentine leukocidin (*PVL*) is considered one of the important virulence factors responsible for white cell destruction and as a marker of community-acquired MRSA (Yang *et al.* 2009; Igbinosa *et al.* 2016).

All *Staphylococcus* species isolated from wastewater sources were negative for the *luk-pvl* virulence gene. This trend has been confirmed in a previous study (Gómez *et al.* 2017). The detection of *mecA* resistance gene in CoNS had been ascribed to possible HGT by mobile genetic components (such as plasmids, intergrons and transposons) from *S. aureus* species, which could facilitate the environmental dissemination of the ARGs to CoNS

(Calero-Caceres *et al.* 2017; Tripathi & Tripathi 2017; Jensen *et al.* 2019). This scenario may be the case in the present study, where the hospital wastewater could be the source of *mecA* and HGT could be responsible for the dispersal of this gene to other staphylococci.

CONCLUSION

This study highlighted the prevalence and diversity of *Staphylococcus* spp. in both clinical and environmental sources. *Staphylococcus* spp. containing the *mecA* gene was detected in the water body receiving hospital wastewaters. Among the *mecA* positive isolates were *S. cohnii* a coagulase-negative staphylococci normally not considered as a pathogen in the clinical settings. All strains of the same *Staphylococcus* species displayed the same antibiotic resistance/susceptibility trend regardless of their isolation sources (clinical or environmental). This potentially shows that there may be a link between the various habitats. These findings on prevalence, diversity and multi-drug resistance staphylococci, particularly CoNS in wastewater and receiving water sources, are worrisome. Environmental water sources are used for agricultural, direct exposure (recreational) and drinking water production purposes. Finding these *Staphylococcus* species potentially from the clinical origin in environmental water is cause for concern. These bacterial species are normally not tested for in microbiological water quality analyses. This raises the issue of regulations and testing regimes of environmental water that receives hospital wastewater. It calls for stringent regulations and proper treatment of hospital effluents before disposal to receiving wastewaters. This would reduce the risks posed to human and animal health and safety and may as well also play an important role in mitigation of global antibiotic resistance, particularly of *mecA* associated resistance.

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ETHICAL STATEMENT

This study was approved by the ethics committee of the hospital used in Ile-Ife, Nigeria, under protocol number: SERC-2016-001-NWU-SA and Health Research Ethics Committee (HREC) of the Faculty of Health Sciences, North-West University, Potchefstroom, South Africa, under ethics number: NWU-00122-17-S1. All clinical staphylococcal isolates evaluated in this study were obtained from the Microbiology Laboratory of the Medical Institution, Ile-Ife, Nigeria, and were anonymized.

HEALTH AND SAFETY

The authors declare that all mandatory laboratory health and safety procedures and protocols were complied with while conducting the experimental work reported in this study.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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