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Enterococcus sp. Contamination Surveillance in Different Levels of Healthcare in eThekwini District, KwaZulu-Natal (KZN) South Africa

2021

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M.MedSci. (UKZN, SA)

Enterococcus sp. Contamination Surveillance in Different Levels of Healthcare in eThekwini District, KwaZulu-Natal (KZN) South Africa

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(**214584066**)

2021

A thesis submitted to the School of Health Sciences, College of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal, Medical School, for the degree of Doctor of Philosophy in Medicine (Medical Microbiology).

This is a thesis in which the chapters are written as a set of discrete research manuscripts accepted, submitted or intended for submission to internationally recognized peer-reviewed journals with an overall introduction and final summary.

This is to certify that the content of this thesis is the original research work of Mrs Christiana Omowunmi Shobo, carried out under our supervision at the Antimicrobial Research Unit (ARU), Discipline of Pharmaceutical Sciences, School of Health Sciences, and the Biomedical Research Unit (BRU), School of Laboratory Medicine and Medical Sciences, College of Health Sciences, Westville campus, University of KwaZulu-Natal (UKZN), Durban, South Africa.

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Declaration

I, **Mrs Christiana Omowunmi Shobo**, declare that

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Signed: 

Date: 14th April, 2021

Dedication

To my dearest parents

Mr John and Mrs Deborah Awojirin

Thank you for being the best parents anyone could ever ask for.

Continue to rest in the bosom of the Lord Almighty.

Acknowledgements

I would like to express my sincere appreciation to:

- The Almighty God for His grace and mercy towards the completion of this PhD study.
- My supervisors, Professor Sabiha Y. Essack and Dr Linda A. Bester for their unequalled mentorship, intellectual leadership and continuous positive support throughout the years of my PhD.
- Dr Daniel G. Amoako, Dr Anou M. Somboro and Dr Akebe L.K. Abia for their constant assistance, professional advice and guidance throughout this research.
- The entire Antimicrobial Research Unit (ARU) and Biomedical Resource Unit (BRU) team, for their continued unconditional support, I am truly blessed to have you all.
- My husband and my mentor, Dr Adeola Obafemi Shobo, for inspiring me throughout my research journey. Thank you for your unwavering support, positivity, and words of encouragement. I will forever be grateful. And to my wonderful kids, Demilade and Damisire, your sacrifices will never be forgotten.
- The Shobo's family, for being a strong pillar of support all these years, for the constant prayers and words of encouragement, thanks for always believing in me.
- My dearest late parents, Mr and Mrs John Awojirin, for teaching in me the core values of respect, humility, honesty and hard work (continue to rest in peace).
- My siblings: Abayomi and Odunayo Awojirin, your patience, support, and sacrifice are greatly appreciated.
- The Young Researchers Grant of the College of Health Sciences, University of KwaZulu-Natal for the financial support.

List of Manuscripts included in this thesis

1. **Christiana Omowunmi Shobo**, Sabiha Yusuf Essack, Linda Antionette Bester. Enterococcal contamination of hospital environments in KwaZulu-Natal South Africa.
   Status: Submitted to Journal of Appl. Microbiol. (submission ID: JAM-2020-2478)

2. **Christiana Omowunmi Shobo**, Arghavan Alisoltani, Akebe Luther King Abia, Philip Senzo Mtshali, Arshad Ismail, Oliver Zishiri, Juliana Deidre Horn, Petra Brysiewicz, Sabiha Yusuf Essack, Linda Antionette Bester. Bacterial diversity and functional profile of microbial populations on surfaces in public hospital environments in South Africa: A high throughput metagenomic analysis. **Sci. Total. Environ. 2020; 719: 137360**. https://doi.org/10.1016/j.scitotenv.2020.137360.
   Status: Published. A journal-specific format is included in the thesis (chapter 3).

3. **Christiana O. Shobo**, Daniel G. Amoako, Mushal Allam, Arshad Ismail, Sabiha Y. Essack, Linda A. Bester. Genome sequence of a novel *Enterococcus faecalis* sequence type 922 strain isolated from a door handle in the intensive care unit of a district hospital in Durban, South Africa. **Microbiol. Resour. Announc. 2019; 8 (35): e00582 – 19.** http://doi.org/10.1128/MRA.00582-19.
   Status: Published. A journal-specific format is included in the thesis (chapter 4).

4. **Christiana O. Shobo**, Daniel G. Amoako, Mushal Allam, Arshad Ismail, Sabiha Y. Essack, Linda A. Bester. Comparative genomics reveals the dominance of major clones of *Enterococcus faecalis* within public hospital environments in South Africa: insights into antibiotic resistome, mobilome and phylogenomics.
   Status: Submitted to Scientific Report (Submission ID: 70b91509-318f-4b9e-bacb-fbf53e8d11d1).

List of Peer-reviewed Publications not Included in this Thesis

1. Bren Kennedy, **Christiana O. Shobo**, Oliver T. Zishiri, Linda A. Bester. Surveillance of *Salmonella* species in the environment of public hospitals in Kwazulu-Natal, South **Africa. J. Hosp. Infect. (2020); 105 (2): 205-212.** https://doi.org/10.1016/j.jhin.2020.02.019.

2. Stephanie Pillay, Daniel G. Amoako, Akebe L. K. Abia, Anou M. Somboro, **Christiana O. Shobo**, Keith Perrett, Linda A. Bester and Sabiha Y. Essack. Characterization of *Campylobacter* spp. isolated from poultry in KwaZulu-Natal, South Africa. **Antibiotics. 2020; 9 (2): 42.** https://doi.org/10.3390/antibiotics9020042.

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List of Abbreviations and Acronyms

AMR	Antimicrobial resistance
AST	Antimicrobial susceptibility testing
ATCC	American Type Culture Collection
CAUTI	Catheter-associated urinary tract infection
CC	Clonal complex
CDC	Centres for Disease Control and Prevention
CGE	Centre for Genomic Epidemiology
CLSI	Clinical Laboratory Standards Institute
COVID 19	SARS-CoV-2 (also known as Coronavirus 19)
CONS	Coagulase-negative *Staphylococcus*
DNA	Deoxyribonucleic acid
ESBL	Extended-spectrum β-lactamase
GI	Gastro-intestinal
GNB	Gram-negative bacteria
GNO	Gram-negative organism
HAI	Hospital-acquired infections
HCW	Healthcare workers
HH	Hand hygiene
HLR	High level resistant
HPV	Hydrogen peroxide vapour
ICU	Intensive care unit
IPC	Infection prevention and control
IPCP	Infection prevention and control program
KEGG	Kyoto encyclopaedia of genes and genomes
KZN	KwaZulu-Natal
LRE	Linezolid resistant enterococci
MDR	Multidrug resistance

MDRO	Multidrug resistant organism
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
NARM	National Antimicrobial Resistance Monitoring system
OR	Operating room
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
PCC	Pearson's correlation coefficient
SA	South Africa
USA	United States of America
USDA	United States Department of Agriculture
UTI	Urinary tract infection
VAP	Ventilator-associated pneumonia
VRE	Vancomycin-resistant enterococci
WHO	World Health Organization
WGS	Whole-genome sequencing

Abstract

Hospital-acquired infections (HAIs) have been identified as long-standing setbacks affecting hospitals' quality of health care. While one of the major challenges related to HAIs is controlling cross-transmission, the role and significance of the inanimate hospital environment chain of transmission are yet to be unequivocally elucidated. Therefore, this study investigated the functional profile and diverseness of bacteria from various inanimate environmental sources, from two different wards in public hospitals at various healthcare levels in eThekwini District, KwaZulu-Natal, South Africa. True to the study focus on investigating the dissemination of bacteria from equipment within the hospital, the study further used *Enterococcus* as well-known HAI as target bacteria and described the molecular and genomic profiles of this specie isolated from the hospital environments.

Samples were collected for a period of three months (September – November 2017) from the four levels of healthcare in eThekwini district, KwaZulu-Natal. The intensive care unit and peadiatic ward were employed in this study. An overall of 620 swabs were collected from areas frequently touched by healthcare workers (HCWs) and patients. These sites include the occupied bed linen, unoccupied bed linen, drip stands, patient files, ward phones, ventilators, nurses' tables, blood pressure apparatus, sinks, linen room door handle and mops. Swabs were placed in Amies transport medium and transported in a cooler box to the laboratory facility to be processed within four hours. The collected swabs (n=620) were pooled and incubated in tryptone soya broth containing 6.5% NaCl at 36.5ºC for 24 hrs and subsequently plated on enterococci chromogenic media. The microbial diversity and functional profiles from the sites were identified using 16S rRNA metagenomics.

Positive colonies were sub-cultured on bile esculin azide agar, and screened using standard microbiological methods, including haemolytic, oxidase and catalase, and API. Identifications were confirmed with polymerase chain reaction (PCR) with the added genus-specific *tuf*-gene and species-specific *sodA*-gene. Antibiotic resistance patterns in the *Enterococcus* spp. isolates were determined by the Kirby-Bauer disk diffusion method against 14 antibiotics as recommended by the Clinical and Laboratory Standard Institute (CLSI) guidelines.

Thirty-seven samples from *E. faecalis* showed intermediate Resistance to vancomycin and were further analyzed using molecular tools viz. whole-genome sequencing (WGS) and bioinformatics analyses. This enabled determining the resistome, mobile genetic elements (MGEs), and clonal lineages circulating across the sites, wards, and hospitals. Metagenomics identified a total of 288 species, 190 genera, 105 families, 50 orders, 29 classes and 11 phyla from the samples analyzed. The dominant functional metabolic pathways implicated in causing human infection discovered were the signal transduction mechanisms, citrate cycle (TCA), transcription-factor bisphenol degradation, tyrosine metabolism. A total of 295 *Enterococcus* spp. isolates were recovered from the hospitals` environmental sites, 83% (n=245) were identified as *Enterococcus faecium*, 13% (n=38) as *Enterococcus faecalis*, 2% (n=6) *Enterococcus gallinarum* and another 2% (n=6) *Enterococcus casseliflavus*. Notably, the pediatric wards had the highest isolation rate compared to ICU, 64% and 36%, respectively. Overall, the sites with the highest isolation rate were occupied beds and mops (to clean ward floors) with 14.9% (n=44) each. The tertiary hospital were the most affected.

The most prominent MDR antibiogram for *E. faecium* was CIP-RIF-NIT-TET-ERY and for WGS analysis of the *E. faecalis* samples confirmed that the *tet*(M) and *erm*(C) genes were the prevalent antibiotic resistance genes found in hospitals. The isolates harboured mobile genetic elements consisting of plasmids (n =11) and prophages (n=14), predominantly clonally specific. The 37 isolates analyzed consisted of 15 clonal lineages with six major sequence types (ST). Phylogenomic analysis showed that major lineages were mostly conserved within specific hospital environments. This study highlighted the inanimate hospital environment as a possible source of opportunistic nosocomial pathogens using *Enterococcus* as an illustrative example and emphasized the urgent necessity to optimize infection prevention and control measures to intercept/moderate the spread of bacteria in the hospital environments.

Chapter 1 – Introduction and Literature Review

1.0 Introduction

Surfaces in hospital environments contain a diverse population of microorganisms that serve as a reservoir of potential pathogens that may be a significant role player in healthcare-associated infections (HAIs) (Haque *et al.*, 2018). The latter term is also commonly referred to as nosocomial infection, representing the variety of infections caused by an extended hospital stay and is the fifth leading threat for severe health problems leading to death (WHO, 2013). Generally, within 24-48 hrs of admission, the new patient's flora gain bacteria that features in the surrounding, making them vulnerable to other infections (Saka *et al.*, 2016). Examples of HAIs are bloodstream infections or urinary tract infections usually caused by invasive devices such as driplines or catheter, respectively, or an infected surgical wound following a surgical procedure (Russo *et al*., 2015).

The inanimate environments in hospitals are often contaminated with microorganisms. These organisms have adaptive features that enable them to live on inanimate surfaces and medical equipment for extended times. (Saka *et al.*, 2016). According to the Centres for Disease Control and Prevention (CDC), contact transmissions can be directly from the body surface or indirectly through contaminated inanimate objects within the hospital surroundings. These are some of the primary routes of pathogenic bacteria transmission in healthcare settings (Siegel *et al*., 2007).

According to Mauldin *et al.*, between five to fifteen percent of in-patients develop an infection during hospitalization, and critically ill patients in intensive care units (ICUs) are five to ten times more vulnerable to be infected with an HAI than patients admitted in general wards (Mauldin *et al*., 2010). HAIs lead to extended hospital stays, which leads to an increase of microbial drug resistance, to a substantial extra financial burden and, finally, to unacceptable deaths. WHO estimates a value is $6.5 billion in the USA and a 7 billion euro loss in Europe annually concerning HAIs (Cabral and Rodriques, 2019).

In South Africa, it is estimated that nearly one in seven patients hospitalized are likely to acquire an HAI (Revelas, 2012). Over the years, HAIs have increasingly become a concern, giving rise to inflated medical costs, lengthy hospital stays, increased complication rate, and a higher morbidity rate (Peleg and Hooper, 2010; Revelas, 2012). According to an incidence survey conducted with the WHO's support in 2002, 55 hospitals located across the globe in 14 countries and representing four WHO regions showed that approximately 9% of hospital patients had HAIs (Popovska *et al*., 2009). Findings on ventilator-associated pneumonia (VAP) and hospital-acquired neonate infections indicated that HAI risks are significantly higher in low-income countries (Allegranzi *et al*., 2011). Unfortunately, very few of these countries have national surveillance systems for HAI (Talaat *et al*., 2016; Turner *et al*., 2016).

Infective agents isolated from hospital environments are recognized to bring about multiple Resistance to antimicrobial agents. Bacteria possessing multi-drug resistance (MDR) include vancomycin-resistant enterococci (VRE), and vancomycin-resistant *S. aureus*, as well as methicillin-resistant *S. aureus* and penicillin-resistant pneumococci (Edosa and Kebede, 2015). Despite emphasizing hand hygiene, this route still serves as a source of MDR organisms in many hospitals (Falagas *et al*., 2011). While quality improvement in hospitals is central to infection prevention and control (IPC) programs (Wiemken *et al*., 2017), most public hospitals in South Africa face numerous. These include limited resources, inadequate staffing, training of infection prevention and control practitioners (IPCPs), high patient movement from ward to ward, insufficient quality control for cleaning services, and sub-optimal implementation of IPC guidelines (Duse, 2009; Pottinger *et al*., 2010). The National Department Of Health only approved the South African new IPC Policy during the coronavirus pandemic (COVID-19) in March 2020 (National Department of Health SA, 2020).

Hospital contamination and HAI are often initiated by non-observance of infection control strategies and dirty/soiled environmental surfaces. Despite the advances in modern medicine, hospital contamination still poses a risk to patients in hospital settings. Therefore, it is vital to identify environmental surfaces that are sources of bacteria and harbour pathogens that could lead to HAIs and hospital outbreaks. This study investigated bacterial dissemination in different healthcare levels, different wards, and places or instruments frequently touched by healthcare

personnel using metagenomics. Focussing on *Enterococcus*, as a target organism, the isolates' prevalence and antibiotic susceptibility patterns were investigated. Using WGS, further analyses were performed on the *E. feacalis* species isolated from the four public hospitals' environments in KwaZulu-Natal in the eThekwini district.

2.0 Literature review

Cross-infection through personnel's hands, patients' endogenous flora, and contamination of the environment postulated as key players causing HAI, especially in ICU's (Schmidt *et al*., 2014). Historically, research has revealed that contamination in hospitals makes a significant contribution in the spread of numerous strategic HAI pathogens, including coagulate negative *Staphylococcus* (CONS), methicillin-resistant *Staphylococcus aureus* (MRSA), *Acinetobacter* spp., *C. difficile*, VRE etc. (Weber *et al*., 2013). This literature review will examine how microorganisms are transmitted within hospitals, the organisms responsible, and those generally isolated from the hospital environments. Furthermore, the review will elucidate bacteria resistance in the hospital environment with specific mention of *Enterococcus* as HAI.

2.1 Routes of transmission

The transmission of pathogens may occur directly or indirectly in the hospital environment. Spread occurs through exchange between patients or via organisms carried by air in proximity and contact with contaminated environmental surfaces (Aly *et al*., 2008). Indirect contamination occurs via health personnel's hands, the most common transmission route in healthcare environments (Weber *et al*., 2013). As shown in figure 1, patients may acquire HAIs endogenously through organisms belonging to their skins` flora, mucous membranes, and female genital tracts (Mehta *et al*., 2014).

HAI acquisition can also be through exogenous means, which may occur either via animate or inanimate. Bacteria are dispensed into the environment from HCW or patients' skin and their oral and nasal cavity during sneezing, talking, etc. These bacteria are transferred to their hands, clothing, and immediate surroundings (Castillo-Rojas *et al*., 2013; Nekkab *et al*., 2017). Contamination may also occur in food, fluids, instruments, equipment due to contamination from human organic waste, pus, blood and blood products. Thereby giving rise to outbreaks, prolonged hospital stays, increased healthcare costs, along with impermanence (Sydnor and Perl, 2011).

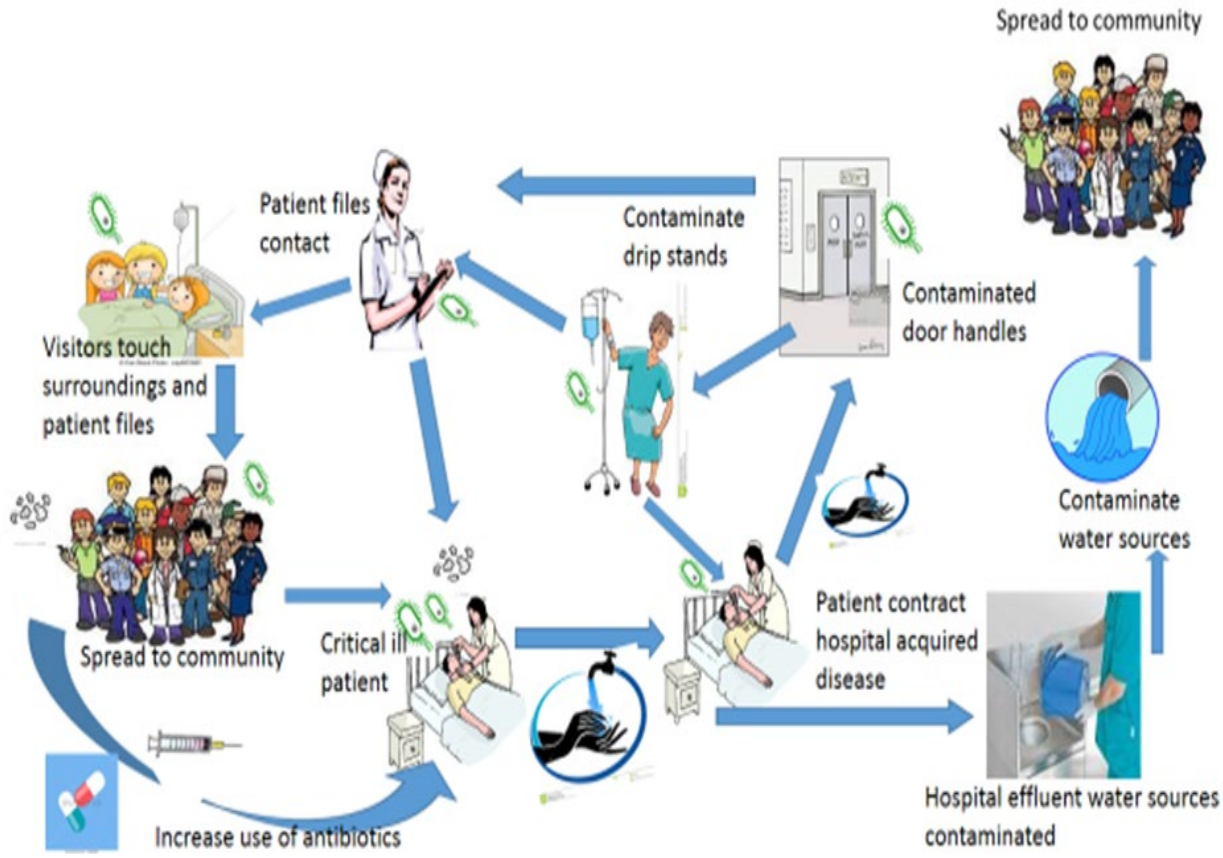

Figure 1: Possible routes for the disseminating pathogenic bacteria within a hospital environment, especially when not following hand washing routines. (with permission from Dr L.A. Bester).

Different contamination routes that could occur in hospital settings are discussed below, with examples of studies given in each section:

2.1.1 Hospital environment and medical equipment

The healthcare environment is a crucial factor in regulating infections. It forms an integral part that needs consideration for effective prevention of HAI and policymaking. Potential highly contaminated hospital equipment used for patient care or maintenance of the patient's surroundings within the hospital includes patient cabinets and bed rails, patient files, visitor chairs, door handles, and the seats of toilets and hospital floors (Siegel *et al*., 2007). Vast amounts of organisms are discharged by patients into their immediate environment, specifically if they have underlying diseases such as respiratory tract infections, wound, skin disease and diarrhoea. These organisms

fall with skin scales, discharge on proximal surfaces, and accumulate in the dust. They are transferred to hospital personnel and patients who touch the contaminated surfaces (Collins, 2008).

In the hospital setting, some hydrophobic organisms have a predominant tendency to spread since they can retain viability for a lengthy period in the environment after being expelled by patients (Weber *et al*., 2013). Bacteria on medical equipment can survive for long periods and disseminated to patients and medical personnel during disease management (Uneke *et al*., 2014). Instruments and utensils can also act as a reservoir of bacteria if not cleaned properly after patients' use. These pathogens may be transferred to other patients using the same instrument and further distributed to other exteriors in the hospital surroundings (Havill *et al*., 2011; Jinadatha *et al*., 2017). Contamination of these surfaces contributes to the dissemination of pathogens and thus, gives rise to the development of horizontal infections (Rodrigues *et al*., 2019)

A descriptive study in Sudan by Nurain *et al.* (2015) was performed on AMR patterns and recurrence rate of HAI isolated from patients who have cancer and their hospital environment from 2010 to 2013. The study found that of the 998 environmental samples collected from infrastructures, furniture, surgical equipment, different laboratories, and the kitchen, 30% showed bacteria belonging to different species. The most prominent spp. isolated were *Bacillus* spp. (n=148), *S. aureus* (n=42), *P. aeruginosa* (n=34), CONS (n=32), *K. pneumoniae* (n=22), and the least were *Proteus* spp. (n=10), Micrococcus (n=6) and *E. coli* (n=2). All the organisms showed complete Resistance to ampicillin, cefotaxime, ceftazidime, and ceftriaxone. *Proteus* spp. and *E. coli* showed no resistance to gentamicin, while *P. aeruginosa* and *K. pneumoniae* showed 24% and 45% resistance, respectively (Nurain *et al*., 2015). Although this study did not analyze the frequencies of bacteria organisms per inanimate site or resistant profile, the obtained data suggested cross-transmission of HAI bacterium from patient care equipment.

Screening for bacteria contamination in the operating and recovery rooms was also performed by Murshed and Kamar (2013) in a Dhaka City Hospital, Bangladesh, from 2008 to 2009. Overall, 120 samples were collected from, among other things, dressing materials, floor, bedsheets, operating theatre devices and invasive materials. The highest organisms isolated were *E. coli* (40%), then *S. aureus* (24%), *Pseudomonas* (12%), and *Proteus* (10%). *S. aureus* and *E. coli*

were predominant on the floor, bedsheets, and trolleys. They also observed that equipment used in the operating theatre (OT) contained fewer organisms than the recovery room (Murshed and Kamar, 2013). The study highlighted many potential pathogenic and opportunistic microorganisms on the sampled equipment, suggesting efficient adherence to cleaning protocols.

Bacterial contamination and susceptibility patterns from inanimate surfaces was determined by Sebre *et al.* (2020) in seven operation rooms and four ICUs at a specialized hospital in Addis Ababa, Ethiopia. Samples collected from areas within patient proximity included nurses' workstations, sinks, ventilators, beds and linens. Their results showed 86% (141/164) positive bacterial growth. The ICU samples showed the highest bacteria contamination rate of 50%, mostly from environmental surfaces and beds with and without linen. Overall, *S. aureus* (34%), *Acinetobacter* spp. (21%), and CONS (15%), including a few samples of *Enterococcus* spp. (2%) were commonly isolated. Susceptibility testing showed high resistance towards penicillin (93%), cefoxitin (84%), and erythromycin (54%). Vancomycin resistance was noted for 19% of *S. aureus*, 18% CONS, and 33% *Enterococcus* spp. (Sebre *et al*., 2020).

A study to assess the potential sources contributing to HAI transmission was carried out in Ghana, Volta Regional Hospital. Thirty-three different samples collected from door handles, taps, desk surfaces, bathrooms, and 15 other surfaces in the theatre before and after cleaning were sampled in the study. One hundred and eighty-seven bacterial isolates obtained from swabs consisted of 56% non-pathogenic isolates, 33% pathogenic isolates, while 14% showed no bacterial growth. Most of the pathogenic bacteria were *S. aureus* (58%) and *E. coli* (39%). The highest bacterial isolation collected from the door handles of the various wards (26%) and bathrooms (25%) (Tagoe *et al*., 2011). Door handles are inevitably a high-touch site that can be a significant contamination source in the hospital environment.

Bacterial contamination and AMR patterns were collected from patient care devices and exterior surfaces in an ICU in a special care medical facility by Darge *et al.* (2019) in Mekelle, Northern Ethiopia. The most common bacterial isolates identified were CONS (35%), *S. aureus* (26%), *Citrobacter freundii* (9%), and *K. pneumoniae* (8%). The antimicrobial analysis revealed more than 50% of the *Staphylococcus* spp. were resistant to erythromycin, amoxicillin-clavulanic acid,

cefoxitin ampicillin and penicillin G. Among the *S. aureus* sampled, 74% was confirmed as MRSA. Also, 74% of the Gram-negative rods showed resistance against nalidixic acid, amoxicillin-clavulanic acid and amoxicillin (Darge *et al.*, 2019). This study noted severe bacterial contamination of medical devices frequently used for patient care and the surrounding surfaces, due to cross-contamination.

A study in Kano, Nigeria, analyzed 100 swabs collected from five different hospitals and various hospital equipment that patients encounter in 2012, confirming 76% positive for bacterial growth. Of the 39 stethoscopes examined, 29 were contaminated, while all the tested x-ray cassettes, stationary grid, and table coaches were also positive for different organisms. The diversity of isolated microorganisms noted showed *Corynebacterium* spp. (10%), *Lactobacillus* spp. (8%), *Streptococcus* spp. (6%) and *Staphylococcus* spp. (52%). The study highlighted the contamination of tools used within the professional healthcare system and liable to HAI's, occasionally with fatal repercussions to both patients and healthcare providers (Yusha'u *et al.*, 2012).

2.1.2 Transmission through health care workers' hands

The significance of cross-transmission through the hands of HCW's were noted by different studies worldwide. Organisms transfer to HCW's hands through contact with patients or excretion from their bodies and during exposure to hand-touching areas in the hospital surfaces polluted with a virus, bacterium, or other microorganisms that can cause disease. Hands become gradually contaminated during patient care unless washed and disinfected regularly (Kelly, 2017; Russotto *et al*., 2015). Mostly, bacteria can stay alive for long periods on their hands and transmitted to other patients in a hectic hospital scenery (Kolmos, 2012).

Fingertip stamps of HCWs who worked in the cardiac ICU of a Heart Institute in Uganda were observed by Ssemogerere and colleagues retrospectively. A total of 56 samples were collected on different hospital staff categories and analyzed. Gram-negative organisms (GNOs) were isolated in 34% HCWs, in which 31% were ICU staff, 42% were not ICU staff, and 26% were other staff of the hospital. Thirty-two isolates were identified, 25% from ICU staff, 47% from non-ICU staff, and 28% from others. The highest organisms isolated were *Acinetobacter* (34%), *Citrobacter*

(22%), and *Pseudomonas* (22%). AMR to carbapenem ranged from 4% to 90% between these organisms (Ssemogerere *et al*., 2019).

An observational study in an ICU of a tertiary hospital in Italy was also carried out by Galazzi *et al.* HCWs had their cell phones swabbed for microbiological assessment before and after work shifts. One hundred swabs collected from 50 cell phones, 43 HCWs admitted to their cell phones' frequent use during working hours. All the phones tested positive for bacteria; the highest frequency of bacteria isolated were CONS (97%), *Bacillus* sp. (56%) and MRSA (17%). Although no patients in ICU, when conducting the study, had infections related to the bacteria found on healthcare workers' cell phones, it was evident that these bacteria were transmittable to the phone through the hands of the HCW (Galazzi *et al*., 2019).

Bacterial contamination on the hands of HCWs was further evident in a study in an Indian Eastern tertiary hospital, which showed that in a total clinical staff complement of 101, 62% (n=63) had bacteria-contaminated hands. Of the 99 supporting staff, 73% (n=72), and 60% of the doctors and 66% of the nursing staff hands were contaminated with bacteria. Clinical staff hands were mostly contaminated with *S. aureus* and non-clinical staff with *A. iwoffii*. In contrast, only one isolate of MRSA was recovered from a doctor's hand (Sarfraz *et al*., 2015). In a study by Kalaiselvi and Padmavathi (2017), 124 samples from HCW's attending to ICU, medical care unit, emergency ward, and operating rooms were collected. Growth was observed in 86% (n=107) of samples, and the bacteria flora isolated were 7% (n=4) for *Micrococcus* spp. and 46% (n=57) CONS, of which 30% (n=17) were MRSA. The highest temporary skin flora organisms found were *S. aureus*, *Enterococcus* spp., and *Acinetobacter* spp. Eleven of the *S. aureus* isolates were MRSA, while 30% of *Pseudomonas* spp. and 75% of *Acinetobacter* spp. were MDR. Notably, 10% of *Pseudomonas* spp., 25% of *Acinetobacter* spp., 20% of *Klebsiella* spp. and 14% *E. coli* also showed resistance imipenem (Kalaiselvi and Padmavathi, 2017). This research paper revealed a high contamination rate of the HCW's hands, which is unfortunate since they attend to vulnerable patients.

Not only can hands be a source of contamination, but the additional liability of multidrug-resistant (MDR) strains can arise. Tajeddin *et al.* (2016) determined the rate of bacteria associated with

HAIs contamination on HCW's hands working in the ICU at a hospital in Tehran, Iran. Out of the 762 swab samples obtained, 35% showed bacterial growth. The most frequent bacteria were *Staphylococcus* spp. with 27%. Nurses' aides (39%), nurses (34%), housekeepers (32%), and doctors (27%) were the most contaminated staff. Multidrug resistance was detected for 52% of *A. baumannii* spp. isolated and 60% of MRSA. MDR phenotypes were noticed amongst 36% of all the HCWs' samples (Tajeddin *et al*., 2010).

2.1.3. Healthcare apparels

The function of HCWs apparel in the spread of infections has been an area of major concern. Studies have demonstrated bacterial contamination on the attires of HCWs while performing patient care activities (Williams *et al.*, 2015; Chiereghin *et al*., 2020). Most of these contaminations occur in frequent hand contact areas, e.g. the cuffs and the pockets of coats, resulting in recontamination of hands after washing (Weiner-Well *et al*., 2010; Uneke and Ijeoma, 2010).

A survey of hospital staff apparel in 238 samples was collected and reported by Weiner-Well *et al.* (2011) that each garment had at least one contaminated area. These bacteria included CONS 50%, *Bacillus* spp. 20% and 18% *Micrococcus* (Weiner-Well *et al*., 2010). This study showed as high as 60% of hospital staff's apparel was contaminated by pathogenic bacteria, together with antibiotic-resistant strains. Uneke and Ijeoma (2010) also reported potential HAI transmission by white coats used by doctors in Nigeria, showing 91% (94/103) of the coats contaminated. The bacteria isolated were diphtheroid 52% (n=49), *S. aureus* 19% (n=18), Gram-negative bacilli 19% (n=18), and *P. aeruginosa* 10% (n=9). Their study confirmed that HCW coat cuffs' had an increased bacterial load compared to the coat pockets' opening edges (Uneke and Ijeoma, 2010).

Contamination of HCWs' attire has been linked with certain care activities in a study by Thom and Johnson (2018) during eight months to analyze HCW scrubs and patient care correlations. Ninety HCW's were provided with frequent new scrubs, sampled in their shift's last hours. From the samples collected, 30% were contaminated with bacteria. The analysis indicated the high contamination risk associated with patient wound care compared to bathing a patient (Thom and Johnson, 2018). The primary factor of HCWs regularly exposed to body fluids and blood is the

quick transferability of microorganisms that instigate infection, including MDROs, e.g. MRSA and *Acinetobacte*r spp., *K. pneumoniae* and *E. coli* (Hawkins *et al*., 2011). HCWs colonized with these bacteria constitute a risk to patients and the environ if wearing the same attire for 24hrs or more. During this time, their hospital garments will directly or indirectly be in touch with co-workers and patients (Mitchell and Edmiston, 2018).

HCW's attire in different departments' acute care settings was researched by Burden *et al.* (2011) and Banu *et al.* (2012). The former compared the extent of bacterial contamination of doctors' standardized short-sleeved laboratory coats after their shift compared with freshly washed coats. A statistically significant difference was not found between bacterial contamination on newly washed ones and those worn for at least eight hours. This study highlighted that after three hours of wearing the newly washed coat, there were almost 50% bacterial contamination, with those counted at eight hours (Burden *et al*., 2011). Then in the Banu *et al*. (2012) study, sides of coats of HCW's were particularly contaminated areas, but also collars and pockets. *S. aureus* 65% (65/100) was the most common species, followed by CONS 10% (7/100). Majority of the Gram-positive cocci showed resistance to clindamycin (59%), penicillin (82%) and erythromycin (71%) (Banu *et al*., 2012). These studies also highlighted healthcare apparels as potential sources in the nosocomial transmission of pathogenic and antibiotic-resistant microorganisms.

2.1.4. Droplet and airborne spreads

Pathogens can also spread by respiratory droplets produced through talking, sneezing, coughing, and respiratory tract. Respiratory droplets higher than five microns do not stay suspended (airborne) for a lengthy period and can be transmitted to patients within a radius of one to two meters. (El-Sharkawy and Noweir, 2014). Organisms such as *S. pneumoniae,* streptococcal pharyngitis, *N. meningitidis*, *M. pneumoniae*, its causative agent plague, and viral microbes instigated by influenza viruses are among the numerous organisms that spread through this route (Duse, 2009).

In the hospital environment, airborne infectious particles vary in their compositions. They include biological material carried by other non-biologic particles (e.g., dust), which harbours bacterial

cells and spores (Fernstrom and Goldblatt, 2013). Generally, hospital indoor airborne microorganisms are shed by staff, patients, and visitors. Consequently, higher bed occupancy had contributed to greater microbial bioburden in the air (D'Alessandro and Gaetano, 2017). Agbash (2010) investigated air samples at labour and paediatric rooms in three hospitals in Khartoum, Sudan. The labour rooms showed 63% (52/79), and the paediatric rooms, 67% (40/60) positivity for bacterial growth (Agbash, 2010).

In six Korean hospital reception areas, airborne bacteria, Gram-negative bacilli (GNB), and fungi were assessed at specific locations for two days. The average levels of bacteria, GNB, and fungi ranged from 1.7×10 cfu/m^3 to 7.7×10 cfu/m^3, indicating that the hospital lobby was highly contaminated (Park *et al*., 2013). This study highlighted the air space contamination in hospital receptions with different bacteria and fungi, hence being an HAI source. A study was conducted to quantify the bacteria load of OT and surgical wards' air environment for four months at a specialized hospital in Southwest Ethiopia. Overall, 108 air samples were collected in 12 rounds. The mean colony counts, 46 cfu/hr and 28 cfu/hr, were above acceptable standards for passive spaces in two different theatre rooms. Likewise, the mean aerobic colony counts in two female wards were 465cfu/hr and 461cfu/hr, greater than the permissible range of 250-450 cfu/hr. Theatres identified *S. aureus*, 66%, as the predominant species, and all these isolates showed complete resistance to methicillin and 83% resistance to ampicillin (Chalachew *et al*., 2011).

Airborne studies also indicate airborne microbiotas and MDRs. In Southern Ethiopia, a referral hospital collected 216 air samples from the delivery room, ICU, and operating theatres, of which 90% were positive for bacteria growth. The pathogens were CONS, *S. aureus*, *E. faecalis*, *E. faecium*, *Acinetobacter* spp., *E. coli,* and *P. aeruginosa*. Antibiotic resistance ranged from 8 to 88%, and 75% of all these organisms were MDR. The study concluded that these organisms were a common source of post-surgical site infection in the studied areas (Solomon *et al*., 2017). Mess *et al.* (2015) reported MDR microorganism's frequency in 48 aerobic samples from indoors, operating rooms, dental surgeries, and waste disposal depots. A total of 280 bacteria isolates collected, predominantly consisting of 38% (n=107) Gram-positive cocci. Ninety-five of the total isolates were MDR as shown by antibiotic susceptibility testing. This study highlighted AMR

bacteria in air samples and the probability of transmission of resistance genes to infectious bacteria in what is referred to as "nosocomial air" (Messi *et al*., 2015).

The spread of infectious agents has postulated by Herfst *et al.,* occur via the air involves four phases: i) The dissemination of the pathogen is linked with dust particles and/or aerosols/fluid condensations when transmitting from one host to another; ii) The pathogen is inhaled by the recipient, leading to an infection of the respiratory tract; iii) In the peripheral tissues or lung, amplification of the organism may occur; and finally, iv) The pathogen emerges at the site of shedding and produce abundant loads capable of discharge (Herfst *et al*., 2010). In the transmission process, the receiver becomes a transmitter when microbial multiplication subsequently results in the pathogen's release (Bunyan *et al*., 2010).

2.2. Infection Prevention and Control

Infection Prevention and Control (IPC) programs were first introduced in the 1950s, focusing on the control of HAIs (Soule *et al*., 2011). In 2012, a National Core Standards for healthcare institutions were introduced in South Africa, with a patient protection focus mandate, including IPC. The National Core Standards' primary purpose was to create a general definition of quality care to be implemented in all healthcare settings in South Africa. The standards guide the staff and executives of hospitals, including the public, establishing a level at which healthcare settings can be evaluated, thus providing national certification of compulsory compliance of healthcare settings (National Department of Health SA, 2012). However, in South Africa, although IPC's importance is well recognized and best practice guidelines published on this topic, infection rates are still on the rise, and IPC remains a challenge (Backman *et al*., 2012).

IPC programmes in developing countries have increased dramatically in the last decade (Huttner *et al.*, 2017). In South Africa (SA), the situation is comparable with other neighbouring countries, with no HAI surveillance agenda and minimal records on child and adult infection (Dramowski and Cotton, 2017). Ineffective and inadequate IPC practices have facilitated the existence, intra/inter-hospital spread, and persistence of MDR organisms (Harbarth et al., 2015). Infection Prevention and Control Program (IPCP) knowledge and proper resources are essential to reduce

the prevalence and unfavourable sequel of HAIs. The objectives of IPCPs are to curtail HAIs impact by contributing to patient safety (Pyrek, 2016; Param, 2016).

Contrary to expectations, in developing countries, IPCPs experiences their resources, either reducing or stay stagnant and, as a result, have failed to accomplish expanding mandate needs (Param, 2016). There has been substantial evolution in standards and laws associated with environmental hygiene management in health care facilities (Carling and Bartley, 2010). According to the CDC, the most imperative measure for counteracting the spread of nosocomial bacterial pathogens is proper handwashing. This refers to hand washing prior to and after contact with patients, prior to and after contact with contaminated inanimate objects (Edosa and Kebede, 2015).

2.3. Hand hygiene (HH)

Hand hygiene (HH) if appropriately implemented, is considered a significant infection control element. Proper HH singly can considerably minimize the danger associated with the cross-transmission of infection in hospitals (WHO, 2009). The hands of HCWs are inhabited predominantly with pathogens that can survive for more than six days, such as MRSA, VRE, MDR-GNBs, *Candida* spp., and *Clostridium difficile* (Cassone and Mody, 2015; Mathur, 2011). Epithelial skin cells comprising viable bacteria are shed regularly from normal healthy skin. Through dissemination, they contaminate hospital environments such as gowns, bed linen, bedside cabinets, sink, and areas within the patient proximity (Jonge *et al*., 2019).

Sasahara and colleagues showed the importance of handwashing when analyzing the relationship between spore-forming bacterial contamination and HH behaviours in a tertiary hospital emergency ward in Japan. The hands of 71 HCWs were evaluated and quantitatively examined after nine working hours. A total of 76% (n=71) of hands were contaminated with bacterial spores, 52% and 51% identified as *B. subtilis* and *B. cereus*, respectively. *C. difficile* was detected on one HCW hand. The results showed a negative interrelationship between the handwashing frequency and the contamination level (Sasahara *et al*., 2015).

Proper HH practices by HCWs can successfully lessen the accusation of HAIs by intersecting the spread of microflora amongst patients (ICPIC, 2017). As incorporated in the global patient safety challenge initiative by WHO, hand hygiene reduces HAI's burden; sadly, compliance rates among HCWs remain low. A review from 2010 to 2014 of some HH studies in developing countries established that compliance ranged from 23% to 69%. If hands are contaminated, HH plays a significant part in lowering the amount transmitted, yet adherence is overall low (Gould *et al.*, 2017; Mathur, 2011). Potential reasons for insufficient commitment to HH include failure to recognize situations requiring hand hygiene, display of non-compliance by co-workers, lack of knowledge of guidelines, absence of proper infrastructure, work overload, insufficient time, and scepticism about the value of HH (Lystsy *et al*., 2006; Protano *et al*., 2019).

Research on HH's awareness and compliance between HCW in a northeast Ethiopia referral hospital was conducted using self-structured questionnaires. The results showed that 66% (n=91) of respondents were familiar with HH, and 34% (n=91) were not. However, out of those familiar with HH, 85% had poor practice, and only a few observed proper HH (Jemal, 2018). An observational study was also conducted in Ethiopia's eastern part on nurses` HH practice using observational tools approved by WHO. Out of 110 participants in the survey, 3902 occasions and 732 HH actions, only 19% overall compliance was observed. This study showed that the observed practice of HH was poor (Awoke *et al*., 2013). This study concluded that although most HCWs were knowledgeable, their compliance with practising HH was low.

In most healthcare facilities, observance of recommended HH procedures remains far too low, hardly surpassing 40% of situations in which HH is specified (Kashyap *et al*., 2011). In 2016, WHO released a guideline on improving HH practice by healthcare personnel and reducing the spread of pathogenic microorganisms to patients (WHO, 2016). These standards emphasized the usage of either alcohol-based hand rub or soapy solutions with water. The 'five moments for hand hygiene' were introduced to explain HH's specific moments concerning an evidence-based model of transmission of microorganisms by the hands of HCWs (Tang *et al.*, 2019).

HH rate compliance among HCWs and its effect on HAI levels in an ICU in a Kuwaiti teaching hospital was investigated by Salama *et al.* (2013). Different methods were used, such as regular

education about HH techniques and consultations with HCW's. Using the WHO HH observation protocol, the overall percentage of those adhering to proper HH increased from 43% before the intervention to 61% after the intervention. Significant reductions observed in the infection rate in the ICU of this hospital, suggesting that the level of recurrence can be affected by dedication to adhering to hand hygiene recommendations (Salama *et al.*, 2013).

2.4. Disinfection and Cleaning

Disinfection and cleaning are part of the preventive measures that assure a risk-free environment for both patients and staff. Through appropriate use of disinfectants, the transmission of microorganisms from inanimate surfaces to patients (mostly *via* HCW's hands) and the hospital environment can be limited (Dancer, 2011). Extreme and continuous cleaning with disinfectant reduces the rate of contamination of medical equipment in hospitals, including environmental surfaces (Quinn *et al*., 2015). The initiation of improved disinfection processes and products is vital in managing and terminating HAI transmission and persisting microbial cells (Abreu *et al*., 2013). According to Moccia *et al.*, an improved sanitization system does not only involve disinfecting surfaces; they also comprise incorporating all actions supplementary to the cleaning and disinfection procedures. These include tracking the operators' technique and training or education (for housekeepers and HCWs) (Moccia *et al*., 2020).

Of recent, the prerequisites with reference to the antimicrobial behaviour of cleaning agents in the hospital have elucidated different European standards (Wales and Davies, 2015). However, new products and equipment with lasting antimicrobial activity minus resistant microorganisms are crucial to substitute the various products from retail with little or no biocidal action (Abreu *et al*., 2013). Studies using a fluorescent targeting method or covert target observation have suggested that only 40% of patients' close proximity were cleaned according to hospital policies {Formatting Citation}.

Researches have revealed that disinfection or cleaning thoroughness can enhance up to 82% (Wiline *et al*., 2010; Clark *et al*., 2010). Regrettably, lethal and routine disinfecting surfaces in rooms by janitors, including medical devices by nursing staff, are often inefficient (Dancer, 2014;

Weber *et al*., 2013). Research has shown patients occupying rooms formerly occupied by patients with MRSA, VRE*, A. baumannii,* and *C. difficile* infections are prone to 50% risk of exposure to similar pathogens than patients not in rooms formerly exposed to mentioned pathogens (Fujitani *et al*., 2011; Shorman and Al-Tawfiq, 2013).

Researchers have also used different methods to describe the role of proper disinfection. Cheng *et al.* (2011) assessed the efficacy of disinfecting against MRSA using wipes in a tertiary hospital's orthopaedic unit in Hong Kong. The proximity surroundings of eight hospitalized patients confirmed to have MRSA were used as a case study. Overall, 56 samples were collected from the bed rails before and after disinfection. Results showed 86% of MRSA colonization before disinfecting bed rails and reduced to 34% after disinfection. A decrease observed in the bacteria load collected five minutes after disinfection confirmed a reduced MRSA survival means. The disinfection process showed reduced bacterial load in the patients' environment and decreased MRSA's survival on the bed rails (Cheng *et al*., 2011).

The investigation of improved disinfectant methods has however, also produced several outcomes. According to a study by Passaretti *et al*. (2020) on six critical-care units in a tertiary hospital, the USA, hydrogen peroxide vapour (HPV) was used to disinfect patients' rooms prone to infections with multidrug-resistant organisms (MDROs). Patients occupying the disinfected room showed a 64% decrease in the acquirement of MDRO and an 80% decline to VRE compared to the initial tests. Using HPV significantly decreases the proportion of rooms contaminated with MDROs by 35% (Passaretti *et al*., 2020).

Datta *et al.* did another analysis on ten ICUs at a USA hospital by improving cleaning measures to estimate the effect of enhanced cleaning and decontamination. The efforts entailed using a black-light marker as a target response, cleaning napkins soaked in a bucket with disinfectant and increased housekeeping staff education. The patient acquisition was compared through a 20-months reference and interference intervals divided by 16 months. MRSA and VRE frequency decreased from 3% to 2 % and 3% to 2%, respectively, during the intervention periods (Datta *et al*., 2020).

In South Africa, IPC practitioners (IPCP's) have numerous challenges that include but are not limited to insufficient resources and inadequate IPC practitioners' staffing to strengthen IPC (Mehtar *et al*. 2014). Difficulties include compromised infection control measures during high patient throughput, high patient movement levels from ward to ward, inadequate infrastructure, and dysfunctional procurement processes (Duse, 2009). The disregard of IPC as a speciality, insufficient expertise (especially microbiological) and improper management of IPC are additional factors that contribute to the challenges faced in South African hospitals. Furthermore, the absence of surveillance data and/or limited reports on IPC associated issues and failure to identify that surveillance is the foundation of this program had caused IPC professionals' work to be more inexpedient (Duse, 2009; Plessis and Monkoe, 2010).

2.5. Common HAI organisms and antibiotic resistance

Bacteria account for approximately 90% of infections, especially in a hospital setting (Khan *et al*., 2015). Bacteria typically considered during HAI infections are *Streptococcus* spp., CONS, *Acinetobacter* spp., *P. aeruginosa,* enterococci, Legionella, *S. aureus, B. cereus*, and enterobacterales family members. The latter includes *P. mirabilis, K. pneumonia, Serratia marcescens,* and *E. coli* which play a pivotal role in HAI (Horan *et al*., 2008; Davies and Davies, 2010). The primary responsible HAI organisms have shifted over time, e.g. *Proteus* sp, *Klebsiella* spp., and *E. coli* were accountable for HAIs in the 1960s, but then again from 1975 to 1980s, *Acinetobacter* spp. with *P. aeruginosa* became clinical challenges (Gordon and Lowy, 2008; Khan *et al*., 2015).

Enterococci are recognized to cause surgical-site infections, while in the blood-borne pathogen, CONS is the primary causative agent. Urinary tract infections (UTI) are frequently instigated by *E. coli*, although less observed in other infection sites. In contrast, *S. aureus* is persistent at different body sites and seldom causes UTI. *P. aeruginosa* accounts for one-tenth of all infections and are evenly disseminated all over the body sites (Khan *et al*., 2015). The routine or overuse of antimicrobial agents is one of the selective pressures contributing to the burden of high-level AMR and MDR antibiotics (Rice, 2010). Risks associated with MDR GNB infection include

immunosuppression, long-term hospitalization and prolonged dialysis or wound care over a month (Aliberti *et al*., 2012).

Provincial outbreaks of MDR Gram-negative infections were most likely movement between different health facilities (Munoz-Price, 2009). Health agencies, such as the World Health Organization (WHO), Infectious Diseases Society of America (IDSA) and the European Centre for Disease Prevention and Control (ECDC) have improved their effort in renovative research into novel antimicrobial combination choices. Besides, it promoted decisively and conscientious undertakings against fatal infections due to a set of MDR and pan-drug-resistant GNB (WHO, 2015). These MDR's, referred to as ESKAPE pathogens (Mulani *et al*., 2019), are described further.

2.6. ESKAPE pathogens and their clinical relevance

The abbreviation ESKAPE comprises six pathogens that reveal MDR with their antibiotics in bracket viz: *Enterococcus faecium* (vancomycin), *Staphylococcus aureus* (methicillin), *Klebsiella pneumoniae* (carbapenem), *Acinetobacter baumannii* (carbapenem), *Pseudomonas aeruginosa* (extended-spectrum β-lactamase), and *Enterobacter* spp. (carbapenem) (Mulani et al., 2019). ESKAPE adeptly "escape" the biocidal action of antimicrobial agents and regularly express novel virulence, transmission, and resistant mechanisms (Navidinia, 2016). AMR genes can be carried on the plasmid, transposons, and bacterial chromosome. Mechanisms of resistance of the ESKAPE pathogen fall into four categories. Firstly, through drug inactivation or alteration (Bush and Jacoby, 2010).

Secondly, through the alteration of drug binding sites as follows; i) positions of drug action may be repositioned by enzymatic alteration, changed by genomic modifications, and/or bypassed metabolically thus, expresses unique penicillin-binding proteins (Tang *et al*., 2014); ii) modifications in cell penetrability, causing reduced intracellular drug build-up, therefore, decreasing the antibiotic available to access the bacterial cell membrane is one approach for bacteria to acquire AMR; iii) stability of antibiotic usage and complete removal affect the bacteria's susceptibility to a specific drug (Santajit and Indrawattana, 2017).

As recently listed by the WHO, these pathogens are prioritized as organisms that urgently require new antibiotics (WHO, 2017). A study on the recommended list of antibiotics in the Clinical and Laboratory Standards Institute (CLSI) guidelines against the different species suggested that the antibiotics used against ESKAPE are fewer and lesser effective (Mulani *et al*., 2019). Over time, the total quantity of antibiotics effective versus ESKAPE declines, predisposing us toward a future with ineffective antibiotics (Laws *et al*., 2019). *Enterococcus* spp. is included in the high priority group; thus, discussing these species further.

2.7. *Enterococcus* species and their dissemination in the hospital environment

Enterococci are Gram-positive bacteria commonly disseminated into the environment, either as innocuous commensals or multifaceted opportunistic pathogens (Teixeira and Merquior, 2013). The genus *Enterococcus* is significant due to its role as the primary causative agents of HAI (García-Solache and Rice, 2019). *Enterococcus* has more than 50 species, but the most commonly isolated species, *Enterococcus faecalis* and *Enterococcus faecium* are of clinical importance since they colonize the human gastrointestinal (GI) tract (Dubin and Pamer, 2017). Unfortunately, enterococci spp. has emerged in both the developing and developed world as serious pathogenic (Fiore *et al*., 2019). Two factors assist the spread of *Enterococcus* in the hospital environment: its capability to survive on the GI tract's exterior and risks associated with HCWs to unintentionally transmit bacteria to patients within proximity (Arias and Murray, 2012).

Enterococcus has revealed to persevere for more than four months in a clinical or hospital environment (Faron *et al*., 2016). *Enterococcus* spp. can also be transmitted by HCWs who may serve as vectors for dissemination between patients in different rooms or wards. VRE can persevere for up to 60 min on HCW's hands and skin in the non-adherence to proper hand hygiene (Jackson *et al*., 2019). A surveillance study of the environment suggested admission to a room formally inhabited by a VRE infected patient was correlated to a significantly increased high hazard ratio of VRE acquisition by the next patients (Drees *et al*., 2008). In the subsequent sections below, we will assess AMR's mode of action and method of resistance in enterococci to rank such mechanisms in perspective within a clinical context.

2.8. Antibiotic resistance mechanism of Enterococci

AMR is a crucial threat to all healthcare bodies and known to globally cause approximately 700,000 annual fatalities (O' Neil Report, 2016). Increasing our knowledge of resistance mechanisms is vital in decreasing first-hand antibiotic therapies that select resistant pathogens (Kristich *et al*., 2014). Due to their intrinsic resistance to numerous antimicrobials, enterococci have entrenched themselves as HAI agents, especially their ability to develop new resistance characteristics. *E. faecalis* (commonly isolated species) is more virulent than *E. faecium* but has a reduced effective level of intrinsic and acquired AMR (García-Solache and Rice, 2019).

The transmissibility of mobile elements and a significant number of antibiotic resistance determinants of *E. faecium* and *E faecalis* are postulated to be linked with inadequacy in the CRISPR (clustered regularly interspaced short palindromic repeats) associated system (*cas*). These *cas* genes exist in a different number of archaea and bacteria. Collectively, they develop an adaptive immunity against plasmids, other mobile genetic elements and phages (Miller *et al*., 2016). Therapeutic treatment of enterococci infections are challenging; the bacterium acquires resistance to many classes of antimicrobial agents. These would include tetracyclines, glycopeptides, and quinolones (Miller *et al*., 2014; Faron *et al*., 2016). Below (Figure 2) is a graphical presentation of the pathways in enterococci's resistance to antibiotics.

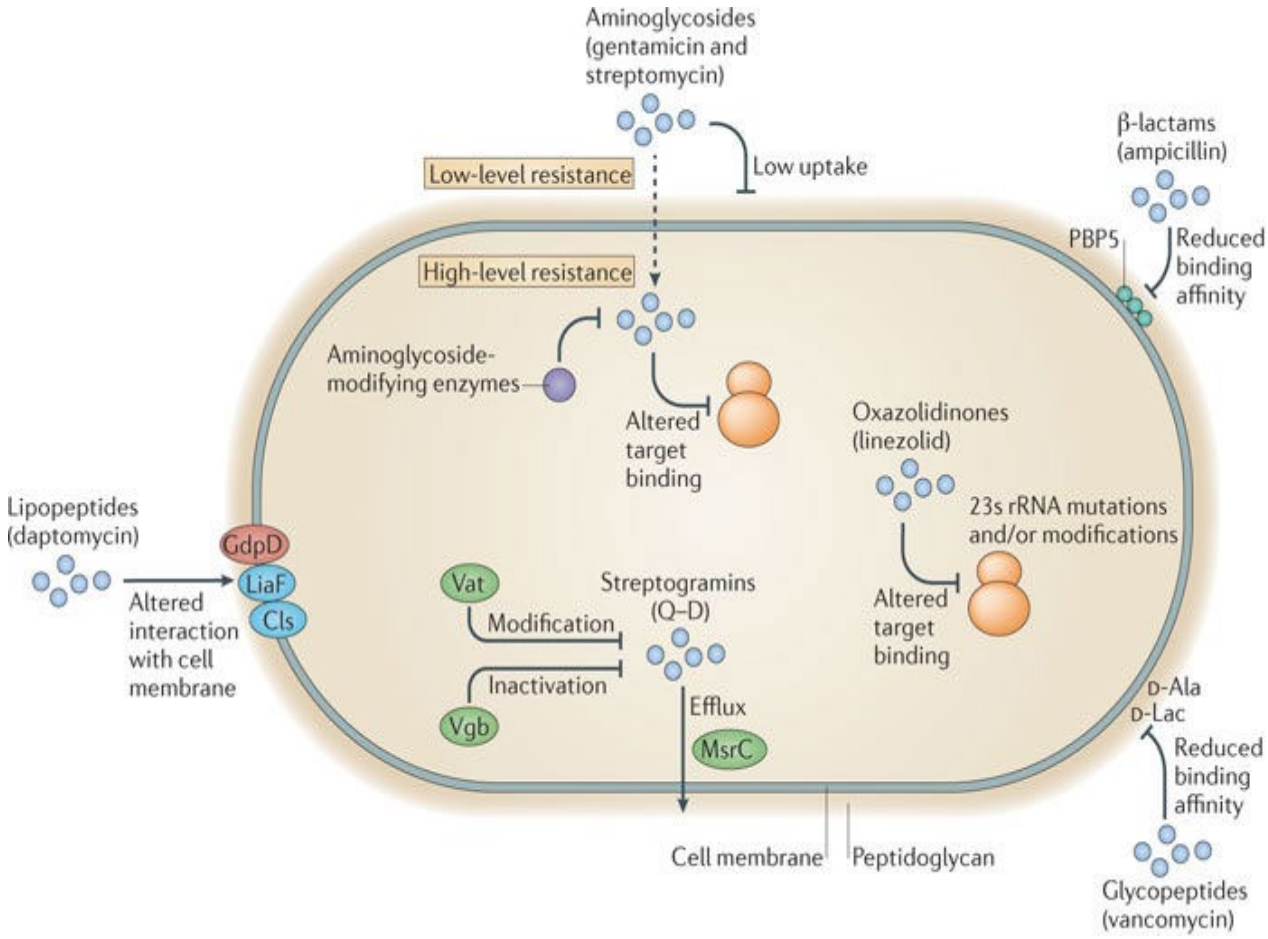

Figure 2: Mechanism of Resistance of enterococci spp. as borrowed from Depardieu *et al*. (2007)

2.8.1. Glycopeptide resistance

The mechanism of action of glycopeptides in enterococci depends on the glycopeptide attaching to the C-terminus end of the D-Ala-D-Ala translocated pentapeptide. This joining inhibits preceding trans-glycosylation, trans-peptization, and carboxypeptidase reactions (Lee *et al*., 2019). Resistance occurs in enterococci by binding to the terminal D-alanine-D-alanine (D-Ala-D-Ala) section of peptidoglycan antecedent, thereby blocking the formation of peptidoglycan chains and preventing the production of the cell wall. Due to a change in the terminal amino acids, resistance to these agents, represented by vancomycin, is high (MIC >64 µg/ml) (Miller., 2014).

The first and most common operon in clinical settings is the *van*A operon (167). Presently, ten distinct vancomycin resistance clusters have been reported in enterococci, *van*A, *van*M and *van*B,

conferring high resistance levels to vancomycin encoded on MGEs. While, *van*F, *van*G, *van*L, *van*C, *van*D, *van*E and *van*N are not infectious and usually confer lesser resistance levels (Lee *et al*., 2019). Vancomycin resistance is mostly mediated through the *vanA* gene, which consists of three major operon components: glycopeptide resistance, accessory genes and regulation, which changes amino acid sediments at the predictable site where vancomycin truss to hinder the cell wall formation (Miller *et al*., 2014).

After its clinical establishment, vancomycin remained effective against enterococci until the early 1980s, first in Europe followed by the USA, strains that exhibit inducible, high-level resistance to vancomycin and teicoplanin (the latter introduced later) (Santajit and Indrawattana, 2017). Surveillance data from the USA from 1995 to 2002 showed that 9% of central line-associated bloodstream infections were instigated by *Enterococcus* species, of which 60% were *E. faecium* and 2% *E. faecalis* (Budavari *et al.,* 1997).

Potential risks to health care facilities are colonized patients who serve as sources to transmit VRE's by shedding within their vicinity and onto HCW's (Faron *et al*., 2016). Hospital environmental surveillance showed VRE isolations from different surfaces in rooms previously occupied by infected patients, such as bed rails, patient gowns, door handles, floors and blood pressure cuffs (Dancer, 2014). It's deduced that risk factors for infection with VRE include sharing a room with someone infected with VRE, elderly/immunocompromised, extended antibiotic usage and presence of invasive equipment (Tacconelli and Cataldo, 2008). The paediatric haematology and oncology unit at a tertiary hospital in South African Cape Town tertiary hospital reported VRE infections and investigated clinical and molecular proportions of the incidents detected. VRE colonization was detected in eight of 55 patients assessed, with the majority carrying the *van*A gene (Lochan *et al*., 2016). This result to effectively curtail VRE colonization, infection and transmission among groups of patients were inconclusive.

2.8.2. Aminoglycoside resistance

Aminoglycosides mode of action hinders the initiation step of protein production in bacteria by attaching to the aminoacyl site of 16S ribosomal RNA within the 30S ribosomal subunit,

interfering with the translation and causing a misread of the codon along the mRNA (McCoy *et al*., 2011). High-level resistance (HLR) to aminoglycosides is mediated by producing aminoglycoside-modifying enzymes or evolving ribosomal attachment sites (Lioy *et al*., 2014). Apart from streptomycin, the transposon aac-6′-Ie-aph-2″, mainly found in Tn4001 in staphylococci and other enterococci variants, also resistance to aminoglycosides. (García-Solache and Rice, 2019).

The prevalence of HLR in hospitals to aminoglycosides regarding the geographical location differs from 40% to 68% among *Enterococcus* species isolates (Higuita and Huycke, 2014; Faron *et al*., 2016). Harada *et al.* (2020) reported HLR to aminoglycoside in their study carried out in Tokyo. One hundred *E. faecalis* isolates were investigated, 30% revealed HLR to gentamicin, and 22% of the samples had HLR to streptomycin. Moreover, of the 40 *E. faecium* isolates examined, 23% of isolates had HLR to streptomycin and gentamicin (Harada *et al*., 2020). In another research by Viderman *et al*. (2019), 546 patients admitted to the ICU of a Kazakhstan specialized hospital had at least one (25%) HAI. Most of them were caused by *E. faecalis*, which were resistant to aminoglycosides in more than 70% of the cases (Viderman *et al*., 2019).

2.8.3. β-Lactam resistance

Due to their ability to prevent important cell-wall peptidoglycan production, penicillin and ampicillin were the most successful β-lactams against *E. faecium* (Lee *et al*., 2020). These drugs' mode of action targets and prevents cell wall synthesis by binding the enzymes involved in the synthesis. The enzymes are anchored in the cell membrane and referred to as penicillin-binding proteins (PBPs) (Kapoor *et al*., 2017). β-lactams intrinsic resistance maintenance in enterococci is due to the excessive production of PBPs with low binding affinity for β-lactams (especially PBP4 and PBP5) (Moon *et al*., 2018).

There are three penicillin's; ampicillin, piperacillin and penicillin, known to have activity against enterococci (Moon *et al*., 2018). High-level resistance to ampicillin was identified in the USA hospitals from the 1970s to 1980s. Within this period, enterococci resistance to vancomycin in the USA was first discovered (Arias and Murray, 2012). Latin American surveillance of AMR

(ReLAVRA) data from 2015 in Argentina, ampicillin resistance rate was 85% in *E. faecium* and 2% in *E. faecalis* (ReLAVRA, 2015)..

The resistance level differs between *E. faecium* and *E. faecalis*, with the former being at least four to 16 fold less susceptible than the latter (Gagetti *et al*., 2019). Despite decreased susceptibility to penicillin, *E. faecalis* isolates remain susceptible to ampicillin. In contrast, approximately 90% of *E. faecium* isolates exhibit reduced susceptibility to ampicillin (Kristich and Rice, 2014), eliminating β-lactams singly as an antibiotic for therapeutic enterococcal infections (Weiner *et al*., 2016). The appearance of β-lactamase is not a reparative challenge since β-lactamase inhibitor combinations impede it. Still, as enterococci strains continue to test for high resistance, it may present a diagnostic challenge (Arias and Murray, 2012).

2.8.4. Linezolid resistance

Linezolid prevents protein production by binding to the 23S ribosomal RNA site of the 50S subunit of the bacteria, inhibiting the 70S ribosomal unit's formation, which inhibits protein synthesis (Etebu and Arikekpar, 2016). Linezolid, belonging to the family Oxazolidinone, is the second of the two compounds approved by the US Food and Drug Administration (FDA) to treat VRE and used worldwide (Stefani *et al.*, 2010; Arias and Murray, 2012). The infection generated by linezolid resistant enterococci (LRE) and HAI spread has been described in some outbreak research with samples from both vancomycin-resistant and enterococci-susceptible (Ntokou *et al*., 2012; O'Driscoll *et al*., 2015). The mechanism of resistance to linezolid is mediated by gene acquisition or mutation. In enterococci, the most common resistance mechanism involves G2576T mutations in genes encoding domain V of the 23S rRNA. This mutation interferes with the initially assigned nucleotide to rRNA genes in *E. coli*, G–to –T alteration at position 2576 (Pfaller *et al*., 2017).

Gawryszewska *et al.* (2017) reported *E. faecium* belonged to the hospital-adapted lineages 1718 and 78 in their analysis of 50 LRE from 2008 to 2015. *E. faecalis* isolates represented ST116 associated with both humans and food-production animals as well as ST6, a hospital-linked strain type. The main reoccurring (94%) mechanism of linezolid and tedizolid resistance observed was

the 23S rRNA (G2576T) mutation (Gawryszewska *et al*., 2017). Ruru *et al. (*2018) also reported the same mutations as well as 5% *cfr* gene in 51% of *E. faecalis* and 81% of *E. faecium*, potentially transferring horizontally between strains. In addition to the *cfr* gene, 32 cases of gene *optr*A-positive LRE were identified (Bi *et al*., 20118).

2.8.5. Resistance to quinupristin-dalfopristin

The mode of action of quinupristin-dalfopristin (Q/D) is through the bacterial ribosome. The former inhibits the late phase of protein synthesis, while the latter inhibits the early protein synthesis phase. Q/D is a combination of two semi-synthetic streptogramins and the FDA's first drug to treat VRE infections (Delgado *et al*., 2000). They inhibit protein synthesis from creating a synergistic effect by linking with the 50S ribosomal subunit. This results in direct interactivity to either compound with a single nucleotide (A2062) at the peptidyl-transferase middle, indicating a major change resulting in bactericidal activity (Harms *et al*., 2004). Due to the action of *Lsa* (a predicted ATP-binding protein), most *E. faecalis* isolates are resistant to dalfopristin (Arias and Murray, 2012).

In *E. faecium*, different mechanisms induce resistance to Q/D, viz; drug alteration, efflux and inactivation via virginiamycin acetyltransferase (Vat) and the ATP-binding cassette protein macrolide-streptogramin resistance protein (*MsrC*) (Stogios *et al*., 2014). Resistant to Q/D reported being as high as 81% in *E. faecalis* clinical samples collected from a different hospital in Tehran, Iran, with 9% in *E. faecium* (Masoumi *et al*., 2020). Haghi *et al*. (2019) also reported a 53% resistance to Q/D of *Enterococcus* spp. from hospitalized patients (Haghi *et al*., 2019).

2.9. Application of metagenomics in the detection of bacterial diversity

Microorganisms dominate the earth in abundance and diversity; although, only a small snippet of this bacterial diverseness (less than 1%) can be cultured in a standardized fashion (Alneberg *et al*., 2018). We employed the use of metagenomics in this study to identify the different microbial diversity of organisms found in the hospital environment investigated. In any biological community, quantifying species diversity is very important as they contribute to ecosystem diversity (either good or bad). The easiest way to quantify and describe a microbial community in

a sample (environmental, eco-genomics, or community genomics), is the abundance of species in a particular region, which refers to the species richness concept (Escobar-Zepeda *et al*., 2015).

The introduction of the metagenomic approach has fundamentally changed the identification of microbial pathogens, and their chromosomal diversity and operation within a particular environment (Girish and Hameeda, 2013). Metagenomics enlightened our knowledge of microbial communities' functional gene composition and further provided a more comprehensive description than phylogenetic surveys (Thomas *et al*., 2012). The advancement of metagenomics reduces, to an extent, the uncertainty of uncultured microorganisms, constituting a large majority of organisms in various environments (Ghosh *et al*., 2019). Approaches such as high-throughput 16S rRNA gene sequencing can profile organisms and their marker genes (Ju and Zhang, 2015). Bacterial diversity is described and compared using the alpha (α), gamma (γ), and beta (β) metrics.

The metric for limited diversity of a community is referred to as the alpha, while gamma determines many communities overall diversity. The beta metric combines alpha and gamma metrics by showing how many contrasting community samples are in an environment (Krebs, 2014). The build-up of species known as operational taxonomic units (OTUs) plots implemented to estimate sample proficiency and correct sampling fallacies, e.g. over or under evaluation of the population's relating factors (Hugerth and Andersson, 2017). This approach assesses the actual species or OTU in the sample and compares samples with different sizes (statistically using rarefaction curves) (Hughes *et al*., 2001). The development of metagenomics provided new insight into a microbial ecosystem and allowed the analysis of large complex communities in a little time (Luana *et al.*, 2018).

There are some limitations to the use of metagenomics; an abundant quantity and high quality of DNA samples are needed to achieve the superior coverage required for metagenomics. Although precaution is taken, human contaminants are found in approximately 50% to 90% of sequences (Wei *et al.*, 2019). The quality of the principal functional annotations of metagenomic sequence fragments is essential to carry out a metagenomic study successfully. Nevertheless, a substantial percentage of data cannot be assigned a function owing to the non-existence of accurate matches

in the reference databases (Qin *et al.*, 2016). Also, when fewer microbiome numbers have closely related species, it may be challenging to assemble these genomes (Wei *et al.*, 2019).

2.10. Whole-genome sequencing (WGS) as a molecular typing method

This study uses WGS to determine the mobile genetic variables of the various clonal lineages and the phylogenomic relationship of *E. faecalis* circulating the different healthcare levels, wards, and multiple sites. Using next-generation sequencing, examining the comprehensive genomes of specific bacterial isolates and tentatively distinguishing strains that differ at only a single nucleotide has been made possible (Salipante *et al*., 2015). In the context of AMR, which is a significant threat to public health, WGS has renewed our knowledge giving new insights into basic resistance genetics (Jasovský *et al*., 2016). WGS gives an improved resolution to report pathogen circulation on a large and global scale (Brodrick *et al.*, 2016). The rapid drop in sequencing cost and a researcher's ability to ask virtually any question related to the genome, transcriptome, or epigenome has made WGS a powerful tool for genomic research (Kulski, 2016).

While WGS was generally related to sequencing the human genome, the accessibility and variable characteristics make it functional for sequencing every species, such as disease-related microbial genomes (Jagadeesan *et al*., 2019). WGS has changed gradually to established procedures in several research laboratories and promptly utilized worldwide by health systems (Jagadeesan *et al*., 2019). Relatively, few statistics are available to complement the existence of resistance factors to phenotypic resistance in *Enterococcus* and other Gram-positive bacteria (Ocheretina *et al*., 2014).

2.11. Classification of hospital-level in South Africa

This section described the different healthcare levels in South Africa and included in this study. The delivery of quality healthcare at all levels is a constitutional obligation in South Africa. According to the Ministry of Health, in terms of Section 35 together with Section 90 of the National Health Act, 2003 (Act No. 61 of 2003) (National Department of Health SA, 2013; National Department of Health SA, 2017), public hospitals are categorized as follows; i) District hospital;

ii) Regional hospital; iii) Tertiary hospital, and iv) Central hospital. These hospitals levels have different functions and explained as follows:

District hospitals are classified as level one in the referral system and the level of healthcare. They have beds no less than 50 and no more than 600 and provide 24 hours care. They serve a specified populace surrounding a health district and assisting primary health care by receiving referrals from primary healthcare clinics and community healthcare centres. Providing in-patient/out-patient services, ambulatory health services, and emergency health services; and where needed, offering education for health care service providers. When they cannot provide further assistance to a patient, they are referred to the Regional hospital for care (National Department of Health SA, 2017).

Regional hospitals are level two hospitals and are like the district hospitals, providing 24-hour healthcare services with 200 to 800 beds. They make available health services in obstetrics, internal medicine, gynaecology, paediatrics and general surgery. They also offer additional specialist services and receives referrals from several district hospitals. In cases of limited resources, the regional hospital could refer patients to the provincial tertiary hospital for further care (National Department of Health SA, 2013).

Tertiary hospitals are level three healthcare that provides specialist level services.; They offer intensive care assistance as supervised by specialists or expert intensivist. They have beds between 400 to 800 and receive a patient's transfer from the regional hospitals not restricted to the provincial limit. In some individual cases, provincial tertiary hospitals could refer patients to a national central hospital for advanced treatment (National Department of Health SA, 2013).

The fourth and highest level is the **central hospitals.** These hospitals comprise highly specialized referral units that collectively deliver an environment for various-speciality, innovation, and research (affixed to a medical school for teaching) and clinical services. They have a maximum of 1200 beds and offer national referral with extraordinarily specialized (National Department of Health SA, 2013).

3.0. Aim and objectives of the study

Although the research was conducted on *Enterococcus* spp. in South Africa, hardly any studies of the hospital inanimate environments across the four healthcare levels sought to understand and subsequently address infection prevention and control. Few (or no) studies have been executed using metagenomics to show the different bacterial diversity and abundance in the hospital environment. WGS is also used in understanding genetic variations to predict the resistome, spread, clonal lineage, and molecular profiles of *E. faecalis* in the hospital environment. Hence, this study contributes to IPCs in hospitals by investigating the microbial diversity and functional profile of bacteria from different sources in various wards at different healthcare levels. Using WGS, the emergence of *E. faecalis*, clonal spread, mobile genetic variable, clonal linage, and antibiotic resistance mechanisms in healthcare facility environments will be better understood, providing evidence to optimize infection prevention in this aspect.

3.1. Research aims

This study investigated environmental samples from different public hospitals as reservoirs of antibiotic-resistant bacteria in terms of their epidemiology, phylogenies, clonality, and antibiotic resistance as an indicator of infection, prevention, and control measures.

3.2. Objectives

- To investigate the microbial diversity and functional profiles of bacteria from different sources in various wards and different healthcare facilities in eThekwini district, South Africa, using 16S rRNA metagenomics.
- To culture, isolate, identify and ascertain the prevalence of *Enterococcus* spp. sampled from selected inanimate surfaces in different wards (intensive care unit and paediatric unit) at different healthcare levels (district, regional, tertiary and central hospital) using selective media, biochemical tests, and polymerase chain reaction (PCR).
- To investigate the antibiotic susceptibility profiles using disc diffusion according to CLSI against 14 antibiotics *viz*., **glycopeptides** [vancomycin, teicoplanin], **quinolones** [ciprofloxacin, levofloxacin], **amphenicols** [chloramphenicol], **penicillins** [ampicillin, penicillin G], **rifamycins** [rifampicin], **tetracyclines** [tetracycline], **macrolides**

[erythromycin], **nitrofurans** [nitrofurantoin], **oxazolidinones** [linezolid] with additional detection of high-level resistance to **aminoglycosides** [gentamycin and streptomycin].

- To identify and characterize the resistance genes and associated genetic mutations (if any) of *E. faecalis* in the hospital environment in the different healthcare levels using WGS and bioinformatics tools.
- To determine the genetic composition and mobile genetic variables of *E. faecalis* in the hospital environment in the different healthcare levels using WGS and bioinformatics tools.
- To determine the clonal and phylogenomic relationship of *E. faecalis* isolated from the hospital environments and the clonal lineages of circulating *E. faecalis* isolated from the different healthcare levels and the various sites between each hospital using WGS and bioinformatics tools.

4.0. Synopsis of methodology

4.1. Ethical considerations

Ethical approval for the study was obtained from the Biomedical Research Ethics Committee (BREC) of the University of KwaZulu-Natal [reference number BE606/16] and the KwaZulu-Natal Provincial Health Research Ethics Committee [reference number KZ 2017RP24 630]. Permission was granted by the relevant provincial Departments of Health, the eThekwini District, and all the hospitals involved to carry out this research.

4.2. General Methodology

This research observed the presence of bacteria at pre-selected hospitals over a short period between September to November 2017 due to this research's fundings. Samples were sourced from four public hospitals at different healthcare levels situated in KwaZulu-Natal provincial district viz. district, regional, tertiary, and central. Sampling was undertaken on three separate occasions of a working week (Monday to Friday) on Mondays, Wednesdays, and Fridays. The wards selected were the ICU and paediatric wards; sample sites viz. the ward phones, ventilators, blood pressure

apparatus, patient files, drip stands, sinks, beds (both occupied and unoccupied), nurses' tables, mops, and the linen room door handle. For statistical representation, 25% of all sample sites present in a particular ward was collected (e.g., if there were eight beds, then two beds were sampled randomly), and pooled (i.e., samples from the same sites were incubated together in the same broth). This study investigated the bacterial isolates using 16S rRNA metagenomics to identify the different microbial diversity in the samples collected.

We narrowed down the study to focus on *Enterococcus* spp. and investigated its contamination in hospital environments, focusing on the paediatric wards and ICUs of public hospitals at four healthcare levels. By employing selective media, biochemical tests and identification of the *tuf* gene confirmed by PCR. The Kirby–Bauer disk diffusion method against 14 antibiotics revealed antibiotic resistance patterns in the enterococcal isolates. Further analyses were conducted on *E. faecalis*, the highest isolated *Enterococcus* specie in the samples collected using WGS. The latter delineated the resistome, mobile genetic support, the clonal and phylogenomic relationship of isolates from the hospital environments and the clonal lineages of circulating *E. faecalis.*

5.0. Outline of the thesis

This project is presented in the form of published and submitted manuscripts and consists of the following chapters

- **Chapter 2**

  Bacterial diversity and functional profile of microbial populations on surfaces in public hospital environments in South Africa: A high throughput metagenomic analysis

  This original research paper explored the variety and operative profiles of the microorganisms found in different healthcare facilities (from the district to the central level), two different wards, and highly touchable surfaces in the hospitals employing the 16S rRNA metagenomics. It also eluded to major pathways and human disease operative classes comprising those involved in AMR. The prospective of diverse hospital environments to aid as reservoirs and potential sources of bacterial pathogens was highlighted.

  A journal-specific published format is presented in chapter 2.

- **Chapter 3**

  Enterococcal contamination of hospital environments in KwaZulu-Natal South Africa

  Here, we described the isolation, identification, and prevalence and antimicrobial susceptibility profiles of *Enterococcus* spp. from selected, frequently touched inanimate surfaces in different wards (intensive care unit and paediatric unit) in the different healthcare levels. This paper highlighted the need for strict sterilization and disinfection protocols, isolation measures, and healthcare and janitorial personnel training on the risk of IPC failures.

- **Chapter 4**

  Genome sequence of a novel *Enterococcus faecalis* sequence Type 922 strain isolated from a door handle in the intensive care unit of a district hospital in Durban, South Africa

  The emergence of this novel *E. faecalis* sequence type 922 (ST922), was recovered from a door handle in the ICU of a district hospital in Durban, South Africa. The sequence type was described as ST922 by the *Enterococcus faecalis* MLST database

https://pubmlst.org/efaecalis/. This indicated that a new strain was emerging and transferred from the hand to the door handle.

A journal-specific published format is presented in chapter 4.

Comparative genomics reveals the dominance of major clones of *Enterococcus faecalis* within public hospital environments in South Africa.

Here we provided a snapshot of the inanimate hospital environment as a reservoir of resistant *E. faecalis*, its associated mobilome (plasmids, prophages, insertion sequences and transposons) revealed an elaborate intra-clonal spread of *E. faecalis* major clones between the sites within each specific hospital setting. We showed that clones were particular to each hospital. This paper improves our knowledge of disseminating *E. faecalis* in hospital environments and will help design optimal infection IPC strategies in clinical settings.

- **Chapter 5**

Conclusion: This chapter captures the work's summary and significance and presents the limitations and recommendations for future work.

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Chapter 2 – Manuscript 1

Bacterial diversity and functional profile of microbial populations on surfaces in public hospital environments in South Africa: A high throughput metagenomic analysis.

Author contributions

- **Christiana Omowunmi Shobo:** Conceptualization the study, methodology, investigation, visualization, writing - original draft.
- Arghavan Alisoltani: Formal analysis, visualization, writing - review and editing.
- Akebe Luther King Abia: Formal analysis, visualization, writing - review and editing.
- Philip Senzo Mtshali: Software, formal analysis.
- Arshad Ismail: Software, formal analysis.
- Oliver Zishiri: Writing - review and editing.
- Juliana Deidre Horn: Writing - review and editing.
- Petra Brysiewicz: Writing - review and editing.
- Sabiha Yusuf Essack: Supervision, Writing - review and editing.
- Linda Antionette Bester: Conceptualization, principal supervision, and funding acquisition.

Objective met: This paper answers objective 3.

Christiana Omowunmi Shobo, Arghavan Alisoltani, Akebe Luther King Abia, Philip Senzo Mtshali, Arshad Ismail, Oliver Zishiri, Juliana Deidré Horn, Petra Brysiewicz, Sabiha Yusuf Essack, Linda Antionette Bester. Bacterial diversity and functional profile of microbial populations on surfaces in public hospital environments in South Africa: A high throughput metagenomic analysis. **Sci Total Environ. 2020; 719: 137360**. https://doi.org/10.1016/j.scitotenv.2020.137360.

Science of the Total Environment 719 (2020) 137360

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Bacterial diversity and functional profile of microbial populations on surfaces in public hospital environments in South Africa: A high throughput metagenomic analysis

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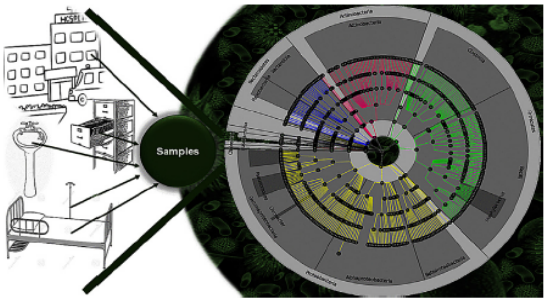
HIGHLIGHTS

- Bacterial community and functional profiles were studied in hospital environments.
- Microbial variation within hospital environments was mainly driven by Hospital type
- ICU surfaces in all the hospitals had the highest number of total and unique OTUs.
- The drip stands had the highest number of pathogenic bacteria signatures.
- The functional profile analysis showed a significant involvement in human diseases.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 27 December 2019
Received in revised form 14 February 2020
Accepted 14 February 2020
Available online 20 February 2020

Editor: Damia Barcelo

Keywords:
Metagenomic analysis

ABSTRACT

With the introduction of the One Health approach to global health advocated by the World Health Organization, the role of the environment as a reservoir and transmission route for diverse microorganisms is increasingly being recognised globally. This study investigated the diversity and functional profiles of bacterial communities using high-throughput metagenomics of the *16S rRNA* gene in samples collected from environmental surfaces in different levels of healthcare in South Africa. A total of 150 samples were collected in three public hospitals [District (A), Regional (C) and Central (B)] from intensive care and paediatric wards. Military hospitals were excluded. Swabs were taken from mattresses, drip stands, ward telephones, patient files and sinks. A total of 7,996,346 reads were found, of which 7,319,569 were quality-filtered reads. Unique (and shared) microbial community structures were identified within the different hospital levels, locations and sample source. A total of 11

Abbreviations: KEGG, Kyoto Encyclopedia of Genes and Genomes; OTUs, Operational Taxonomic Units; PICRUSt, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States.

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https://doi.org/10.1016/j.scitotenv.2020.137360
0048-9697/

Environmental reservoirs
Bacterial diversity
Functional profiles
Human diseases
Hospital setting

phyla, 29 classes, 50 orders, 105 families, 190 genera and 288 known species were identified. The primary phyla identified were Proteobacteria, Firmicutes and Actinobacteria. The dominant class identified was Gammaproteobacteria, followed by *Bacilli* and Actinobacteria. *Acinetobacter* (16.08%), *Citrobacter* (13.64%), *Staphylococcus* (9.65%) and *Corynebacterium* (6.15%) were predominant genera. Although the functional profile analysis identified citrate cycle (TCA), signal transduction mechanisms, bisphenol degradation, tyrosine metabolism and transcription-factors as the dominant pathways, human disease functional classes, including involvement in antibiotic resistance, were significantly identified. The drip stands, patient files and ward telephones in all the wards of Hospitals A and C contained a higher number of human diseases functional classes. These findings highlight the potential of different hospital environments to serve as reservoirs and possible sources of bacterial pathogens; thus, the need for better monitoring and hygienic practices within the hospital environment.

1. Introduction

The environment is recognised as the largest reservoir of microorganisms, including pathogens and the survival of these microbial species in diverse environmental settings, including on inert surfaces, presents a challenge for microbiologists. Only a small fraction of the microorganisms found in the environment have been identified to date due to the limitation of conventional methods used such as cultures, which are often biased and favour the growth of selected organisms at the expense of others (Al-Awadhi et al., 2013). These organisms may spread and colonise different settings either naturally or through human activities (Merikanto et al., 2014).

While most of these microorganisms could be harmless to humans and other animals, a significant number of them have been implicated in infections, especially in humans (Aarts and Margolles, 2014; Martínez, 2014; Pigłowski, 2019). Although the study of the diversity of microbial communities, and in some cases, their functional attributes, have been studied in soil (Maron et al., 2011), air (García-Mena et al., 2016), water (Bai et al., 2019) and other environments commonly encountered by humans, such studies in hospital settings are limited. Hospitals represent complex environmental settings compared to other natural settings. Due to the continuous movement of people with different disease conditions into and out of hospitals, the chances of transmission of infection in such an environment would likely be higher (Revelas, 2012). This is more pronounced in low-and middle-income countries, where it has been demonstrated that poor environmental conditions within healthcare facilities were among the leading causes of increased hospital-acquired infection (Cronk and Bartram, 2018). Specifically, in South Africa for example, it was revealed that lack of cleanliness among other factors, accounted for poor service delivery within healthcare facilities (Maphumulo and Bhengu, 2019). Similarly, Mulogo et al. (2018) reported poor environmental conditions in hospitals in Uganda. Thus, the contamination of the hospital environment has been identified as a significant contributor to several critical healthcare-associated pathogens, patient colonisation and infection (Dancer, 2014; Russotto et al., 2017), with the environmental transmission of organisms such as vancomycin-resistant enterococci and methicillin-resistant *Staphylococcus aureus* being reported in these settings.

Environmental assessment has confirmed the continual contamination of items, equipment, and general sites in bed spaces, wards and throughout multiple clinical areas in hospitals (Dancer, 2014; Lemmen et al., 2004). As such, strategic measures aimed at maintaining hygienic conditions in such places have been among the approaches employed to prevent environmental transmission of these microorganisms. Ensuring cleanliness in hospitals is considered one of the most crucial aspects required to address patient safety issues within hospitals. Although cleaning and disinfection of environmental surfaces are essential elements within infection prevention and control (IPC) programs in hospital settings, achieving the desired levels of surface disinfection remains a challenge (Boyce, 2016).

Given that organisms on hospital environmental surfaces could have adverse health implications for hospital personnel and already immunocompromised patients, a thorough understanding of the microbial populations and their possible functional attributes is crucial. Studying these organisms has been achieved by conventional culture procedures (Wolfe, 2018). Nevertheless, these methods are extremely challenging, as over 99% of the microorganisms present in various environments are not readily culturable (Stewart, 2012). Culture techniques are usually based on isolation of indicator organisms, and this could lead to selective bias, thus, preventing the identification of organisms that could be unique within a given environment such as a hospital setting (Mora et al., 2016). It is estimated that of the >61 bacterial phyla known to date, approximately 50% have no culturable representatives (Wonyong, 2012).

With the advent of advanced molecular, culture-independent techniques such as metagenomics, scientists are gradually gaining a better understanding of larger microbial communities, compared to single species or group of species. The introduction of these techniques has revolutionised the detection of microbial pathogens, including the resolution of their molecular phylogenetic background together with their genetic diversity and function within given environments (Oulas et al., 2015). These techniques allow for the identification and exploitation of previously unknown bacteria, even in the most complex ecosystems (Simon and Daniel, 2011). Where these techniques have been used in hospital environments, they have mostly focused on the indoor and outdoor air quality (Lai et al., 2015), hospitals effluent (Singh et al., 2019) and ambulances (O'Hara et al., 2017). Metagenomic studies on different surfaces within different hospital wards are limited. Two studies conducted in Brazil explored the microbiome on hospital surfaces using metagenomics and reported the abundance and diversity of diverse microbial species (King et al., 2016; Ribeiro et al., 2019). However, these studies did not involve an analysis of the functional profiles of the microbial communities and did not consider the potential effect of the different levels of healthcare.

This study thus sought to investigate the microbial diversity and functional profiles of bacteria from different sources, in different wards and at different levels of healthcare facilities in Durban, South Africa, using 16S rRNA metagenomics.

2. Materials and methods

2.1. Ethical approval

Ethical approval for the study was obtained from the Biomedical Research Ethics Committee (BREC) of the University of KwaZulu-Natal under the reference number BE606/16. Permission to conduct the study was obtained from the relevant provincial Department of Health, the eThekwini District and all hospitals involved in the study.

2.2. Study site, sample collection and sample size

The selected sites included public hospitals situated in KwaZulu-Natal, South Africa but excluding military hospitals. For non-disclosure reasons, the names of the hospitals were withheld and referred to as

A, B and C representing the District, Central and Regional Hospital respectively. Central hospitals in South Africa, also known as specialised hospitals (bed size 800 upward), offer tertiary hospitals services and serve as referral hospitals for both the district and regional hospitals. The regional hospital provides services to a specific regional population and receives referrals from several district hospitals. It has a 743-bed size. The district hospital serves a health district and supports primary health care services on a 24-hour basis with a 300-bed size. Samples were collected from the general ward (male), the intensive care unit (ICU) and paediatric ward except for Hospital B, where samples were only collected from the ICU due to limited resources. Samples were taken from five sources that included ward phones, drip stands, patient files, sinks and patient beds.

All samples were collected by randomly swabbing approximately 5 cm of the respective surfaces using pre-labelled Nylon flock swabs with transport media (FLOQSwabs™ COPEN diagnostics Inc., USA). The swabs were then transported to the Biomedical Research Laboratory at the University of KwaZulu-Natal in iceboxes and processed immediately upon arrival within 3 to 4 h of sampling. A total of 150 samples were collected from all the hospitals and samples from the same site in each hospital were pooled. After pooling, a total of 28 samples (13 from hospital A, four from hospital B and 11 from hospital C) were further analysed.

2.3. DNA extraction and high throughput sequencing

The collected swabs were homogenised in Brain Heart Infusion (BHI) broth to separate the bacteria from the swabs. Bacteria were then harvested from the broth solution by centrifugation at 13000 ×*g* (HERMLE Z233M-2 Labortechnik GmbH), and DNA was extracted from the resultant pellet using the Purelink™ microbial DNA purification kit (Thermofisher Scientific, CA. USA) as per the manufacturer's instructions.

The concentration and purity of the isolated DNA were measured spectrophotometrically using the NanoDrop (ND-1000 Spectrophotometer, Wilmington, DE USA) at wavelengths of A260 and A280. The extracted DNA was sent to the Sequencing Core Facility, National Institute for Communicable Diseases (NICD) of the National Health Laboratory Service, Sandringham, Johannesburg, South Africa, where sequencing was carried out on an Illumina MiSeq machine (Illumina, USA), targeting the V3–V4 variable region of the *16S rRNA* gene in the genomic DNA (Klindworth et al., 2013).

2.4. Data analysis

Previously described analysis pipelines (Abia et al., 2019, 2018), with slight modifications, were used for the analysis of NGS-based *16S rRNA* gene sequencing data. Briefly, the CLC Genomics Workbench (CLC Bio Qiagen) version 11.0.1 together with CLC Microbial Genomics Module version 3.6.1 was used to identify operational taxonomic units (OTUs). Sequence quality control, filtering, and trimming were all conducted to obtain clean data. Clustering was done at a 99% similarity threshold using Greengene database, and OTUs were assigned to sequences based on 99% identity. The Venn plotter was used to plot Venn diagrams (http://bioinformatics.psb.ugent.be/webtools/Venn/).

Diversity within and between samples (Alpha and Beta diversities) was estimated and plotted using the R Studio package (ggplot2) Phyloseq (Mcmurdie and Holmes, 2013). The ANOVA and Moderated *t*-test, implemented in "limma" Bioconductor R package, were applied to identify significant differences between OTUs and functional classes (Ritchie et al., 2015). ANOVA was used to ascertain the presence of general differences between the hospitals, wards and sample sources. The relative abundance of OTUs was considered as the response variable, while the hospital level was the explanatory variable. In the case where the results of ANOVA were significant, the moderated t-test for pairwise comparison of hospitals was used (False Discovery Rate adjusted p-value ≤.05). The same statistical procedure was used to assess the role of locations and sampling sources on the diversity of OTUs.

GraPhlAn on Galaxy was run to draw general microbial composition across all samples. A heatmap of the 500 most abundant OTUs was constructed using the R package "ComplexHeatmap" to cluster the samples. Principal Component Analysis (PCA) was undertaken to investigate the clustering and diversity between samples using the mixOmics R package. PICRUSt, based on the KEGG (Kyoto Encyclopedia of Genes and Genomes) database, was used to predict the function of the OTUs, (Langille et al., 2013). To characterise the key OTUs and functional classes between hospital samples, the supervised learning Random Forest Algorithm (mtry = p/3 and bootstrapping = 1000) was applied using the R package "randomForest".

3. Results

3.1. Shared and unique bacteria between hospital samples, as determined by 16S metagenomics

The 16S metagenome was applied to compare bacterial composition and diversity of different hospital levels, locations and sample sources. A total of 7,996,346 reads were generated, of which, 7,319,569 quality-filtered reads were obtained (Table 1). In general, the average number of reads, the length of reads (nt) and the number of OTUs across all samples were estimated at 285, 583 and 364, respectively.

A total of 2030 OTUs were characterised across all samples using the CLC Genomics Workbench (QIAGEN) based on Greengene database (Table S1). There were 462, 658 and 325 reads shared OTUs across the different hospital levels, locations and sample sources, respectively (Fig. 1). The highest numbers of total and unique OTUs across the samples obtained from the different hospital locations were recorded in the intensive care unit (ICU) (Fig. 1B), while the drip stands and bed harboured the highest number of both total and unique OTUs among all the hospital sample sources (Fig. 1C). In contrast, hospital C, the paediatric unit and patient files contained the lowest numbers of total and unique OTUs (Fig. 1).

The bacterial community structure distinctly differed between the hospital samples (Fig. 2). The number of known taxonomic levels for each sample is given in Table 2. A total of 11 phyla, 29 classes, 50 orders, 105 families, 190 genera and 288 known species were characterised for all samples. Whilst most of the samples were dominated by Proteobacteria, Firmicutes was the most abundant phylum in four samples (Fig. 2A). At the class level, Gamma-proteobacteria was dominant in most of the hospital samples, followed by Bacilli and Actinobacteria. Furthermore, the bacterial diversity and composition of the hospital samples differed at the genus and species levels (FDR adjusted p-value ≤.05), although many genera and species were identified as unknown (uncultured) bacteria. The four genera *Acinetobacter* (16.08%), *Citrobacter* (13.64%), *Staphylococcus* (9.65%) and *Corynebacterium* (6.15%) were recorded as the predominant genera in all samples (Table S1). *Staphylococcus sciuri*, *Acinetobacter lwoffii*, and *Acinetobacter johnsonii* were found among the most abundant known species (Fig. 2B).

Taxa richness was estimated based on Chao1 and Shannon indices (Fig. 3). Results indicated that samples obtained from the same hospital had almost similar richness (Fig. 3A). Based on Chao1, the general trend of alpha diversity was higher for general ward units, followed by paediatric and intensive care units. In most cases, the bacterial community of drip stand and sink generally showed higher alpha bacterial diversity compared to other sampling sites (Fig. 3B).

Beta diversity analysis (diversity between samples) was most evident between the hospital levels (except Hospital B), although it was also linked with the location within the hospital; in other words, most of the samples obtained from the same hospital were clustered together as demonstrated using the heatmap of the most abundant OTUs. These

Table 1
General characteristics of sequencing data for each hospital sample.

Hospital	Wards	Sampling Site	Total number of reads	Avg. length of reads	Number of predicted OTUs
A	General ward	Bed	280,148	287.8	513
		Drip stand	355,302	233.4	492
		Patient File	276,968	283.1	447
		Ward Phone	310,228	225.4	466
	Intensive care unit	Bed	186,988	259.8	397
		Drip stand	229,200	266.5	446
		Patient File	117,572	258.9	339
		Sink	970,312	215	493
		Ward Phone	222,142	242	343
	Paediatric ward	Bed	211,618	291.3	454
		Drip stand	263,498	199.5	416
		Patient File	238,152	257.6	238
		Ward Phone	308,148	214.9	393
B	Intensive care unit	Bed	129,386	299.7	181
		Drip stand	999,620	254.6	706
		Patient File	126,544	299.7	156
		Sink	305,038	296.2	465
C	General ward	Ward Phone	243,464	287.5	309
		Drip stand	296,260	290.9	301
		Patient File	215,210	298.6	289
	Intensive care unit	Drip stand	183,254	291.3	319
		Patient File	340,484	279.8	338
		Ward Phone	206,310	290.8	263
	Paediatric ward	Bed	180,714	299.5	274
		Drip stand	190,842	293.3	296
		Patient File	222,192	275.6	308
		Sink	226,848	299.8	308
		Ward Phone	159,904	297.5	263

findings were further validated using PCA based on the Euclidean distance matrix (Fig. 4).

3.2. Key OTUs and pathogenic genera identified in hospital samples

Since the primary driver of variation between samples was the hospital level, multiple pairwise comparisons were applied between two hospitals using the moderated *t*-test implemented in the limma package. Findings demonstrated significant (FDR adjusted *p*-value ≤.05) differences between Hospitals A and C, whereas no significant (FDR adjusted *p*-value ≤.05) differences were evident between Hospital B (which could be as a result in differences in pooled sample sizes) and the two other hospitals (Tables S2-S4). Six OTUs were among the top significant identified OTUs (FDR adjusted *p*-value >.05) in Hospital C compared to A, and these included "g_Proteus.685373", "f_Alcaligenaceae.6864", "s_stationis.650615", "g_Corynebacterium.218364", "g_Oceanimonas.751128", and g_Proteus.204864 (Table S3). The ANOVA test revealed no significant (*p*-value >.05) changes in OTU abundances between the three studied wards (Table S5). However, pairwise comparison (FDR adjusted *p*-value ≤.05) revealed a significantly higher abundance of "g__Aerococcus.1091624" in the ICU compared to the general ward unit. Regarding sampling sites, only "g_Wautersiella.547181" significantly (*p*-value ≤.05) changed between five sample sources (Table S6). Similar to the results of PCA and beta-diversity, these findings also indicate that the main driver of OTU diversity is the hospital level.

This study then focussed on human pathogenic genera. *Corynebacterium*, *Acinetobacter*, *Citrobacter*, *Staphylococcus* were among the dominant pathogenic genera across all hospital samples (Table 3). Interestingly, the drip stands contained the highest number of pathogenic bacteria in the studied hospitals and hospital locations. Moreover, a mean decrease of accuracy and mean decrease of Gini index were estimated to determine the key OTUs between hospital-level samples, where a higher amount of both indices indicated the importance of the specific OTUs (S-Fig. 1). Two "s_geniculata.256155" and "g_Proteus.10205" OTUs had the highest mean decrease Gini, which could associate samples obtained from the three different hospital levels (Out-of-bag error rate: 25%) (S-Fig. 1A). Similarly, "g_Aerococcus.851916" and "g_Rhizobium.559108" were recorded as the most important OTUs between the three hospital locations and five sample sources, respectively (S-Fig. 1B and C).

3.3. Functional profile analysis of the predicted bacterial community in the hospital samples

The functional profiles of OTUs were predicted from amplicon data using PICRUSt on Galaxy. A total of 279 functional groups at level three KEGG Orthology (Table S7) were found. For all samples, the most abundant pathways involved transporting and environmental information processing, although poorly characterised functions were also among the top functional classes (Table S7). Like OTUs, pairwise comparisons of functional classes between every two hospitals were conducted using the moderated *t*-test implemented (FDR adjusted *p*-value ≤.05). This analysis demonstrated significant differences between Hospital C and the other hospitals, while there were no significant changes between Hospital A and B (Tables S8-S10). Citrate cycle (TCA cycle), signal transduction mechanisms, bisphenol degradation, tyrosine metabolism and transcription factor related pathways were among the top significantly different functional groups in Hospital C. ANOVA analysis revealed no significant changes in OTU abundances between the three studied hospital location and five sample sources (Table S11 and S12). Based on the higher mean decrease of Gini index, key pathways between hospital levels were tyrosine metabolism, G protein-coupled receptors, protein processing in the endoplasmic reticulum and TCA cycle (Fig. 4A). DNA repair and recombination proteins were the top functional groups that could separate hospital locations (Fig. 4B).

Human disease functional classes were also identified to investigate the hospital environment as a potential route for the transmission of human infections. Infectious diseases, neurodegenerative diseases, cancers, metabolic diseases and immune system diseases were among the most abundant predicted functional classes (Table S7). In general, Hospital A and C (Fig. 5A), ICU and paediatric units (Fig. 5B), drip stands, patient files and ward phones (Fig. 5C) contained a higher number of human diseases functional classes.

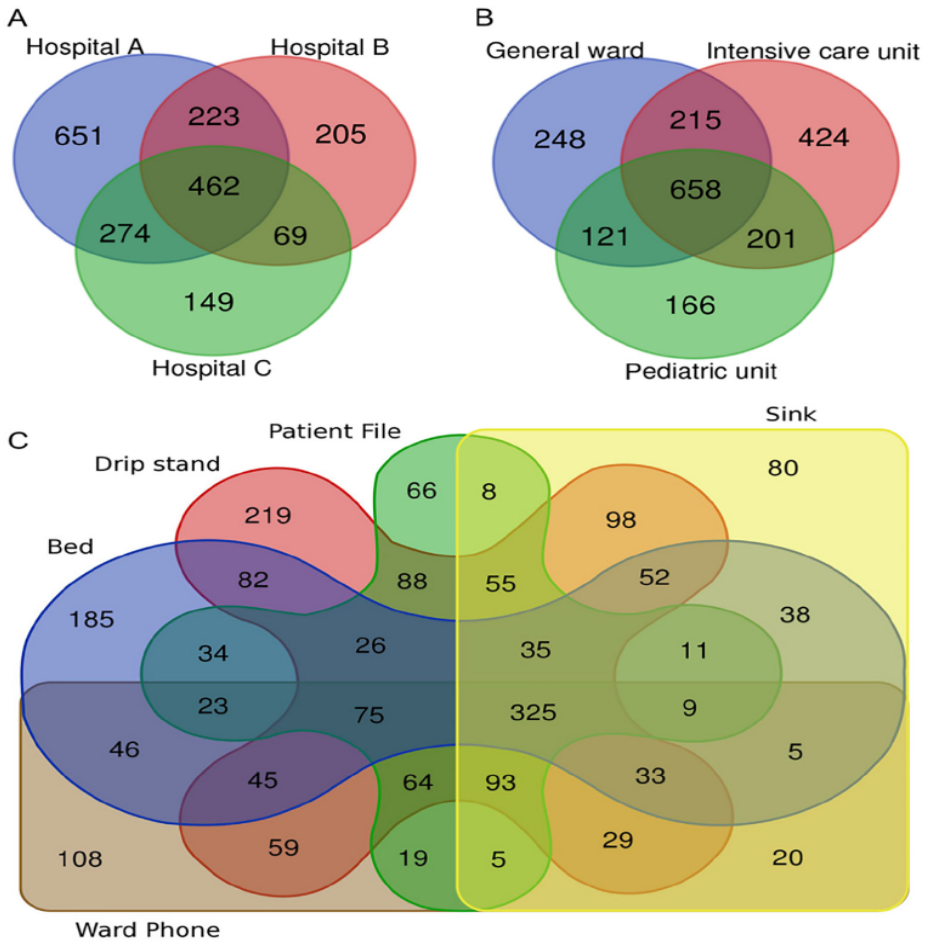

Fig. 1. Venn diagram illustrating shared and unique bacteria between studied hospital levels (A), hospital locations (B) and sample sources (C).

4. Discussion

This study investigated the bacterial population diversity and their associated functional profiles in samples collected from the hospital environment in three different hospital levels in South Africa, using high-throughput *16S rRNA* gene-based metagenomics. The different hospitals displayed unique bacterial diversity profiles, albeit with some similarities across the locations and the sample sources within the wards. *Corynebacterium*, *Acinetobacter*, *Citrobacter*, *Staphylococcus* were the most abundant genera in all hospitals while *Staphylococcus sciuri*, *Acinetobacter lwoffii*, and *Acinetobacter johnsonii* were the most abundant pathogenic species identified. The functional profiles of the identified bacteria revealed involvement in diverse human diseases, including cancers (Fig. 6).

4.1. Bacterial composition within the different levels of healthcare

The overall bacterial composition within any given environment could be influenced by numerous factors. Although hospitals are generally characterised by the movement of patients, visitors and healthcare workers in and out, as well as within the hospitals, other factors could affect the composition and diversity of microorganisms within these settings. For example, it has been demonstrated that the design of the hospital building, notably the source of ventilation, plays a significant role in the microbial diversity and composition in any given hospital environment (Chen et al., 2017). These factors make the hospital environment a complex one in which the microbial composition and diversity need proper understanding (Collins, 2008). In this study, although a considerable number of OTUs was shared between hospitals, wards and sampling units, a significant proportion of unique OTUs were also observed (Fig. 1). The highest number of unique OTUs (651) was observed in Hospital A (District Hospital) while the least (149) was recorded in Hospital C (Regional Hospital). According to the South African National Health Act of 2003, hospitals are categorised in increasing order of specialisation as District, Regional, Tertiary, Central and Specialised hospitals (National Department of Health SA, 2013).

This means that the District Hospital is the entry point for a potential patient who may then be referred to a Regional followed by a Central Hospital in cases where treatment cannot be provided in the District Hospital. As a result, District Hospitals are characterised by more frequent visits (patients, caregivers and the general public), increased movement of personnel, and the increased use and exchange of materials. This could explain the highest microbial population (highest number of unique OTUs) recorded at the District Hospital that was observed. Also, the higher the hospital level, the higher the infrastructural development. Thus, a Central Hospital would have better infrastructure

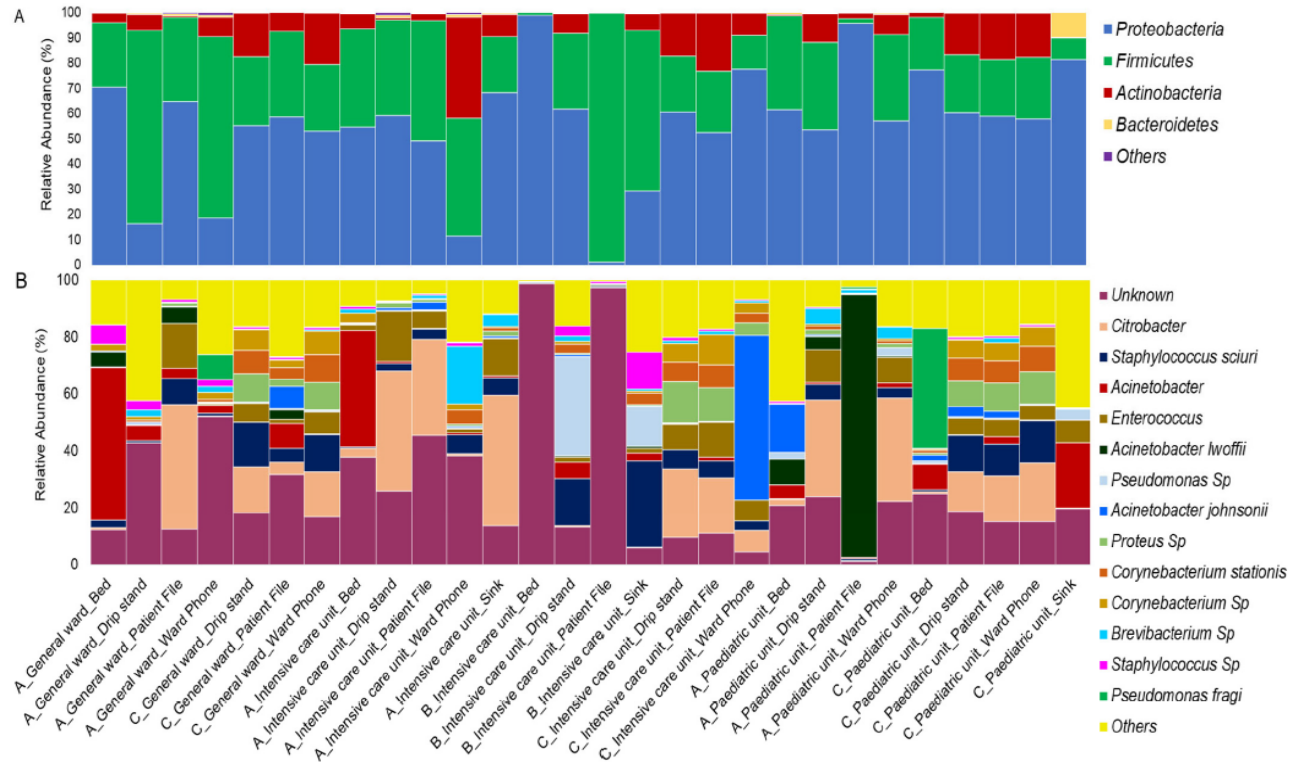

Fig. 2. Comparison of bacterial communities obtained from hospital samples. A and B illustrates the relative abundances (%) of OTUs at phylum and species levels, respectively.

Table 2
The number of identified taxonomic levels for each sample, including phylum, class, order, family, genus and species levels.

Hospital	Unit	Sampling site	Phylum	Class	Order	Family	Genus	Species
A	General ward	Bed	6	12	22	47	70	98
		Drip stand	6	15	28	62	83	113
		Patient File	7	16	26	54	83	113
		Ward Phone	7	13	26	54	80	114
	Intensive care unit	Bed	7	12	24	44	64	84
		Drip stand	7	15	25	54	77	109
		Patient File	5	10	21	48	61	82
		Sink	5	12	25	53	68	93
		Ward Phone	6	12	26	46	59	81
	Paediatric unit	Bed	6	10	19	35	50	71
		Drip stand	7	16	26	53	72	96
		Patient File	5	10	21	43	61	81
		Ward Phone	6	11	21	49	62	86
B	Intensive care unit	Bed	3	5	9	15	17	26
		Drip stand	8	19	33	65	88	125
		Patient File	4	6	12	21	25	33
		Sink	7	19	33	62	82	114
C	General ward	Drip stand	7	11	22	45	57	80
		Patient File	4	7	12	26	38	58
		Ward Phone	7	12	23	45	59	79
	Intensive care ward	Drip stand	7	16	28	46	57	82
		Patient File	5	13	27	53	70	96
		Ward Phone	7	12	23	43	56	80
	Paediatric ward	Bed	4	6	13	28	35	47
		Drip stand	6	12	22	48	62	79
		Patient File	6	11	21	46	58	80
		Sink	4	7	16	28	40	57
		Ward Phone	6	14	24	43	55	74

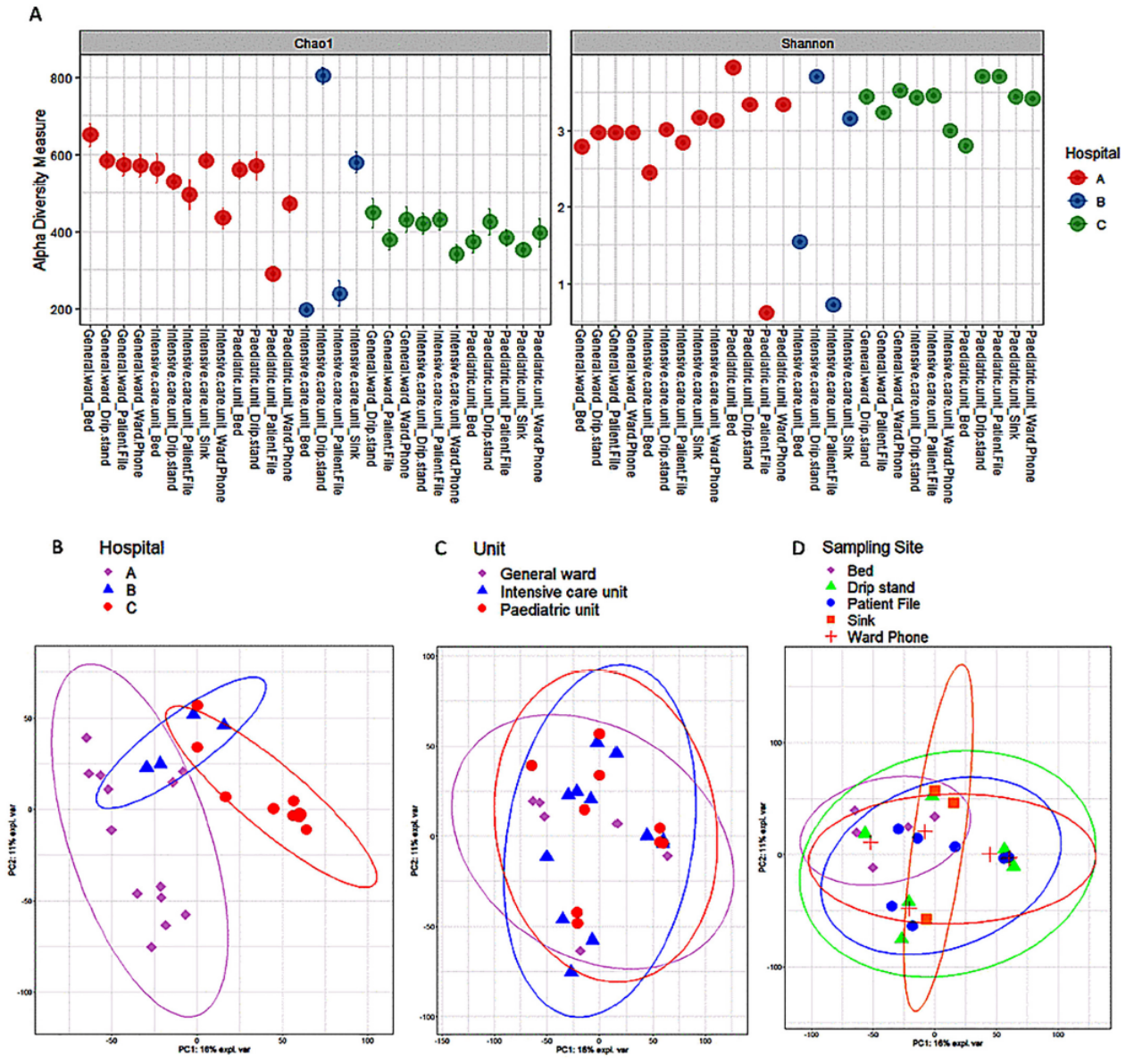

Fig. 3. Bacterial diversity within samples (alpha diversity) based on Cho1 and Shanon indices (A). B Principal component analysis (PCA) obtained based on the 500 most abundant OTUs. A, B, and C represent clustering based on the hospital level, hospital location and sample sources, respectively.

than the Regional and District hospitals. As such, the different hospital levels in this study reflect the case-mix/nature of diseases treated in the hospitals and is indicative of the quality of the IPC programme – raising the question of whether the surfaces and equipment are adequately decontaminated and if health care workers wash their hands between patients.

In this study, the environment in the ICUs in all the hospitals had the highest number of total and unique OTUs (Fig. 1B). These findings are particularly disturbing as patients within an ICU are generally more immunocompromised (Pickkers and Hotchkiss, 2016) and this, therefore, increases their chances of acquiring hospital infection. Patients in the ICU are up to 10 times more likely to contract an HAI than those in other units (Poza et al., 2012). Similar findings have earlier been reported by Poza et al. (2012), who concluded that the patients in ICUs were more vulnerable to infection than in other environments.

4.2. Bacterial diversity and potentially pathogenic genera and species in hospital samples

There was a distinctive difference in the bacterial communities between the three hospitals investigated (Fig. 2). The identification of Firmicute and Proteobacteria as the predominant phyla in the current study corroborate the findings of Poza et al. (2012) who reported these phyla as the most predominant in a hospital in Spain. Although four main genera *Corynebacterium*, *Acinetobacter*, *Citrobacter*, *Staphylococcus* (Table S1) and three species (*Staphylococcus sciuri*, *Acinetobacter*

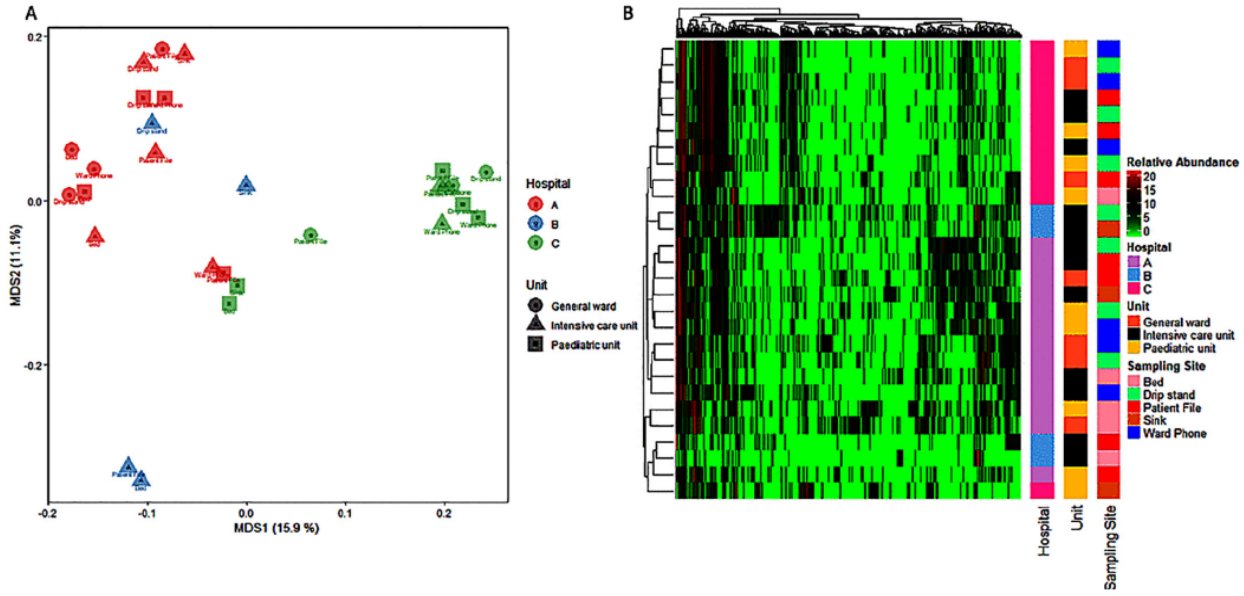

Fig. 4. Bacterial diversity among (Beta diversity) hospital samples (A) and sample clustering using Heatmap based 500 most abundant OTUs (B).

lwoffii, and *Acinetobacter johnsonii*) (Fig. 2B) were the most abundant in all the samples, taxa richness showed that samples from the same hospital had almost similar richness with the ICU having the least alpha diversity (Fig. 3).

The findings of this study were in line to those of Tang et al. (2015) who found skin-associated genera (*Propionibacterium*, *Corynebacterium*, *Staphylococcus*, *Streptococcus*, and *Bradyrhizobium*) to be highly abundant on medical devices and workstations (Tang et al., 2015). Poza et al. (2012) also suggested that although bacteria may be abundant in an ICU, a lower level of diversity may be due to the selective pressure arising from the confined area compared to places like general wards which receive a multitude of diverse patients and visitors.

Four bacterial genera (*Corynebacterium*, *Klebsiella*, *Pseudomonas*, *Staphylococcus*) that contain potentially pathogenic species were detected among the top genera in this study. *Corynebacterium* spp. are known to cause infection in both immunocompromised and healthy patients, although they form part of the normal human skin flora and are also found in animals, food products and the soil (Carr, 2017). Their survival in a hospital environment could be attributed to their ability to persist in the presence of toxic compounds, to acquire resistance

Table 3
The most abundant common pathogenic genera across hospital samples. Data are given in relative abundance (%).

Hospital	Ward[a]	Sampling site	*Bacillus*	*Bacteroides*	*Corynebacterium*	*Klebsiella*	*Pseudomonas*	*Staphylococcus*	*Streptococcus*
A	G	Bed	0.1839	0.0076	2.4406	0.6410	0.6163	18.9043	0.0436
		Drip stand	0.3837	0.0688	1.8475	0.0542	0.1022	25.2810	0.2231
		Patient File	0.0481	0.3659	0.1129	0.0230	0.1819	10.2776	1.8356
		Ward Phone	0.5384	0.0241	3.2915	0.0197	8.8392	7.0710	0.2451
	I	Bed	0.8903	0.0719	3.6298	0.0205	0.0616	5.6638	0.1164
		Drip stand	0.0540	0.5238	0.1836	0.0675	0.3213	2.8727	1.0125
		Patient File	0.1498	0.1395	0.4959	0.1395	0.4236	4.1327	0.8730
		Sink	0.3493	0.0868	1.5918	0.2564	0.3323	6.3019	0.7777
		Ward Phone	0.3781	0.0000	7.1529	0.0773	0.8422	8.3675	0.6990
	P	Bed	0.1127	0.0000	0.1859	1.3183	8.3157	7.8509	0.0394
		Drip stand	0.7988	0.0176	2.0000	0.0969	0.4200	5.8767	1.0837
		Patient File	0.1131	0.0000	0.3987	0.0079	1.4617	1.1186	0.0377
		Ward Phone	1.9127	0.1283	1.7097	0.1073	7.1049	3.7997	1.4042
B	I	Bed	0.0070	0.0000	0.0387	0.0035	0.1266	0.7560	0.0035
		Drip stand	1.0208	0.0000	4.4222	0.4857	34.7542	20.3204	0.0550
		Patient File	0.0513	0.0000	0.1026	0.0032	0.2437	1.3305	0.0000
		Sink	0.1951	0.0000	4.7840	0.0954	13.9534	43.5206	0.0509
C	G	Drip stand	0.3444	0.0000	16.6904	0.0181	0.4298	16.5739	0.5023
		Patient File	0.0626	0.0000	7.1808	0.0330	0.6426	5.7901	0.2505
		Ward Phone	0.3086	0.0000	19.5754	0.0374	0.8386	13.8297	0.4146
	I	Drip stand	2.8259	0.0000	15.1720	0.0549	0.2744	8.0695	0.0720
		Patient File	0.5124	0.0000	20.8997	0.0288	0.2529	6.5969	0.0577
		Ward Phone	0.3412	0.0000	7.5784	0.0117	0.0991	3.8023	0.0292
	P	Bed	0.0357	0.0000	1.7617	5.1552	42.8609	0.7202	0.0195
		Drip stand	0.2944	0.0037	15.8630	0.0484	3.2345	13.3999	0.4099
		Patient File	0.0000	13.1753	0.0000	0.0000	11.9918	0.0000	0.0000
		Sink	0.0057	0.0000	0.2438	12.5882	3.6736	0.2013	0.0057
		Ward Phone	0.2495	0.0000	17.1304	0.0208	0.5282	15.5916	0.5656

[a] G: General ward, I: Intensive care unit, P: Paediatric unit.

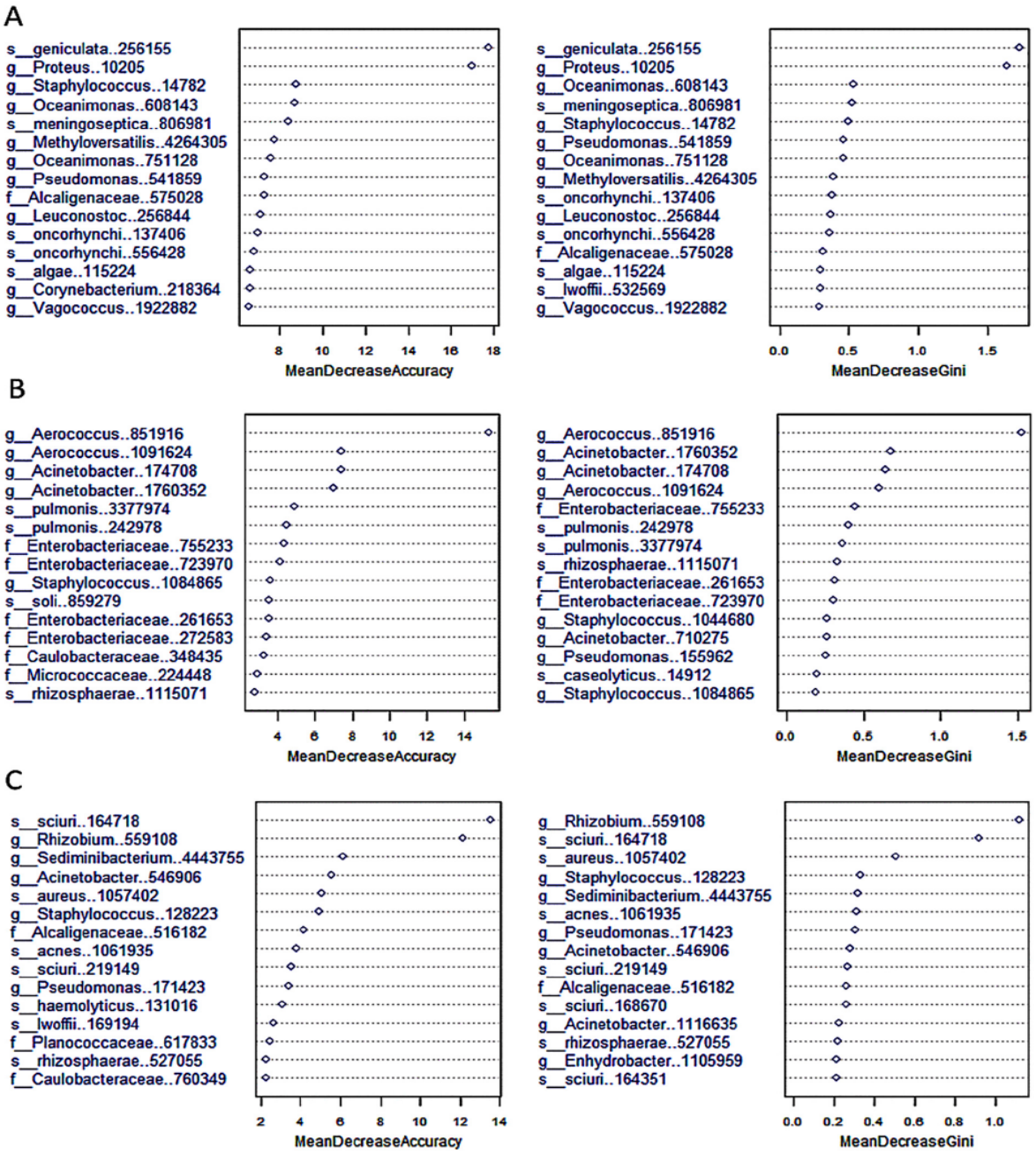

Fig. 5. Mean decrease of Gini index calculated based on functional categories associated with the type of hospital (A), ward (B) and sampling site (C) using Random Forest supervised learning models.

mechanisms and live on dry surfaces for lengthy periods (Poza et al., 2012). *Klebsiella* and *Pseudomonas* were also highly represented. According to the Centre for Diseases Control (CDC), members of these genera can be spread through person-to-person contact (from one patient to another through the contaminated hands of healthcare workers or visitors) or via contaminated equipment or surroundings not adequately cleaned (CDC, 2014). Also, although *Staphylococcus* spp. usually infect immunocompromised individuals through the mucosae and skin, these bacteria have developed extensive resistance to many antimicrobial agents making them one of the leading causes of HAI (Hanberger et al., 2011; Carr, 2017).

Of public health concern, were the three most abundant species identified in the current study. *Staphylococcus sciuri* is responsible for many human infections such as endocarditis, peritonitis, septic shock, urinary tract infections, pelvic inflammatory disease and wound infections (Chen et al., 2007). The presence of this bacterial pathogen has previously been reported in a hospital in Serbia (Shittu et al., 2005) and it has been shown to possess remarkable antibiotic resistance,

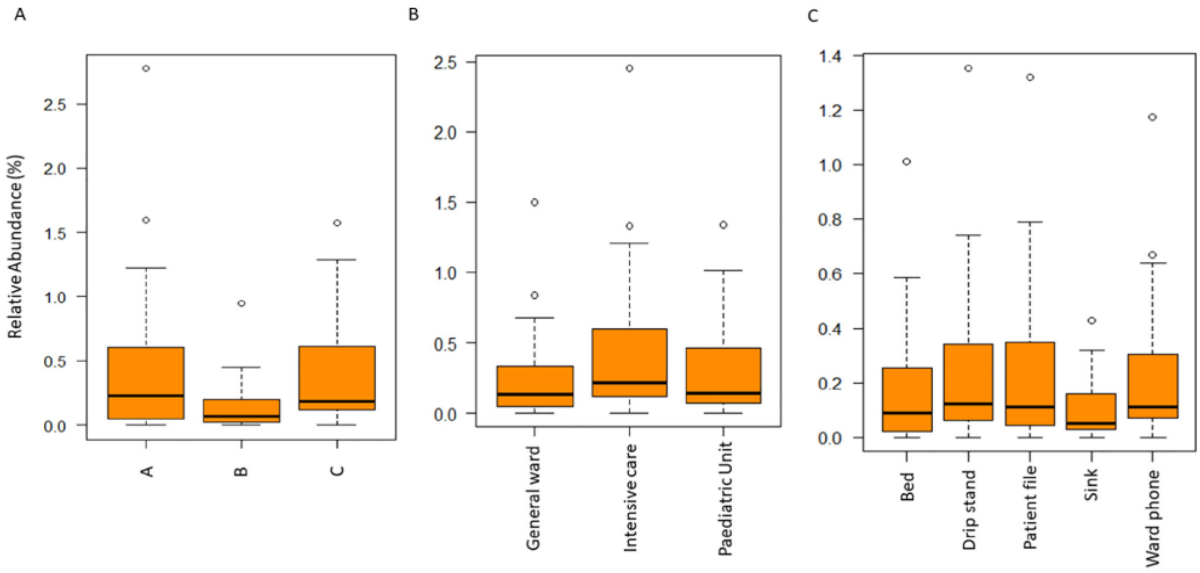

Fig. 6. Boxplots depict the distribution of human diseases related pathways between hospital levels (A), hospital locations (B) and sample sources (C).

including against methicillin (Garza-Gonzlez et al., 2011). Like the *Corynebacterium* spp. that are also found in animals, this organism has also been isolated in pigs making it an important zoonotic pathogen with potentially severe adverse effects to human medicine, especially in a hospital setting (Chen et al., 2007).

Acinetobacter lwoffii is an aerobic, non-fermentative and Gram-negative bacillus ordinarily present in the oropharynx and skin of approximately 25% of healthy individuals, but has also been associated with blood, urinary tract, skin and wound infections in immunocompromised individuals in hospital settings (Regalado et al., 2009). It has been classified as an emerging pathogen and has also been associated with automated peritoneal dialysis-linked peritonitis (Tas et al., 2017), pyogenic liver abscess in diabetic patients (Pal Singh et al., 2016), and sepsis (through intravascular catheters) and pneumonia (through prolonged mechanical ventilation) in premature neonates (Nakwan et al., 2011).

Apart from being pathogenic, some members of the Acinetobacter genus have become extremely drug resistant (XDR), accounting for about 70% of deaths due to XDR-linked infections globally (Wong et al., 2017). One example is *A. johnsonii*, which was among the most abundant species isolated in the current study. Although this species is often found in hospital environments, and rarely causes infections in humans, a recent study on the genomic analysis of this organism revealed 108 potential virulence proteins, some of which were homologous to known virulence proteins found in the pathogenic *Acinetobacter baumannii* (Tian et al., 2016). More importantly, another genomic study in China revealed that some *A. johnsonii* strains had horizontal genetic transfer (HGT) potential and harboured several resistance determinants like *strA*, *strB*, *ereA*, *sul1*, *aacC2* $bla_{\text{PER-2}}$, $bla_{\text{OXA-58}}$, $bla_{\text{TEM-1}}$, and a variant of $bla_{\text{OXA-211}}$, ($bla_{\text{OXA-498}}$) (Montaña et al., 2016). These authors concluded that *A. johnsonii* could actively transform by acquiring exogenous DNA from other bacterial species in the environment, thus becoming a reservoir of resistance genes (Montaña et al., 2016).

4.3. Functional profile analysis of the microbial populations in the hospital samples

Unlike conventional community metagenomics, functional profile screening of microbial populations has improved the understanding of the role played by microbial communities in different environments such as the gut of humans (Langille, 2018), animals (Lamendella et al., 2011) and environmental samples (Abia et al., 2019).

In this current study, PICRUSt, based on the KEGG database, revealed several functional classes mainly connected with metabolic pathways. However, from a human health perspective, a substantial fraction of the bacterial community was involved in human infectious diseases (such as tuberculosis, cholera and pertussis) as well as neurodegenerative diseases (such as Alzheimer's and Huntington disease) (Table S7; Supplementary material). Also important was the identification of pathways related to resistance to the β-lactam class of antibiotics. *Acinetobacter lwoffii*, one of the most abundant species identified in this study, has been reported to harbour genes responsible for extreme drug resistance and has the potential of horizontal gene transfer as previously mentioned. Thus, the presence of this organism together with the molecular signatures suggest that the hospital environment is a more complex one than currently perceived and this could have severe consequences on the health of hospitalised patients, hospital personnel and visitors, in addition to compromising the control of HAIs.

It should, however, be noted that this study was limited by the number of samples collected and that not all the units within the hospitals were investigated. Thus, the results presented could underestimate the actual diversity and composition within the study area. More extensive studies involving all hospital levels and all locations within the hospitals would give a more comprehensive overview of the bacterial communities involved in hospital settings. Also, the accuracy of functional inferences based on 16S metagenomics findings using PICRUSt is limited, and shotgun metagenomics/metatranscriptomics could provide more robust results.

5. Conclusion

This study revealed the existence of unique microbial communities in the environments within and between the different hospital levels and locations. The most abundant genera and species had pathogenic potential, as supported by the identification of human disease functional profiles among the bacterial communities. Also, pathways associated with antibiotic resistance were identified, indicating that the hospital environment could be an important reservoir of resistant bacteria. These findings call for review and strict observation of infection

control programmes within the hospital environment as neglecting these principles could pose substantive public health risks.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.137360.

CRediT authorship contribution statement

Christiana Omowunmi Shobo: Conceptualization, Methodology, Investigation, Visualization, Writing - original draft. **Arghavan Alisoltani:** Formal analysis, Visualization, Writing - review & editing. **Akebe Luther King Abia:** Formal analysis, Visualization, Writing - review & editing. **Philip Senzo Mtshali:** Software, Formal analysis. **Arshad Ismail:** Software, Formal analysis. **Oliver Zishiri:** Writing - review & editing. **Juliana Deidre Horn:** Writing - review & editing. **Petra Brysiewicz:** Writing - review & editing. **Sabiha Yusuf Essack:** Supervision, Writing - review & editing. **Linda Antoinette Bester:** Conceptualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

Professor Essack is Chairperson of the Global Respiratory Infection Partnership, sponsored by an unrestricted educational grant from Reckitt and Benckiser, UK. All the other authors declare no conflict of interest.

Acknowledgement

The authors would like to thank the Provincial Department of Health and the hospitals and staff that participated in this study.

Funding

The principal investigator would like to thank the Young Researchers Grant Competition of the College of Health Sciences, University of KwaZulu-Natal for funding the study.

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Chapter 3 – Manuscript 2

Enterococcal contamination of hospital environments in KwaZulu-Natal, South Africa

Authors contributions

Christiana Omowunmi Shobo: Co-conceptualised the study, Carried out the sample collection, performed the laboratory work, analysed the data and wrote the manuscript, Vetted the results, Undertook critical revision of the manuscript.

Sabiha Yusuf Essack: Co-conceptualised the study, Vetted the results, Undertook critical revision of the manuscript

Linda Antionette Bester: Co-conceptualised the study, Vetted the results, Undertook critical revision of the manuscript

Objective met: This paper answers objective 1 and 2

Enterococcal contamination of hospital environments in KwaZulu-Natal, South Africa

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Running title: *Enterococcus* contamination in public hospitals

Keywords: *Enterococcus*, *E. faecalis*; *E. faecium*; antibiotic resistance; infection prevention and control (IPC); hospital contamination.

This article has been submitted to Journal of Applied Microbiology

Abstract

Enterococci are implicated in hospital-acquired infections and show high tenacity on inanimate objects in the hospital environment. This study investigated the prevalence of *Enterococcus* spp. in selected wards in public hospitals at four levels of healthcare from a district in KwaZulu-Natal, South Africa.

Swabs were collected from frequently touched areas in the paediatric wards and intensive care units (ICUs). Presumptive *Enterococcus* spp. were isolated and confirmed to genus and species levels, followed by Kirby-Bauer disk diffusion against 14 antibiotics. The results showed that enterococci were recovered from all 11 surfaces tested with the highest contamination rate observed on occupied beds and mops used to clean floors. A total number of 295 *Enterococcus* was identified. PCR identified *Enterococcus faecalis* 245 (83.1%) and *Enterococcus faecium* 38 (12.9%), while WGS identified *Enterococcus gallinarum* 6 (2%) and *Enterococcus casseliflavus* 6 (2%). Significant prevalence was observed in pediatric wards (64.1%) compared to the intensive care units (35.9%), $p < 0.05$, in central, regional and district hospitals. Collectively, 82.0% of enterococci isolates were multidrug-resistant, and 80 different antibiograms observed. The most prominent antibiogram for *E. faecium* was CIP-RIF-NIT-TET-ERY and for *E. faecalis* CIP-TET-ERY. *E. faecalis* was the most frequent enterococcal species isolated in all the hospitals investigated and correlates with studies conducted elsewhere. A substantially greater number of isolates were recovered from the paediatric wards compared to ICU, and thus improved strategies should be developed to manage infection control practices.

It is suggested that the elevated use of antibiotics contributed to the increased non-susceptible isolates observed from ICUs. This study highlighted the high recovery rate of enterococci in the hospital environment even in a non-outbreak setting. Enterococci had a high prevalence rate on the surfaces within the hospitals studied. This study gives an insight into the possible roles janitorial staff may play in infection control intervention, including proper handling of hospital cleaning equipment and ignorant contribution to bacteria dissemination.

1.0 Introduction

Hospitals are the main reservoirs for many pathogenic bacteria consisting of community and residential initiated strains (Miller *et al.,* 2014). Hospital environments contribute to disseminating the bacterial pathogen, causing healthcare-related infections, which is an issue of paramount distress for healthcare facilities and patients (Revelas, 2012). These are spread via hand contact with the body or environmental surfaces carrying the bacteria. The organisms can survive for lengthy periods, weeks, sometimes as long as four months (Ndubuisi *et al*., 2017). Other sources of cross-contamination in hospitals include surgical instruments (Dancer, 2014), procedure/trash carts, computer keyboards (Page *et al*., 2009), soft surfaces (seat cushions, bed cloths, carpets), patients' environment (beds, bedside tables, bed cloths, chairs) (Kumarasamy *et al*., 2010), patients' charts/medical reports, medical tapes, diagnostic equipment and hospital kitchens equipment (Nurain *et al*., 2015).

Enterococci are opportunist Gram-positive cocci that can survive in different environments and are able to instigate severe infection in humans and animals (Olawale *et al.*, 2011). *Enterococcus* transmission occurs endogenously from the gut and exogenously through the hand of healthcare workers (Castillo-Rojas *et al*., 2013; Nekkab *et al*., 2017), thereby leading to outbreaks, extended hospital stays, increased healthcare costs, and even mortality (Sydnor and Perl, 2011). Transient carriage of enterococci on healthcare workers' hands were reported in other studies (Montoya *et al*., 2019; Rosenthal *et al*., 2013). They can survive for as long as 60 minutes on hands and as long as four months on inanimate surfaces, where they may form a reservoir for cross-transmission unless there is proper decontamination (Kramer *et al*., 2006).

Most of these infections are attributable to *Enterococcus faecalis* and *Enterococcus faecium,* especially in clinical samples (Castillo-Rojas *et al*., 2013). Acquisition of enterococci resistance occurs when exposed to antibiotics or from other organisms in their immediate environments (Hollenbeck and Rice, 2012; Miller *et al*., 2014) via plasmids or transposons (Bertelloni *et al*., 2015; Hollenbeck and Rice, 2012). There are minimal studies in South Africa describing enterococcal contamination and antibiotic resistance, especially in the hospital environment at different levels of healthcare. This study investigated enterococcal contamination of hospital

environments in the paediatric wards and ICUs of public hospitals at four levels of healthcare from a provincial district in South Africa.

2.0 Methods

2.1 Ethical approval

The Biomedical Research Ethics Committee (BREC) of the University of KwaZulu-Natal granted permission with reference BE606/16 and the KwaZulu-Natal Provincial Health Research Ethics Committee, reference KZ 2017RP24 630. The Management of each participating hospital further provided gatekeeper approval.

2.2. Study design, hospital setting, and sample sites

We performed an observational study on surfaces commonly in contact with healthcare workers in the hospital environment from September to November 2017. Samples were collected from the four different levels of healthcare in South Africa and referred to as central hospital, tertiary hospital, regional hospital and district hospital. The Intensive-care unit and paediatric unit of each hospital were investigated in this study. Sites of sample collections included ward phones, ventilators, blood pressure apparatus, patient files, drip stands, sinks, beds (both occupied by patient and unoccupied), nurses' tables, mops, and linen room door handle. **Supplement file 1** gives the details of the different hospital level and the reasons for the ward and site included in this study.

2.3. Sample collection/ transportation

All the environmental samples were obtained using moistened cotton swabs. Approximately 5cm^2 of surface areas where appropriate were swabbed. For the door handles, the swab was rotated over the whole handle surface. Swabs were placed in a transport boxes and processed within four hours of sampling.

2.4. Isolation and identification by multiplex PCR

Swabs were incubated in tryptone soya broth (TSB) (Oxoid, Basingstoke, England) containing 6.5% NaCl and incubated at 36.5ºC overnight. Broth samples were, then streaked onto enterococci

chromogenic media plate (VRE chromogenic agar Sigma-Aldrich, Louis, USA). Blue-coloured colonies were confirmed as enterococci by sub-culture on Bile Esculin Azide agar (HiMedia, Mumbai, India). Suspected colonies were further verified by standard biochemical tests, including haemolysis on 5% sheep blood agar, oxidase and catalase tests. Presumptive enterococcal cultures were stored in TSB 20% glycerol solutions at -80°C until further analyses. The isolate was identified to the species level using multiplex PCR following the boiling method for DNA extraction, as previously described (Englen and Kelley, 2000). For each strain, the DNA concentration and purity were estimated using a Nanodrop spectrophotometer (ND-1000, Thermo Scientific), and DNA probity was verified using a 1% agarose gel.

Identification of enterococci was based on the detection of the genus-specific *tuf*-gene (product size 112 bp), as described by Ke *et al*. (1999). Amplifications of species-specific genes were performed to identify both *E. faecalis* and *E. faecium* using the *sod A*-gene according to Jackson *et al.* (Jackson *et al*., 2004). Primers, sourced from Inqaba Biotec, South Africa, are shown in **supplementary table 1**. The reaction mixture (25 µL) contained: 2.5 µL of the DNA template, 12 µL of Master Mix (Inqaba, South Africa), 1 µL of each primer- Ent1, Ent2, FL1, FL2, FM1 and FM2, and 4.5 µL of nuclease-free water. The PCR cycling conditions consisted of the initial denaturation 94°C/3 min, amplification - 30 cycles (94°C/30 s, 53°C/45 s, 72°C/60 s), final extension 72°C/7 min. Amplification was verified by gel electrophoresis in 1% agarose stained with ethidium bromide and visualised with a UV transilluminator (Uvitec, Cambridge UK).

2.5. Antibacterial susceptibility tests

Susceptibility testing was carried out using the Kirby-Bauer disc diffusion method according to the CLSI guidelines (CLSI, 2018) and results reported as susceptible or non-susceptible (for intermediate or resistant isolates) against 14 antibiotics viz., glycopeptides [vancomycin (30µg), teicoplanin (30µg)], quinolones [ciprofloxacin (5µg), levofloxacin (5µg)], amphenicols [chloramphenicol (30µg)], penicillins [ampicillin (10µg), penicillin G (10µg)], rifamycins [rifampicin (5µg)], tetracyclines [tetracycline (30µg)], macrolides [erythromycin (15µg)], nitrofurans [nitrofurantoin (300µg)], oxazolidinones [linezolid (30µg)] with additional testing for high-level resistance to aminoglycosides [gentamicin (120µg) and streptomycin (300µg)]

(Hudzicki., 2009). *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 25923 was used as controls (CLSI, 2018).

2.6. Statistical analysis

The isolation rates of the different species of enterococci and their antimicrobial resistance profiles were compared between the different wards and hospitals investigated. Statistical analysis was carried out using the Fisher-Free- man-Halton exact test (Z test for proportion) for contingency tables. All analyses were performed using Stata statistical software IBM SPSS version 25 with a p-value < 0.05 indicative of statistical significance. Due to the small sample size, the confidence level was analysed at 90% to produce a broader confidence interval.

3.0 Results

3.1 Isolation rates in the hospitals

A total of 620 swab samples were collected over a period of three months from the four levels of healthcare facilities. Of the 620 swab samples collected, which include 120 isolates from the central hospital, 174 from the tertiary hospital, 231 from the regional hospital, and 95 from the district hospital. Overall, 295 *Enterococcus* spp were identified from all the hospitals. Viz; 54 of from the central hospital, 111 from the tertiary hospital, 101 from the regional hospital and 29 from the district hospital. Species identified using sodA amplification were *E. faecalis* with (245, 83.1%) while (38, 12.9%) were *E. faecium.* After next-generation sequencing (NGS), 6 (2.0%) isolates previously identified as *E faecium* (possibly mixed cultures) were re-identified as *E gallinarum* and 6 (2.0%) as *E casseliflavus.* **Table 1** below shows the detailed distribution of all the species isolated at various levels of healthcare in KwaZulu-Natal, South Africa.

3.2 Isolation rate in the wards

An overall sample size of 64.1% of isolates were obtained from sites in paediatric wards and 13.9% from ICUs. The tertiary hospital had the highest isolation rate with paediatric and ICU at 58 (52.3%) and 53 (47.7%) respectively, followed by the regional hospital with 61 (60.4%) and 40 (39.6%) isolated from the paediatric and ICU, respectively. Fewer samples were collected from the central hospital, but most isolates (50, 92.6%) were from the paediatric ward samples, with only (4, 7.4%) from the ICU. The district hospital also showed a higher prevalence for the

paediatric ward with 21 (72.4%), but only 8 (27.6%) from ICU. The number of isolates from each site showed a significant p-value of < 0.00001, 0.02144 and 0.01314 respectively in the paediatric ward compared to ICU. The full distribution of enterococcal isolates by species, hospital, ward and sample sites are presented in **Table 1**.

3.3 Isolation rates from the samples collected

The sites with the overall highest isolation rates were the occupied beds and the mops with 44 (14.9%) isolates each. In the central hospital, the sites with the highest isolation rate were the occupied beds 12 (22.2%), followed by the nurses' tables 11 (20.3%). For the tertiary hospital, the sites with the highest isolation rates were the ward phones and mops, with 16 (14.4%) each. In the regional hospital, the mops and the occupied beds were frequently harboured enterococci (14, 13.8%) each. For the district hospital, most positive samples identified were from the door handles 8 (27.5%) followed by the nurses' tables with (7, 24.1%) and the mops with (6, 20.6%).

Table 1: A detailed distribution of *E. faecium*, *E. faecalis*, *E. casseliflavus* and *E. gallinarum* isolates by hospitals, wards and sample sites for public hospital A – D.

Sites	**Central (A)**					**Tertiary (B)**					**Regional (C)**						**District (D)**				
	ICU		**Paediatric**			**ICU**		**Paediatric**			**ICU**		**Paediatric**				**ICU**		**Paediatric**		
	Ef	*Ec*	*Ef*	*Ec*	*Es*	*Ef*	*Ec*	*Ef*	*Ec*	*Es*	*Ef*	*Ec*	*Ef*	*Ec*	*Es*	*Eg*	*Ef*	*Ec*	*Ef*	*Ec*	*Eg*
Phone	-	4	-	3	-	-	5	-	11	-	-	2	-	6	-	-	-	1	-	-	-
Drip stand	-	-	-	-	-	-	3	3	6	-	4	1	3	-	-	-	-	-	-	-	-
Bp apparatus	-	-	-	-	-	-	6	4	-	-	2	3	-	3	1	-	-	-	-	-	-
Patient file	-	-	-	6	-	-	5	2	3	2	1	3	-	4	-	1	-	-	-	-	-
Ventilator	-	-	-	-	-	-	3	-	-	-	-	-	-	4	-	-	-	-	-	-	-
Mop	-	-	-	8	-	2	6	2	6	-	-	5	-	9	-	-	-	-	-	6	-
Sink	-	-	-	4	-	-	2	-	3	-	-	2	-	7	-	-	-	-	-	-	-
Occupied bed	-	-	-	12	-	2	6	1	3	1	-	7	-	7	-	-	-	-	-	5	-
Unoccupied bed	-	-	3	3	-	-	3	2	4	-	-	-	1	3	-	-	-	-	-	-	2
Nurses table	-	-	1	8	2	2	4	-	3	-	-	4	-	8	-	-	-	3	-	4	-
Door handle	-	-	-	-	-	2	2	-	2	-	-	6	-	2	-	2	-	4	-	4	-

Keys: *Ef* = *Enterococcus faecium*, *Ec* = *Enterococcus faecalis*, *Es* = *Enterococcus casseliflavus* *Eg* = *Enterococcus gallinarum*. ICU = Intensive care unit and Paediatric = Paediatric ward

3.4 Antimicrobial susceptibility

The highest non-susceptible percentiles of all the species were to macrolides (91%), followed by tetracyclines (83%), rifampicin (75%) and quinolones (72% to ciprofloxacin but a lesser value of 14% to levofloxacin). **Figure 1** describes the antibiotics susceptibility profile of all the isolates recovered from the various hospitals. *E. faecium* showed the highest non-susceptibility to the following antibiotics: erythromycin (90%), tetracycline (82%), ciprofloxacin (82%), rifampicin (71%) and nitrofurantoin (50%). A low level of non-susceptibility was recorded for penicillin (11%), ampicillin (11%), gentamicin (8%) and linezolid (2.6%). All *E. faecalis* isolates showed a non-susceptibility rate of 92% to erythromycin, 83% to tetracycline, 78% to rifampicin, 69% to ciprofloxacin, 42% to linezolid, 33% to chloramphenicol with less resistance to levofloxacin (11%), streptomycin (10%), nitrofurantoin (9%) and gentamicin (3%). All isolates of *E. faecium* and *E. faecalis* were completely susceptible to vancomycin.

A high non-susceptibility rate was recorded for *E. faecium* and *E. faecalis* from the ICU and paediatric units in all the hospitals to tetracycline, erythromycin, ciprofloxacin and rifampicin (>50%). Across the healthcare level, *E. faecium* was not isolated from any samples from the district hospital. There was significant resistance (70.0% of isolates) to erythromycin, ciprofloxacin and tetracycline was noted in the regional, tertiary and central hospitals. All *E. faecium* (**Figure 1A**) isolates in the regional hospital were completely susceptible to the following antibiotics: gentamicin, levofloxacin, ampicillin, streptomycin, linezolid and penicillin while in the tertiary hospital, there was a 48% non-susceptibility to levofloxacin, 9% to ampicillin, 4% to linezolid and penicillin with complete susceptibility to gentamicin and streptomycin. On the contrary, the central hospital showed a non-susceptible rate of 75% (gentamicin), 75% (levofloxacin), 50% (ampicillin), 25% (streptomycin), 75% (penicillin) and total susceptibility to linezolid.

For *E faecalis* isolates (**Figure 1B**), the district, tertiary and central hospitals samples showed complete susceptibility to gentamicin, ampicillin, teicoplanin and penicillin. The regional hospital showed a non-susceptible rate of 8% to gentamicin, 5% penicillin and 2% to both ampicillin and teicoplanin. All isolates of *E. gallinarum* and *E. casseliflavus* were 100% non-susceptible to vancomycin and ciprofloxacin with 95% non-susceptible to erythromycin.

There was no significant difference in the resistance rate between *E. faecium* and *E. faecalis*. P-value (90%) of the species against the antibiotics showed a significant difference to some antibiotics. A significantly higher rate of non-susceptibility to gentamicin, ampicillin, levofloxacin, nitrofurantoin, streptomycin and penicillin was detected in *E. faecium* and *E. faecalis* ($p < 0.05$) in the central hospital, while greater resistance of *E. faecalis* and *E. faecium* ($p < 0.05$) to ciprofloxacin, rifampicin, streptomycin and tetracycline was found in the tertiary hospital. Collectively, 82% of all the enterococci isolates were multidrug-resistant (resistant to three or more antibiotic classes). A total of 80 antibiograms were observed (**table 2**).

The district, regional, tertiary and central hospitals showed different antibiograms with a total of 26 (90%), 74 (73%), 93 (84%) and 48 (89%), respectively. The most common resistant profile discovered in all the hospitals was the CIP-TET-ERY. This bio-gram was discovered in the central hospital (nine isolates), tertiary hospital (three isolates), regional hospital (ten isolates) and district hospital (two isolates), with the majority of the resistant pattern found in the pediatric unit. Among the *E. faecium* isolates, the most prominent profile was resistant to CIP-RIF-NIT-TET-ERY (24% of the isolates), and among the *E. faecalis* isolates, the common antibiogram was CIP-TET-ERY (10% of the isolates).

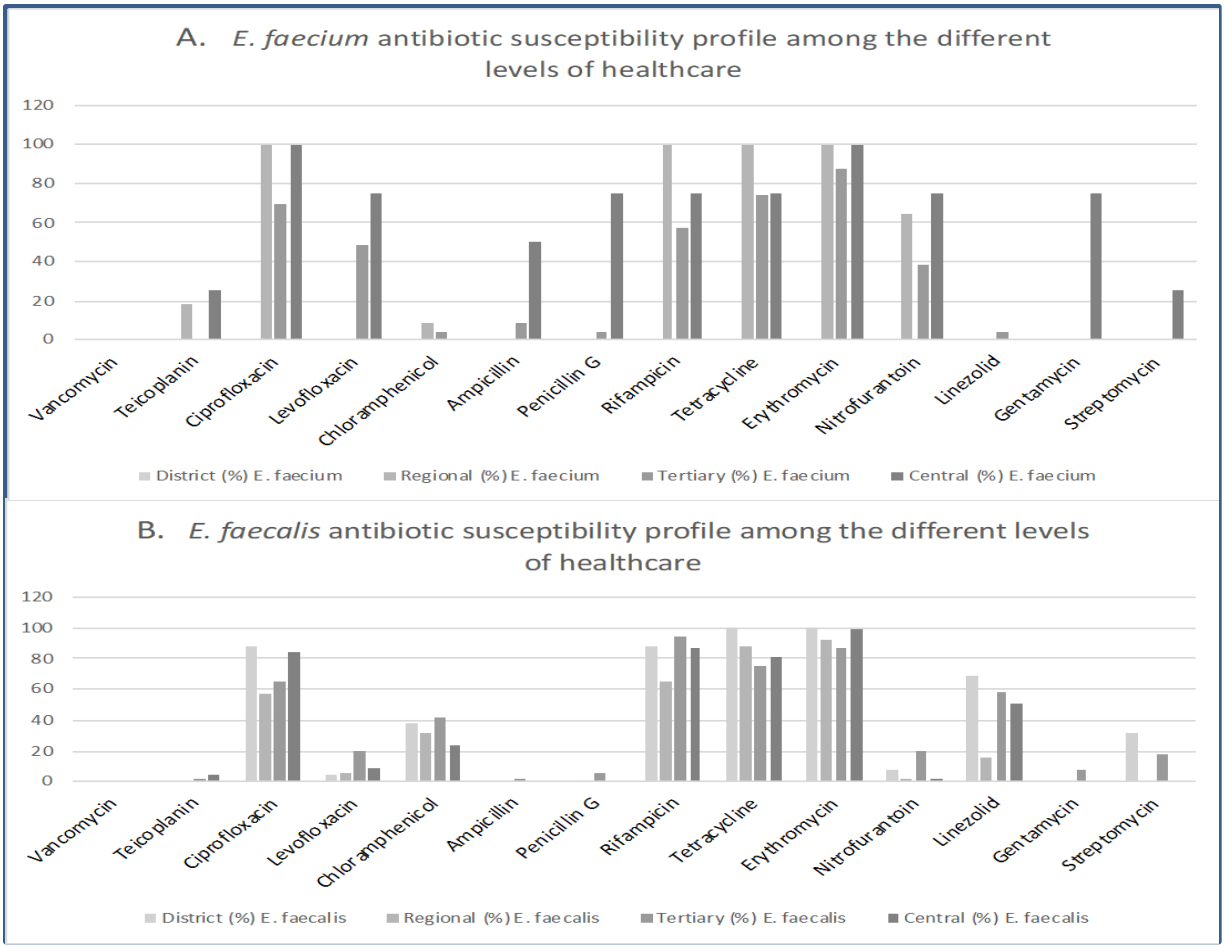

Figure 1: Distribution of the antibiotic susceptibility profiles across the levels of healthcare:

A] *E. faecium* antibiotic susceptibility profile among the different levels of healthcare.

B] *E. faecalis* antibiotic susceptibility profile among the different levels of healthcare.

Table 2: Table showing the susceptibility profile of all the *Enterococcus* isolates recovered at the different levels of healthcare

	Central N=54		Tertiary N=111		Regional N=101		District N=29		Total S N=295	Total Non-S N=295
	S	**Non-S**	**S**	**Non-S**	**S**	**Non-S**	**S**	**Non-S**		
Vancomycin	52	2	107	4	97	4	27	2	**94%**	**6%**
	96%	4%	96%	4%	96%	4%	93%	7%		
Ciprofloxacin	7	47	37	74	37	64	3	26	**28%**	**72%**
	13%	87%	33%	67%	37%	63%	10%	90%		
Gentamycin	51	3	104	7	101	0	29	0	**97%**	**3%**
	94%	6%	94%	6%	100%		100%			
Chloramphenicol	43	11	76	35	71	30	19	10	**71%**	**29%**
	80%	20%	68%	32%	70%	30%	66%	34%		
Levofloxacin	47	7	82	29	97	4	27	2	**86%**	**14%**
	87%	13%	74%	26%	96%	4%	93%	7%		
Ampicillin	52	2	107	4	101	0	29	0	**98%**	**2%**
	96%	4%	96%	4%	100%		100%			
Rifampicin	10	44	27	84	32	69	4	25	**25%**	**75%**
	19%	81%	24%	76%	32%	68%	14%	86%		
Nitrofurantoin	49	5	85	26	92	9	27	2	**86%**	**14%**
	90%	10%	76%	23%	91%	9%	93%	7%		
Teicoplanin	51	3	109	2	99	2	29	0	**98%**	**2%**
	94%	6%	98%	2%	98%	2%	100%			
Streptomycin	53	1	96	15	100	1	22	7	**92%**	**8%**
	98%	2%	86%	14%	99%	1%	76%	24%		
Tetracycline	12	42	27	84	10	91	2	27	**17%**	**83%**
	22%	78%	24%	76%	10%	90%	7%	93%		
Linezolid	28	26	63	48	89	12	10	19	**64%**	**36%**
	52%	48%	57%	43%	88%	12%	34%	66%		
Erythromycin	1	53	16	95	8	93	2	27	**9%**	**91%**
	2%	98%	14%	85%	8%	92%	7%	93%		

Table 3: Multi-drug resistant profile among the different level of healthcare

ANTIBIOGRAM	Central n=54		Tertiary n=111		Regional n=101		District n=29	
	ICU	PEAD	ICU	PEAD	ICU	PEAD	ICU	PEAD
TET-LZD-ERY	0	5	0	2	0	0	0	0
CIP-TET-ERY	0	9	0	3	6	4	0	2
CIP-LZD-ERY	0	3	0	0	0	0	0	0
CIP-RIF-LZD-ERY	0	1	0	0	0	1	0	0
CIP-TET-LZD-ERY	1	4	0	0	0	1	3	3
CIP-RIF-TET-ERY	0	1	2	0	5	5	0	2
CIP-RIF-ERY	0	2	2	0	0	1	0	0
CIP-RIF-TET-LZD-ERY	0	2	4	2	0	3	0	1
CIP-RIF-TET	0	1	0	0	0	0	0	0
CIP-RIF-ERY	0	2	0	2	0	1	0	0
CHLO-RIF-TET-LZD-ERY	0	1	0	0	0	0	0	0
CIP-CHLO-RIF-TET-ERY	0	1	0	2	2	0	0	0
CIP-CHLO-RIF-TET-LZD-ERY	4	2	3	3	0	3	0	0
CIP-RIF-NIT-TET-ERY	0	0	0	1	3	4	0	0
CIP-CHLO-RIF-STREP-TET-LZD-ERY	0	0	5	0	1	0	2	2
CIP-CHLO-TET-LZD-ERY	0	1	0	0	0	3	0	2
CHLO-TET-ERY	0	0	0	0	3	6	0	0
CIP-LEV-TET-LZD-ERY	0	1	0	0	1	1	0	0
CIP-GEN-LEV-RIF-NIT-TET-ERY-PEN	0	1	0	0	0	0	0	0
CIP-GEN-CHLO-LEV-RIF-TET-LZD-ERY	0	0	3	0	0	0	0	0
CIP-LEV-TET-ERY	0	1	0	3	0	0	0	0
CIP-CHLO-RIF-LZD-ERY	0	2	1	0	0	0	0	0
CIP-GEN-LEV-AMP-RIF-NIT-TEC-TET-ERY-PEN	0	1	0	0	0	0	0	0
CIP-CHLO-STREP-TET-LZD-ERY	0	0	0	0	0	0	0	2
CIP-CHLO-TET-ERY	0	0	0	0	3	2	0	0
RIF-TET-LZD-ERY	0	0	0	3	1		0	1
RIF-NIT-LZD-ERY-PEN	0	0	2	0	0	0	0	0
CIP-CHLO-LEV-TET-LZD-ERY	0	0	1	0	0	1	0	0
RIF-TET-ERY	0	0	0	5	0	1	0	0
CHLO-TET-LZD-ERY	0	0	1	0	0	1	0	0
CIP-LEV-RIF-NIT-ERY	0	0	1	2	0	0	0	0
CIP-RIF-NIT-ERY	0	0	1	0	1	0	0	0
CIP-LEV-RIF-NIT-TET-ERY	0	0	1	2	0	0	0	0
CHLO-RIF-TET-ERY	0	0	1	1	0	1	0	1
CIP-RIF-NIT-LZD-ERY	0	0	1	0	0	1	0	0
CIP-CHLO-STREP-LZD-ERY	0	0	2	0	0	0	0	0
CHLO-RIF-NIT-TET-LZD-ERY	0	0	1	1	0	0	0	0
VAN-CIP-TET	0	0	0	1	1	0	0	0
VAN-TET-ERY	0	0	0	1	0	0	0	0
CIP-STREP-ERY	0	1	0	0	0	0	0	0
VAN-CIP-ERY	0	1	0	0	0	0	0	0
VAN-CIP-LZD-ERY	0	1	0	0	0	0	0	0
VAN-CIP-RIF-ERY	0	0	0	0	0	0	1	0
VAN-CIP-RIF	0	0	0	0	0	0	1	0
CIP- RIF-STREP-TET-LZD-ERY	0	0	0	0	0	0	1	0
CHLO-LEV-RIF-STREP-TET-LZD-ERY	0	0	0	0	0	0	0	1

CIP-RIF-NIT-TET-LZD-ERY	0	0	0	0	0	0	0	1
CIP-CHLO-LEV-RIF-TET-ERY	0	0	0	0	0	1	0	0
CIP-RIF-TET-ERY	0	0	0	0	0	1	0	0
CIP-TET-LZD	0	0	0	0	0	1	0	0
VAN-CIP-CHLO-LEV-TET-ERY	0	0	0	0	0	1	0	0
VAN-CIP-CHLO-TET-ERY	0	0	0	0	0	1	0	0
CIP-RIF-LZD	0	0	0	0	0	1	0	0
VAN-CIP-TET-ERY	0	0	0	0	0	1	0	0
RIF-NIT-LZD-ERY	0	0	1	0	0	0	0	0
CIP-CHLO-LEV-RIF-STREP-TET-LZD-ERY	0	0	1	0	0	0	0	0
CIP-GEN-LEV-AMP-RIF-NIT-STREP-TET-ERY-PEN	0	0	2	0	0	0	0	0
RIF-NIT-LZD	0	0	1	0	0	0	0	0
LEV-RIF-TET-ERY	0	0	0	1	0	0	0	0
CIP-CHLO-LEV-TET-ERY	0	0	0	1	0	0	0	0
VAN-CIP-LEV-TET-ERY	0	0	0	1	0	0	0	0
CIP-NIT-LZD	0	0	0	1	0	0	0	0
CIP-LEV-NIT-STREP-TET-LZD-ERY	0	0	0	1	0	0	0	0
CIP-CHLO-LEV-RIF-LZD-ERY	0	0	0	1	0	0	0	0
CIP-LEV-RIF-LZD-ERY	0	0	0	1	0	0	0	0
CIP-CHLO-RIF- NIT-LZD-ERY	0	0	0	1	0	0	0	0
VAN-CIP-TET-ERY	0	0	0	1	0	0	0	0
AMP-TET-ERY-PEN	0	0	1	0	0	0	0	0
LEV-NIT-TET	0	0	1	0	0	0	0	0
CIP-AMP-TET-LZD-ERY	0	0	1	0	0	0	0	0
CIP-GEN-CHLO-RIF-TET-LZD-ERY	0	0	1	0	0	0	0	0
CIP-GEN-CHLO-LEV-RIF-STREP-TET-LZD-ERY	0	0	1	0	0	0	0	0
CHLO-RIF-STREP-TET-LZD-ERY	0	0	1	0	0	0	0	0
STREP-TET-ERY	0	0	1	0	0	0	0	0
CIP-CHLO-RIF-NIT-TET-LZD-ERY	0	0	1	0	0	0	0	0
RIF-NIT-TET-ERY	0	0	1	0	0	0	0	0
CIP-LEV-RIF-TET-LZD-ERY	0	0	0	1	0	0	0	0
CIP-LEV-RIF-TET-LZD-ERY-PEN	0	0	0	1	0	0	0	0
CHLO-LEV-RIF-TET-LZD-ERY	0	0	0	1	0	0	0	0
CIP-LEV-RIF-TET-LZD-ERY	0	0	0	1	0	0	0	0
	5 (9%)	44 (81%)	46 (41%)	47 (42%)	27 (27%)	47 (47%)	8 (28%)	18 (62%)

Listed abbreviations: PEAD pediatrics; VAN vancomycin; AMP ampicillin; PEN penicillin; ERY erythromycin; CHL chloramphenicol; CIP ciprofloxacin; GEN gentamicin; NIT nitrofurantoin; STREP streptomycin; TEC teicoplanin; TET tetracycline; LEV levofloxacin; and RIF rifampicin

4.0 Discussion

The evaluation of hospital environments (air, water and surfaces) has become vital for good healthcare quality and patient safety (Gonsu *et al*., 2015). Thus, this study investigated the presence of *Enterococcus* spp contamination on frequently touched surfaces by healthcare workers and patients in South African public hospitals. Microbial contamination persistently populate of communities, and hospital environments can be derived from flora shed by visitors, patients and healthcare workers. Thus contaminating environmental surfaces and acting as a potential reservoir for the spread of microbial agents in hospitals as well as in the community, thereby increasing the risk of infection among susceptible hosts (Bhatta *et al*., 2018; Boyce, 2007).

4.1 Prevalence of enterococci in different types of hospitals

Hospitals are categorised in decreasing order of specialisation as district, regional, tertiary and central/specialised hospitals as stipulated by the South African National Health Act of 2003 (National Department of Health SA, 2013). This suggests that the district and regional hospitals are the entry point for a patient who may be then referred to a tertiary followed by a central hospital in instances when specialised care is needed. Thus, there is high traffic of patients (in and out-patients), caregivers, healthcare personnel, and exchange of materials within the district and regional hospitals, increasing the possibility of contamination. In this study, the regional (also local) and the tertiary hospitals showed a high recovery rate of 34% and 38% respectively of the *Enterococcus* spp. while a lesser rate was observed in the central and district hospitals. This could be explained by the higher levels of expertise and practice expected in a central hospital (e.g. effective compliance of IPC) while the lower rate detected in the district hospital might be due to lesser beds available and consequently fewer number of patients.

4.2 Isolation of the different species in the different level of care

It is well-documented that the hospital environment facilitates the spread of numerous significant healthcare-associated pathogens, especially vancomycin-resistant enterococci (VRE) (Dancer, 2014). These organisms can be shed by colonised patients and staff, thereby contaminating the surfaces and escalating the possibilities of acquisition by other patients. Enterococci are opportunistic organisms that cause infections in immunocompromised hosts (Agudelo and Huycke, 2014). Enterococcal infection has been documented in medical units, especially the ICU (Moemen *et al*., 2015; Moses *et al*., 2012), can be transferred between

hospitals (Olawale *et al*., 2011; Zhang *et al.*, 2017) and by hospital inanimate objects (Hayden *et al*., 2008; Huslage *et al*., 2010). The species most frequently detected, *E. faecium* and *E. faecalis* are implicated in most enterococcal infections (Lebreton *et al*., 2014). *E. faecalis* was by far the most frequent enterococcal species isolated in all the hospitals investigated in this study. Our results correlate with a study carried out at some selected tertiary and secondary care hospitals in Abuja, Nigeria, which found *E faecalis* (57.8%) as the predominant contaminant followed by *E faecium* (23.5%) and then other species (18.6%) (Ndubuisi *et al*., 2017).

E. gallinarum and *E. casseliflavus* are not commonly found in clinical specimens and are therefore not considered significant pathogens. However, such as in immunocompromised hosts, these species have shown to cause severe infections, including several hospital-acquired outbreaks of meningitis, endocarditis and bacteremia (Corso *et al*., 2005; Dargere *et al*., 2002; Eshaghi *et al*., 2015; Tan *et al*., 2010) and spontaneous bacterial peritonitis (Luz Narciso-Schiavon *et al*., 2014). Our study showed a 2.0% prevalence of both *E. casseliflavus* and *E. gallinarum,* with the highest percentage of *E. casseliflavus* in the tertiary hospital and *E. gallinarum* in the regional hospital. Although enterococci can survive for months on dry surfaces, they can be readily removed using routine hospital disinfectants (Jia *et al*., 2014). Our study showed a 2% prevalence *E. gallinarum* and *E. casseliflavus* are recognised as normal flora organisms making it difficult to establish their role in infctions (Luz Narciso-Schiavon *et al*., 2014). Thus, they are not usuallyof primary concern in investigating hospital infections (Shirano *et al*., 2011).

4.3 Prevalence of enterococci in the different wards

Our study showed a high contamination rate of *Enterococcus* in the three hospitals' paediatric units (central, regional and district hospitals). This high rate of contamination is alarming since paediatric wards patients are very vulnerable (Moore, 2001) and are known to be predisposed to numerous infections because of the immature immune system of children (Simon *et al*., 2015). Similar studies carried out within the paediatric ward in a Nigerian hospital by Saka *et al.* showed bacterial isolates on non-critical surfaces within paediatric wards at a prevalence of 67.7% (Saka *et al*., 2016). The continuous movement of active children outside of their allocated beds within the wards, as observed during sampling, could also facilitate the spread of microbial contamination in this ward. Behavioural personalities of children/infants, such as

regular mouthing of hands and objects, drooling and sharing of toys with each other while playing may also facilitate the spread of contamination (Moore, 2018).

4.4 Prevalence of *Enterococcus* on different sites

Contamination of hospital inanimate environments occurs either by direct patient shedding of microorganisms into their surrounding environment (especially patient's bed, sink) or transfer of microorganisms to health workers hands and subsequent contamination, for example, ward phones, computers and patient files (Russotto *et al*., 2015). Hospital textiles such as draperies/blinds, staff apparels and bedspreads may play a vital role in the spread of pathogenic bacteria (Fijan and Turk, 2012). The distribution of bacterial contamination of the selected surfaces in our study shows that the most contaminated sites were the beds (both occupied and unoccupied). The high contamination of occupied beds is anticipated since skin scales, stool, blood, and urine originating from patients can contain a high number of microorganisms. However, the high-level contamination of the unoccupied beds is a source of concern since a new patient assigned to this bed might be at risk of exposure to enterococci which might facilitate infection. Literature in the field of microbial survival on hospital textiles after laundering showed that *E. faecium* could survive at 60°C and some other strains of enterococci at 71°C for 10mins) (Orr *et al*., 2002), which suggests that the hospital linen is a possible source of enterococcal infection.

Hand hygiene is among the most important measures to prevent the transmission of microorganisms and the acquisition of hospital infections (Gesser-Edelsburg *et al*., 2019; Pittet *et al*., 2006). Hospital healthcare workers hands may become colonised with multidrug-resistant bacteria, which in turn may be transmitted to their environments and patients during routine care (Morgan *et al*., 2012) in cases of poor hand hygiene compliance. The patients' files are one of the most shared objects handled by all healthcare givers in hospitals for following daily patient management progress. Suppose proper high-quality hand hygiene is not performed before attending to the next patient. In that case, they are liable to transmit these multidrug-resistant microorganisms to other surfaces in the health care facilities (Panhotra *et al*., 2005). The overall prevalence of bacterial contamination on the nurses' tables, phones, patients' files revealed by our study was 13%, 11% and 9%, respectively. A high contamination rate has been reported in a survey carried out in a tertiary hospital in Taiwan by Chan *et al.,* which showed the contamination rates of the medical chart to be as high as 83.2% (Chen *et al*., 2014). Panhotra *et al.* (2005) also revealed in their study patient file contamination of 85.2%

in ICU and 24.7% in surgical wards with pathogenic and potentially pathogenic bacteria. Such contamination is suggestive use of a variety of factors which may include poor sanitation practices or ineffective disinfectants.

Hence, ensuring proper hand washing and disinfection of non-critical hospital environment/surfaces is essential for infection prevention and control. Suitable hygiene protocols on appropriate cleaning procedures of surfaces is recommended to help eradicate dirt, control the spread of pathogens and also eliminate some of the resident flora in hospital environments (Andersen *et al*., 2009). While performing janitorial tasks (especially when using mops), mop heads and towels are common sources of cross-contamination. Our study showed a 15.0% contamination rate from a wet mop. A study carried out by Exner *et al.* demonstrated the transfer of contamination between surfaces because of failure in cleaning procedures (Exner *et al.*, 2004). While repeated cleaning and the use of disinfectants are expected to reduce bacterial contamination, this is contingent on the use of the correct concentration and replacement of the disinfectant solution once the bioburden exceeds the disinfectant capacity of the disinfectant solution. Interventions focused on education or training janitorial staff are likely to reduce contamination and improve infection control in these hospitals.

4.5 Antibiotic susceptibility of the *Enterococcus* spp

The emergence and spread of antibiotic resistance in clinical settings is a significant health concern, but antimicrobial resistance in clinical isolates that have spread from environmental sources and contaminants have mostly been overlooked (Moges *et al*., 2014). Compared to *E. faecalis, E. faecium* is intrinsically more antibiotic-resistant , with the majority of its pathogenic isolates expressing resistance to ampicillin, vancomycin and a high concentration of aminoglycosides (Agudelo and Huycke, 2014). In this study, more than 75% of the isolates were non-susceptible to erythromycin, tetracycline, rifampicin and ciprofloxacin. Nonetheless, isolates were highly susceptible to ampicillin, teicoplanin and penicillin. All isolates of *E. faecalis* and *E. faecium* were susceptible to vancomycin, while all isolates of *E. gallinarum* and *E. casseliflavus* were non-susceptible to vancomycin. The latter is expected since they are intrinsically resistant to low-level vancomycin (Dutka-Malen *et al*., 2014).

We found that the prevalence of antimicrobial resistance differs in the enterococci species isolated from the two wards of the hospitals. A higher number of non-susceptible isolates were detected in the enterococci isolated from the ICU wards compared to those from pediatric

wards of the hospitals. This may be associated with higher use of antibiotics in ICU since most patients could have a critical illness that demands long-term antibiotic use. Isolates of *E. faecium*, *E. faecalis, E. gallinarum* and *E. casseliflavus* in all the healthcare settings were not susceptible to erythromycin, ciprofloxacin or tetracycline. A high number of non-susceptible were detected in the different hospitals level; this may be attributed to patterns of antibiotic use. Other factors could include specific characteristics of each hospital, such as patient case-mix as well as other external factors like the influx of resistant pathogens originating in that community (Li and Webster, 2018).

The occurrence of MDR in hospitals is a real danger to public health, especially if nothing is done to combat its spread (Chaoui *et al*., 2019). The highest MDR patterns observed in this study were in the paediatric units of all the hospitals, with the central hospital showing the highest (81%). Three of the hospitals demonstrated the same multi-drug resistance patterns. Since these hospitals receive referrals within the same provincial area, it could be deduced that the same resistant clone is spreading amongst these hospitals due to the constant transfer of patients between them. We thus recommend further studies using more sensitive typing techniques such as whole genome-sequencing to prove this hypothesis.

There are minimal studies on the contamination and antibiotic resistance of *Enterococcus* spp. in hospital environments in South Africa. Although this study was performed in a non-outbreak setting, *Enterococcus* spp was isolated from all sites and equipment in two different wards at four different healthcare levels. There is a need for more stringent sterilisation, cleaning, disinfection and isolation measures, and healthcare and janitorial personnel training on the risks of bacteria contamination and IPC failures. Intelligible procedures should be implemented for hand hygiene, laundry and care of mops.

Acknowledgement

The authors would like to acknowledge the funding received from the University of KwaZulu-Natal, College of Health Sciences Young Research Grant and the South African National Research Foundation. We would also like to express gratitude to the participating hospitals along with the IPC staff.

Conflict of interest

Professor Sabiha Essack is the chairperson of the Global Respiratory Infection partnership sponsored by an unconditional educational grant from Reckitt and Benckiser, UK.

Author Contributions

Co-conceptualised the study: CO, SE, LB Carried out the sample collection, performed the laboratory work, analysed the data and wrote the manuscript: CO. Vetted the results: CO, LB Undertook critical revision of the manuscript: CO, SE, LB.

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Supplementary Table1: List of primers used for the identification of *Enterococcus* to specie level used in this study.

Primer	Gene	Sequence (5'-3')	Product size	Reference
Ent 1	*tuf*	5'-TACTGACAAACCATTCATGATG-3′	112	(Ke *et al*., 1999)
Ent 2		5'-AACTTCGTCACCAACGCGAAC-3'		
FL1	*sodA*	5' ACTTATGTGACTAACTTAACC3'	360	(Jackson *et al*., 2004)
FL2		5' TAATGGTGAATCTTGGTTTGG3'		
FM1	*sodA*	5' GAAAAAACAATAGAAGAATTAT3'	215	(Jackson *et al*., 2004)
FM2		5' TGCTTTTTTGAATTCTTCTTTA3'		

Chapter 4 – Manuscript 3 and 4

Manuscript 3 and 4 met objectives 4 and 5.

Manuscript 3

Genome Sequence of a novel *Enterococcus faecalis* sequence type 922 strain isolated from a door handle in the intensive care unit of a district hospital in Durban, South Africa

Authors contributions

- **Christiana O. Shobo,** co-conceptualized the study, undertook sample collection, microbiological laboratory and bioinformatic analyses and drafted the manuscript.
- Daniel G. Amoako, undertook bioinformatics analyses, data interpretation and a critical revision of the manuscript.
- Mushal Allam and Arshad Ismail performed whole-genome sequencing analysis and critical revision of the manuscript.
- Sabiha Y. Essacks and Linda A. Bester, co-conceptualized the study, provided funding, supervised the study and undertook critical revision of the manuscript.

Christiana O. Shobo, Daniel G. Amoako, Mushal Allam, Arshad Ismail, Sabiha Y. Essack, Linda A. Bester. 2019. Genome Sequence of a Novel *Enterococcus faecalis* sequence type 922 strain isolated from a door handle in the intensive care unit of a district hospital in Durban, South Africa. Microbiology Resource Announcement. 8 (35). e00582-19. Doi: 10.1128/MRA.00582-19.

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GENOME SEQUENCES

Genome Sequence of a Novel *Enterococcus faecalis* Sequence Type 922 Strain Isolated from a Door Handle in the Intensive Care Unit of a District Hospital in Durban, South Africa

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ABSTRACT Herein, we highlight the genome sequence of a novel *Enterococcus faecalis* sequence type 922 (ST922) strain isolated in South Africa. The 3,564,442-bp genome harbored defense systems, a resistome, a virulome, and genetic support, which is of importance to the control of hospital-acquired infections. The genomics of *Enterococcus faecalis* yields greater understanding into its pathogenesis.

Enterococcus faecalis is one of the leading causes of hospital-acquired infections (HAIs) (1). Patients acquire HAIs from contaminated hospital environments, including inanimate objects and direct or indirect contact with the hands of health care workers (HCWs) (2, 3). Here, we present the emergence of sequence type 922 (ST922), a novel *E. faecalis* sequence type found in strain 2SIL2 isolated from a door handle in the intensive care unit of a district hospital in Durban, South Africa. Ethical approval was granted by the Biomedical Research Ethics Committee (BREC) of the University of KwaZulu-Natal, reference BE606/16.

The 2SIL2 strain was isolated and confirmed as *Enterococcus faecalis* using *Enterococcus* selective agar base (Sigma-Aldrich, St. Louis, MO, USA) and an API 20 strep kit (bioMérieux, Marcy-l'Etoile, France). It was streaked onto a tryptic soy agar (TSA) (Sigma-Aldrich) plate and incubated at 37°C for 24 h. Following incubation, genomic DNA was extracted from 1 CFU of a pure culture of the isolate using the GenElute bacterial genomic DNA kit (Sigma-Aldrich). A paired-end library (2 × 300 bp) was prepared using an Illumina Nextera XT DNA sample preparation kit and sequenced on a MiSeq machine (Illumina, San Diego, CA, USA). The generated sequenced reads (2,003,064 reads) were quality assessed and trimmed using the next-generation sequencing (NGS) core tools in the CLC Genomics Workbench version 11.0.1 (CLC bio/Qiagen, Aarhus, Denmark). Default parameters were used for all software unless otherwise specified. The genome was *de novo* assembled using SPAdes version 3.11 (4), and 927 contigs (99× coverage) were obtained, the largest being 59,734 bp and having an N_{50} value of 8,774 bp. The CheckM tool version 0.9.7 (5) was used with lineage-specific marker sets from other genetically well-characterized closely related *E. faecalis* strains to verify that the sequence reads were not from mixed species. The NCBI Prokaryotic Genome Annotation Pipeline (PGAP) version 4.3 (available at https://www.ncbi.nlm.nih.gov/) and RAST Server version 2.0 (available at http://rast.nmpdr.org) were used for annotation.

The genome features were as follows: genome size, 3,564,442 bp; GC content, 36.80%; total coding sequences (CDS), 4,101; number of coding genes, 3,920; number of RNA genes, 57; number of rRNAs, 3; and number of tRNAs, 49. The novel sequence

Citation Shobo CO, Amoako DG, Allam M, Ismail A, Essack SY, Bester LA. 2019. Genome sequence of a novel *Enterococcus faecalis* sequence type 922 strain isolated from a door handle in the intensive care unit of a district hospital in Durban, South Africa. Microbiol Resour Announc 8:e00582-19. https://doi.org/10.1128/MRA.00582-19.

Editor Vincent Bruno, University of Maryland School of Medicine

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Received 17 May 2019
Accepted 5 July 2019
Published 29 August 2019

type was defined as ST922 by the *Enterococcus faecalis* MLST database (https://pubmlst.org/efaecalis/). BURST algorithmic analysis identified ST93 (a double-locus variant) as the potential parent ancestry to the clone. With the CRISPRCasFinder (6) at default settings, 5 putative CRISPR arrays were identified on contigs 45, 46, 47, 48, and 129, with 2 associated Cas clusters on contigs 48 and 129 of the genome. The antibiotic resistome included those for aminoglycosides [*aph(3′)-III*, *ant(6)-Ia*, *str*], macrolide-lincosamide-streptogramin [*lsa(A)*, *erm(B)*, and *lnu(G)*], oxazolidinone (*optrA*), phenicol (*cat*, *fexA*), tetracycline [*tet*(M), *tet*(L)], and trimethoprim (*dfrG* and *dfrK*) (according to the ResFinder tool Web platform, version 3.1) (7).

PlasmidFinder version 1.3 (8) identified three plasmid replicons (rep7, rep9, and rep11). The PHAge Search Tool (PHAST) (9) detected two intact phages (*Entero_phiFL3A_NC_013648* and *Paenib_Xenia_NC_028837*). The insertion sequences in the genomes (IS*3*, IS*66*, IS*1634*, and IS*701*) were predicted by BLAST searches against contigs on the ISfinder database (10). The GoSeqIt VirulenceFinder database version 2.0 discovered the following putative virulence determinants (using a threshold identity of ≥95% and a minimum length of 60%): aggregation substance (*agg*), pheromone precursor lipoproteins (*cad*, *camE*, *cCF10*, and *cOB1*), endocarditis- and biofilm-associated pili (*ebpA*, *ebpB*, and *ebpC*), endocarditis antigen A (*efaAfs*), enterococcal leucine-rich internalin-like protein A (*elrA*), gelatinase (*gelE*), hyaluronidase (*hylA* and *hylB*), sortase A (*srtA*), and thiolperoxidase (*tpx*), potentially contributing its ability to adhere to surfaces, invade the immune system, colonize, and cause harmful effects to the host (11). The genomics of *Enterococcus faecalis* highlights the need for effective infection control systems in health care facilities to prevent HAIs.

Data availability. This whole-genome sequence project has been deposited in DDBJ/ENA/GenBank with the BioProject and BioSample numbers PRJNA523601 and SAMN10984490 under the accession number SIYF00000000. The described version is SIYF01000000. The raw reads have been submitted to the SRA (accession number SRR9021436).

ACKNOWLEDGMENTS

We thank Janetta Top and the team of curators at the *Enterococcus faecalis* PubMLST databases for curating the data and making them publicly available at https://pubmlst.org/efaecalis/.

We are grateful to the Sequencing Core Facility, National Institute for Communicable Diseases, National Health Laboratory Service (Johannesburg, South Africa), the South African Research Chairs Initiative of the Department of Science and Technology and the National Research Foundation of South Africa (grant number 98342), and the College of Health young researcher grant (University of KwaZulu-Natal) for sponsoring this research. Sabiha Y. Essack is the chairperson of the Global Respiratory Infection Partnership sponsored by an unrestricted educational grant from Reckitt and Benckiser. All other authors declare that they have no competing interests regarding the publication of this paper.

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Manuscript 4

Comparative genomics reveals the dominance of major clones of *Enterococcus faecalis* within public hospital environments in South Africa: Insights into antibiotic resistome, mobilome and phylogenomics

Author Contributions

- **Christiana O. Shobo,** co-conceptualized the study, undertook sample collection, microbiological laboratory and bioinformatic analyses, interpreted results, and drafted the manuscript.
- Daniel G. Amoako, undertook bioinformatics analyses, data interpretation and a critical revision of the manuscript.
- Mushal Allam and Arshad Ismail performed whole-genome sequencing analysis and critical revision of the manuscript.
- Sabiha Y. Essacks, co-conceptualized the study, supervised the study and undertook a critical revision of the manuscript.
- Linda A. Bester, the principle investigator, funded the study, co-conceptualized the study, supervised the study and undertook a critical revision of the manuscript

Comparative genomics reveals the dominance of major clones of *Enterococcus faecalis* within public hospital environments in South Africa: Insights into antibiotic resistome, mobilome and phylogenomics

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Keywords: Genomics, *Enterococcus faecalis*, Sequence type, Contamination, Hospital Environment, South Africa

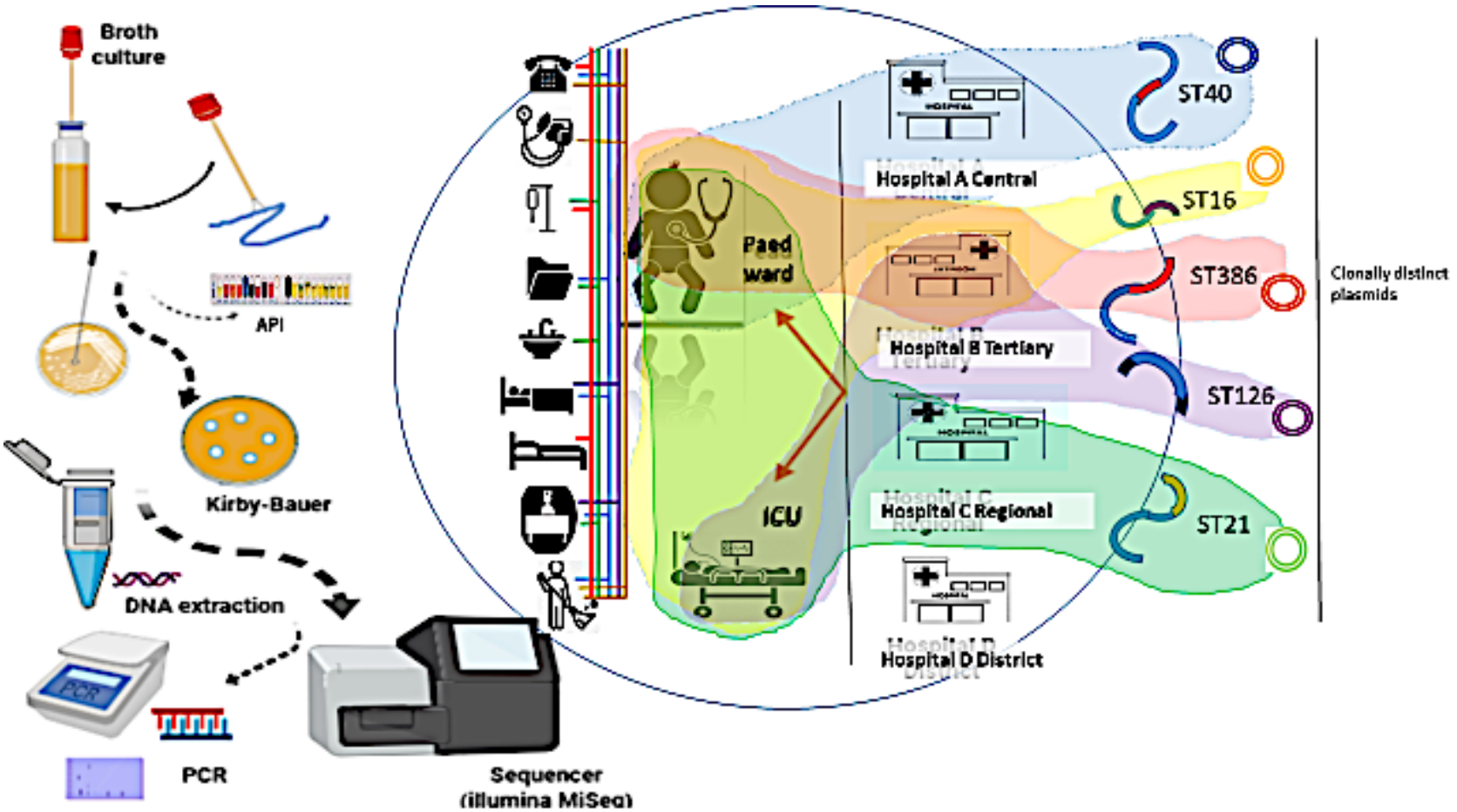
Running title: Genomic analysis reveals the dominance of major clones of *E. faecalis*

Submitted to the Journal of Hospital Infection

Highlights

- WGS was used to study *E. faecalis* in four hospital environments in South Africa
- The *tet(M)* and *erm(C)* genes were the most common antibiotic resistome
- The isolates harboured clonally distinct mobile genetic elements
- Major clones were mostly conserved within specific hospitals
- Intra-clonal spread between the sites within each hospital setting was evident
- The study calls for the need of optimal contamination control strategies in hospitals

Graphical Abstract



Abstract

Enterococci are among the most common opportunistic hospital pathogens. This study used whole-genome sequencing (WGS) and bioinformatics to determine the antibiotic resistome, genetic support, clones and phylogenetic relationship of *Enterococcus faecalis* isolated from hospital environments in South Africa. Isolates were recovered from 11 frequently touched sites by patients and healthcare workers in different wards at 4 levels of healthcare (A, B, C and D) in Durban, South Africa. Following microbial identification and antibiotic susceptibility tests. Of the 245 isolates identified, 38 were subjected to WGS on the Illumina MiSeq platform. WGS confirmed that none of the isolates harbour vancomycin resistance genes. The tet(M) (82%) and erm(C) (42%) genes were the most common antibiotic resistance genes found in isolates originating from the different hospital environments. The isolates harboured mobile genetic elements consisting of plasmids (n=11) and prophages (n=14), that were mostly clone-specific. Of note, a myriad of insertion sequence (IS) families were found with the IS3 (55%), IS5 (42%), IS1595 (40%) and Tn3 Transposon been the most predominate. Microbial typing using WGS data revealed 15 clones with 6 major sequence types (ST) belonging to ST16 (n =7), ST40 (n = 6), ST21 (n =5), ST126 (n = 3), ST23 (n =3) and ST386 (n=3). Phylogenomic analysis showed that the major clones were mostly conserved within specific hospital environments. However, further metadata insights revealed the complex intra-clonal spread of these *E. faecalis* major clones between the sampling sites within each specific hospital setting. The genomic data and analyses further our understanding of *E. faecalis* in the hospital environments and are broadly relevant in the design of optimal infection prevention strategies in hospital settings.

1.0. Introduction

The surveillance of hospital environments can be a useful tool better to understand the opportunistic microbial communities within the hospital (Comar *et al*., 2019), to identify the source of an outbreak (Gilchrist *et al*., 2015), and to evaluate the efficacy of environmental disinfection or other infection prevention and control measures (Bani-Yaghoub *et al*., 2012). Inadequate control practices have played a significant role in disseminating, persistence, intra- and inter-hospital spread of drug-resistant organisms. Regrettably, promising clinical trials comparing the different approaches to, and the impact of infection prevention and control interventions on the control of drug-resistant bacteria in hospitals and other healthcare facilities are minimal (Dusé, 2005; Harbarth *et al*., 2015). Accurate identification of resistant bacterial reservoirs and modes of transmission help inform such interventions.

The recent accomplishment in tracking worldwide epidemics (McGann *et al*., 2016) and hospital-acquired outbreaks (Quainoo *et al.*, 2017) attributes to whole-genome sequencing (WGS). Genomic similarities have broadened our understanding of the advancement and spread of infectious agents, antibiotic resistance genes, and their genetic support in bacterial species and the extent of genomic variation, resulting in varied phenotypes (Balloux *et al*., 2018; Quainoo *et al*., 2017). *Enterococcus faecalis (E. faecalis)* is a good indicator bacteria in hospital environment monitoring (Zaheer *et al*., 2020). Antibiotic resistance is either intrinsic or through sporadic mutation or the acquisition of foreign genetic material, by horizontal gene exchange occurring with the aid of mobile genetic elements plasmids, prophages and insertion sequences (Hollenbeck and Rice, 2012; Miller *et al*., 2014).

A number of previous surveillance studies involving *E. faecalis* in Africa have focused either on wastewater treatment plants (WWTPs) and hospital effluent but not on the internal hospital environment (Ekwanzala *et al*., 2020; Iweriebor *et al*., 2015). Moreover, in South Africa, studies on the contamination of *E. faecalis*, using high discrimination resolution typing are scarce. Therefore, this study uses WGS in delineating the resistome, mobile genetic support, the clonal and phylogenomic relationship of *E. faecalis* isolated from the hospital environment in places frequently touched by patients and healthcare workers at four different levels of healthcare in the metropolitan city of Durban, South Africa.

2.0. Materials and Methods

2.1 Ethical approval

Ethical clearance was received from the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (Ref. BE606/16). The study was also registered on the Health Research and Knowledge Management database (HRKM 098/17) of the KwaZulu-Natal Provincial Health Research Ethics Committee. Participating hospitals further granted gate keeper's approval.

2.2. Study setting

The selected hospitals were all public hospitals situated in the eThekwini region in Durban, South Africa. For non-disclosure reasons, the hospitals' names were withheld and referred to as A, B, C and D representing central, tertiary, regional and district facilities respectively. The central hospital (A), with a 1200 bed-size, offers tertiary level sub-specialist services and serves as a referral hospital for the district, regional and tertiary hospitals. The tertiary hospital (B) with 800 beds also s specialist services and receives referrals from regional and district hospitals not limited to provincial boundaries. The regional hospital (C), with a 743 bed-size, provides services to a specific regional population and receives referrals from several district hospitals. The district hospital (D) has 300 beds and serves as a health district and supports primary health care services on a 24-hour basis.

Samples were collected in the intensive care unit (ICU) and paediatric ward from 11 sites that included the telephones, ventilators, blood pressure apparatus, patient files, drip stands, sinks, beds (both occupied and unoccupied), nurses' tables, mops and the door handle of the linen room. A total number of 620 samples were collected over a period of three months from the four levels of healthcare. All samples were collected by randomly swabbing approximately 5 cm of the site using pre-labelled Nylon flock swabs with transport media (FLOQSwabs™ COPEN diagnostics Inc, USA). The swabs were then transported to the laboratory in iceboxes and processed within three to four hours after sampling.

2.3. Isolation and identification of *Enterococcus*

2.3.1. Phenotypic determination of *Enterococcus*

The samples were inoculated separately into tryptone soya broth (TSB) (Oxoid, Hampshire, England) and incubated at 37 °C for 2 hrs with shaking at 100 rpm. Following incubation, 1 ml of each culture was inoculated into 9 ml of TSB supplemented with 6.5% NaCl and incubated at 37°C for 24 hrs with shaking at 100 rpm. All 24 hrs cultures were sub-cultured by spread plating 100 µl onto Bile Esculin Azide agar (Himedia, Mumbai, India). Plates were incubated for 24 hrs at 37 °C, and brown-grey colonies surrounded by black halos were considered presumptive enterococci. Presumptive colonies were streaked onto Bile Aesculin agar (Lab M, Lancashire, UK), and incubated at 37 °C to obtain pure colonies. For characterisation of haemolysis, cultures were prepared on 5% Sheep Blood agar (Oxoid, Hampshire, England), and on Tryptone Soya Agar (TSA) (Oxoid, Hampshire, England) for biochemical characterisation and the Gram string test (Gregersen, 1978). Phenotypic identification was undertaken using API 20 Strep kits (Biomerieux SA, Marcy I 'Etoile, France). *Staphylococcus aureus* American Type Culture Collection (ATCC) 29213 and *E. faecalis* ATCC 29212 were used as controls. Presumptive enterococci were stored in 10% glycerol stock solution at - 80 °C until further processing.

2.3.2. Molecular confirmation of isolates

DNA was extracted from a 24hrs culture using the heat lysis method as formerly described (Englen and Kelley, 2000). A multiplex polymerase chain reaction (PCR) was performed to confirm isolates at the genus and species level. Genus-specific and species-specific primer used in all the reactions were as previously described (Jackson *et al*., 2004; Ke *et al*., 1999) (Table S1). The PCR reaction mixtures and thermal cycling conditions used were as previously described (Molechan et al., 2019). All reactions included a positive control (Table S1) and a "no template control (NTC)". The PCR products were electrophoresed at 90 V and run on a 1.8% gel having 0.5 µg/ml ethidium bromide and visualized using the Gel Doc™ XR+ imaging system (Bio-Rad, Hercules, California, USA).

2.4. Antibiotic susceptibility testing (AST)

The Kirby-Bauer disk diffusion method was used to determine the antibiotic susceptibility of the isolates according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI,

2017). The following antibiotics were used: erythromycin (15 µg), chloramphenicol (30 µg), linezolid (30 µg), ampicillin (10 µg), penicillin (5 µg), vancomycin (30 µg), teicoplanin (30 µg), tetracycline (30 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), nitrofurantoin (300 µg) and rifampicin (5 µg). All antibiotics were sourced from Oxoid (Basingstoke, United Kingdom). *Staphylococcus aureus* ATCC 25923 was used as the control. High-level aminoglycoside resistance was determined using gentamicin (120 µg) and streptomycin (300 µg) discs on Mueller-Hinton agar (Oxoid, Hampshire, England) with *E. faecalis* ATCC 29212 as the control isolate. All samples showing intermediate resistance to vancomycin were further chosen for WGS analysis.

2.5. DNA isolation, genome sequencing, assembly and annotation

Genomic DNA (gDNA) was extracted using GenElute® bacterial genomic DNA kit (Sigma–Aldrich, St. Louis, Missouri, United States) according to the manufacturer's instructions. The quantification of extracted gDNA was determined on a Nanodrop ND1000 spectrophotometer (Thermo Scientific™, Waltham, USA), Qubit® 2.0 fluorometer (Invitrogen, Oregon, USA) and verified on an agarose gel electrophoresis. Multiplexed paired-end libraries (2 × 300 bp) were prepared using the Nextera DNA Flex sample preparation kit (Illumina, San Diego, California, United States) and sequences determined on an Illumina MiSeq platform with 100× coverage at the National Institute of Communicable Diseases Sequencing Core Facility, South Africa. The resulting raw reads were checked for quality, trimmed and de novo assembled into contigs using the CLC Genomics Workbench version 10 (CLC, Bio-QIAGEN, Aarhus, Denmark). Default parameters were used for all software unless otherwise specified. The CheckM tool version 0.9.7 (Parks *et al*., 2015) was used to verify that the sequence reads were not from mixed-species using lineage-specific marker sets from other genetically well-characterised closely-related *E. faecalis* isolates. The *de-novo* assembled reads were uploaded in GenBank and annotated using National Centre for Biotechnology Information (NCBI) prokaryotic genome annotation pipeline and Rapid Annotations using Subsystems Technology (RAST) 2.0 server (Aziz *et al*., 2008).

2.6. WGS-based molecular typing of E. faecalis isolates

Multilocus sequence typing (MLST) typing was performed in-silico using the WGS data online platform tool MLST 1.8 (Larsen *et al*., 2012) which also predicted the allelic profiles of the

seven housekeeping genes, *aroE, gdh, gki, gyd, psts, xpt, and yqil* of *E. faecalis* as described previously (Feil *et al*., 2003).

2.7. Phylogenomic analysis of *Enterococcus faecalis* isolates

The de novo-assembled contigs were uploaded, and the analysis was submitted to CSI (Call SNPs and Infer) Phylogeny-1.4 (https://cge.cbs.dtu.dk/services/CSIPhylogeny-1.2), an online service which identifies single-nucleotide polymorphism (SNPs) from WGS data, filters and validates the SNP positions, and then infers phylogeny based on concatenated SNP profiles (Ahrenfeldt *et al*., 2017). The pipeline was run with default parameters: a minimal depth at SNP positions of 10 reads, a minimal relative depth at SNP positions of 10%, a minimal distance between SNPs of 10 bp, a minimal *Z-score* of 1.96, a minimal SNP quality of 30 and a minimal read mapping quality of 25. A bootstrapped with 100 replicates indicator was applied to identify recombined regions and provide the phylogenetic accuracy in groups with little homoplasy. The Figtree (http://tree.bio.ed.ac.uk/software/figtree/) was used to edit and visualize the phylogenetic tree. The phylogeny was visualised alongside metadata for isolate demographics (including hospital, source, ward), sequence type and antibiotic resistome using Phandango (Hadfield *et al*., 2017) to provide a comprehensive analysis of the generated phylogenomic tree.

2.8. Genomic identification of the antibiotic resistome and mobile genetic elements (MGEs)

The bacterial analysis pipeline of GoSeqIt (https://www.goseqit.com/web-services/) via ResFinder (Zankari *et al*., 2012), Antibiotic Resistance Gene-Annotation (ARG-ANNOT) database (Gupta *et al*., 2014) and the Comprehensive Antibiotic Resistance (CARD; https://card.mcmaster.ca) (Jia *et al*., 2017) database tools were also used to annotate and identify antibiotic-resistant genes. Plasmid replicons were predicted through PlasmidFinder (Carattoli *et al*., 2014) (https://cge.cbs.dtu.dk/services/PlasmidFinder/). The PHAge Search Tool (PHAST; http://phast.wishartlab.com/) (Zhou *et al*., 2011) server was used for the identification, annotation, and visualization of prophage sequences. The assembled genomes were further analysed for insertion sequences and transposons using ISFinder (https://isfinder.biotoul.fr/) (Siguier, 2006). RAST SEEDVIEWER (https://rast.nmpdr.org/seedviewer.cgi) (Overbeek *et al*., 2014) and Integrall database

(http://integrall.bio.ua.pt/) (Moura *et al*., 2009) was also used to annotate and identify the investigated genomes for integrons and associated gene cassettes.

2.9. Data analysis, interpretation and availability

Descriptive statistics were used to describe the frequency of *E. faecalis* that was isolated from different point of sampling (and sources). The prevalence of *E. faecalis* from different point of sampling (and sources) was compared with the Pearson's correlation coefficient (PCC) test using the GraphPad Prism software version 5.0. A value of $p < 0.05$ was considered statistically significant. The raw read sequences and the assembled whole-genome contigs have been deposited in GenBank. The data is available under project number **PRJNA523601**.

3.0. Result

3.1. Identification of bacterial isolates, phenotypic antibiotic resistance and selection

Of the 620 samples taken, 298 *Enterococcus* spp. were obtained, of which 245 were confirmed as *Enterococcus faecalis* via phenotypic and molecular assays. Antibiotic susceptibility testing revealed that none of the 245 identified *E. faecalis* isolates was vancomycin resistant (VRE). However, a total of thirty-eight (38) were of intermediate susceptibility to vancomycin and were therefore selected for genotypic characterization by WGS and bioinformatics analysis (Table 1). The highest and lowest number of such isolates were recovered from tertiary (B) (n=15) and district hospital (D) (n=3), respectively (Table 1). The paediatric ward harboured the highest number of isolates (n=25), followed by the ICU (n=13) while the site with the highest isolation rate within all the hospitals was the nurses' table (n=8).

PCC analysis revealed a positive correlation (r^2=0.755) between the different levels of care (hospital size/number of beds) and the number of isolates obtained however this was not statistically significant (*p* value= 0.245) (Table S3 and Figure S1). Isolates showed high-level resistance to both tetracycline (n=30, 79%) and a moderate resistance rate to erythromycin (n=18, 47%). A small number of the isolates showed aminoglycoside resistance (gentamicin [n=4] and streptomycin [n=6]). Majority of the isolates were susceptible to ampicillin, penicillin, teicoplanin and levofloxacin whiles all the isolates were susceptible nitrofurantoin (Table S2).

3.2. WGS-based species confirmation and molecular typing

The identification of *E. faecalis* isolates was confirmed with generated genomic data via the Global Platform for Genomic Surveillance (Pathogenwatch). MLST-analyses (ST) revealed that the *E. faecalis* in the provincial public health-care facilities were multiclonal belonging to 15 different STs with the six major STs belonging to ST16 (n =7), ST40 (n = 6), ST21 (n =5), ST126 (n = 3), ST23 (n =3) and ST386 (n=3), with diverse allelic profiles (Table 1). Moreover, one isolate belonged to the novel sequence type (ST922) (Shobo *et al*., 2019).

3.3 Resistance profiling of *E. Faecalis* isolates

In total, 14 antibiotic resistance genes (ARGs) and variants were detected (Table 1). There were no specific differences in the resistome with regards to their hospital levels and wards. The frequency of ARGs ranged between 2–13 genes, with fifteen isolates carrying three resistance genes. Acquired ARGs conferring resistance to tetracycline [*tet(M)* and *tet(L)*], macrolide-lincosamide-streptogramin B (MLS_B) [*erm(B)* and *mphD*], aminoglycosides (*sat4A, aph3-lll, ant6-la, aac6-aph2*), trimethoprim-sulfamethoxazole (*dfrG* and *dfrK*) and phenicols (*catA* and *optrA*) were found in the isolates as shown in Table 1. The *tet(M)* and *erm(B)* genes were found in 82% (31/38) and 42% (16/38) of the isolates, respectively. The *dfrG* gene predominately caused resistance to trimethoprim-sulfamethoxazole (Table 1 and Table S2).

3.4. WGS detection of mobile genetic support

WGS analysis revealed 11 different plasmid replicons from seven *rep* families in different combinations in the *E. faecalis* isolates (Table 2). pTEF2 (rep9), pTEF3 (repUS13), pAD1 (rep9) and pEFC1 (rep6) were the most predominant replicon types occurring in 14 (37%), 13 (34%), 13 (34%) and 9 (24%) isolates respectively. Of note, two isolates 2SIL2 and 2SPJ101 from hospital D concomitantly harboured unique plasmid replicons (pk214 (rep7), pEFR (rep11), pPD1 (rep9), pRE25 (rep2), pUB110 (repUS14) and pKH7 (rep7)) that were absent in the other isolates (Table 2). Eight (21%) of the isolates did not possess any plasmid replicons. The replicons harboured by the isolates were clonally related. For instance, major replicon pTEF2 harboured by isolates belonging ST21 while the replicon set pTEF3 (repUS13), pAD1 (rep9) and pEFC1 (rep6) were harboured in ST40 isolates. Furthermore, most of the isolates (n=5) belonging to ST16 lacked plasmids.

The prophage analysis revealed all isolates hosted at least one intact bacteriophage except for three isolates belonging to different STs (Table 2). The predominant intact bacteriophages found were the Entero_phiFL1A (42%; n=16), Entero_phiFL3A (16%; n=6), Entero_vB_IME197 (16%; n=6) and Entero_phiEf11 (13%; n=5).

Table 1: Summary of the population, hospital level, sample source, sample type, and genotypic characteristics of the *E. faecalis* isolates

	Isolate demographics			WGS in-silico typing	Resistance genes
	Hospital	Source	Ward	MLST (15)	
1MPA1	Central	PHONE	PEAD	ST40	TetM, MphD, Isa(A)
1MPA3	Central	PHONE	PEAD	ST40	TetM, MphD, Isa(A)
1MPD4	Central	PATIENT FILE	PEAD	ST16	ErmB, TetM, MphD, Isa(A), CatA, dfrG, dfrK, Str
1MPF1	Central	MOP	PEAD	ST498	MphD, Isa(A)
1MPF3	Central	MOP	PEAD	ST498-LIKE	TetM, MphD, Isa(A)
1MPJ101	Central	OCCUPIED BED	PEAD	ST40	TetM, MphD, Isa(A)
1MPK2	Central	NURSES TABLE	PEAD	ST40	TetM, MphD, Isa(A)
1MPK3	Central	NURSES TABLE	PEAD	ST40	TetM, MphD, Isa(A)
1MPK4	Central	NURSES TABLE	PEAD	ST40	TetM, MphD, Isa(A)
2MPJ104	Central	OCCUPIED BED	PEAD	ST23-LIKE	TetM, MphD, Isa(A)
3MPH1	Central	SINK	PEAD	ST610	ErmB, TetM, MphD, Isa(A), TetL
3MPJ101	Central	OCCUPIED BED	PEAD	ST258	MphD, Isa(A)
2UIJ104	Tertiary	OCCUPIED BED	ICU	ST126	TetM, MphD, Isa(A)
2UIK2	Tertiary	NURSES TABLE	ICU	ST126	TetM, MphD, Isa(A)
2UIK3	Tertiary	NURSES TABLE	ICU	ST21	ErmB, TetM, MphD, Isa(A)
2UPA3	Tertiary	PHONE	PEAD	ST386-LIKE	MphD, Isa(A)
2UPC4	Tertiary	BP APPARATUS	PEAD	ST386-LIKE	MphD, Isa(A)
2UPF4	Tertiary	MOP	PEAD	ST314	MphD, Isa(A)
2UPJ202	Tertiary	UNOCCUPIED BED	PEAD	ST386-LIKE	MphD, Isa(A)
3UIA2	Tertiary	PHONE	ICU	ST16	TetM, MphD, Isa(A), CatA, dfrG, Str
3UIC1	Tertiary	BP APPARATUS	ICU	ST16	ErmB, TetM, MphD, Isa(A), CatA, dfrG, Sat4A, Aph3-lll, Ant6-la, Aac6-Aph2
3UIE2	Tertiary	VENTILATOR	ICU	ST268	TetM, MphD, Isa(A)
3UIJ202	Tertiary	UNOCCUPIED BED	ICU	ST282	ErmB, ------ , MphD, Isa(A), CatA, dfrG, Sat4A, Aph3-lll, Ant6-la, TetL

3UPF3	Tertiary	MOP	PEAD	ST16	ErmB, TetM, MphD, Isa(A), dfrG
3UPF4	Tertiary	MOP	PEAD	ST16	ErmB, TetM, MphD, Isa(A), dfrG
3UPH1	Tertiary	SINK	PEAD	ST23	TetM, MphD, Isa(A)
3UIC2	Tertiary	BP APPARATUS	ICU	ST16	ErmB, TetM, MphD, Isa(A), CatA, dfrG, Sat4A, Aph3-lll, Ant6-la, Aac6-Aph2
1CIB1	Regional	DRIP STAND	ICU	ST21	ErmB, TetM, MphD, Isa(A)
1CID1	Regional	PATIENT FILE	ICU	ST21	ErmB, TetM, MphD, Isa(A)
1CIH3	Regional	SINK	ICU	ST21	ErmB, TetM, MphD, Isa(A)
1CPK2	Regional	NURSES TABLE	PEAD	ST21	ErmB, TetM, MphD, Isa(A)
1CPK3	Regional	NURSES TABLE	PEAD	ST126	TetM, MphD, Isa(A)
2CPF3	Regional	MOP	PEAD	ST41	TetM, MphD, Isa(A)
2CPH2	Regional	NURSES TABLE	PEAD	ST16-LIKE	ErmB, TetM, MphD, Isa(A)
3CPH1	Regional	SINK	PEAD	ST23	TetM, MphD, Isa(A)
2SIL2	District	DOOR HANDLE	ICU	ST922	ErmB, TetM, MphD, Isa(A), CatA, dfrG, dfrK, Sat4A, Aph3-lll, Ant6-la, TetL, FexA, OptrA
2SPJ101	District	OCCUPIED BED	ICU	ST6	ErmB, TetM, MphD, Isa(A), CatA
2SPL2	District	DOOR HANDLE	PEAD	ST314	ErmB, TetM, MphD, Isa(A)

Four prophages were identified in one *E. faecalis* ST16 (3UPF4) strain isolated from the mop of a paediatric ward in hospital B with a peculiar bacteriophage (Lactoc_PLgT_1). The isolates 1C1H3, 1MPD4, 2U1K2 and 2UPF3 from different hospitals hosted three prophages. Figure 1 illustrates the circular structure of the most predominant prophage Entero_phiFL1A in the *E. faecalis* ST40 (IMPA3). The prophage harboured by the isolates were clonally related (Table 2).

Many IS families were found in the isolates with no association concerning the hospital and ward. The five major IS families were IS3 (predicted to be linked with *Enterococcus faecium/Streptococcus agalactiae* sources), IS5 (predicted to be associated with *Cyanotheca sp.* sources), IS1595 (predicted to be linked with *Bacillus subtilis*), ISL3 (predicted to be linked with *Streptococcus mutans/thermophilus*) and IS607 (predicted to be linked with both *Campylobacter sp.* and *Virus NY2A*), (Table S4). The transposase (Tn3) linked with *Bacillus thuringiensis* was found in 7 of the isolates identified from different sources (Table S4). All the isolates lacked integrons and their associated gene cassettes.

3.5 Phylogenomic and metadata analysis

A phylogenetic tree reconstructed to analyse genetic relationships between the isolates revealed a high divergence of isolates according to the different care levels (Figure 2). For instance, each hospital was generally associated with specific dominant clones (i.e., ST40 and ST498 were mostly found in hospital A; ST16, ST126, and ST386 were found in hospital B; and ST21 was predominately found in hospital C (Table 1 and Figure 2).

Phylogenomic trees coupled metadata visualization analysis provided a more in-depth insight into the characteristics and distinctions between isolates and revealed the intra-clonal spread of *E. faecalis* strains between different sources within the same hospitals (Figure 2). Specifically, ST21 was found on the drip stand, patient file, sink and nurses table in both ICU and paediatric ward of hospital C. Similarly, ST40 was observed on the ward phone, patient file, mop, occupied bed and nurses table of the paediatric ward hospital A. The ST16 clone was isolated on the mop (paediatric ward), phone and BP apparatus (ICU) of hospital B. Moreso, ST386 linked with the phone, BP apparatus, and unoccupied bed in the paediatric ward of hospital B. At the same time, ST126 was found on the occupied bed and nurses table in the ICU of the same hospital.

Table 2: Genomic analysis of mobile genetic elements (MGEs) of *E. faecalis* isolates

NB. All the isolates lacked integrons and associated gene cassettes.

Strain ID	Hospital	MLST (n=15)	Mobile Genetic Support	
			Plasmids replicons (n=11)	Intact prophage (n=18)
1MPA1	Central	ST40	pTEF3, pAD1, pEF47	Entero_phiFL1A
1MPA3	Central	ST40	pTEF3, pAD1, pEFC1	Entero_phiFL1A
1MPD4	Central	ST16	-	Entero_phiFL1A, Entero_EFC_1, Lactob_PLE2
1MPF1	Central	ST498	pTEF3	Entero_phiFL3A
1MPF3	Central	ST498-LIKE	pTEF3, pAD1, pEFC1	Entero_phiFL3A, Entero_phiFL1A
1MPJ101	Central	ST40	pTEF3, pAD1, pEFC1	Entero_phiFL1A
1MPK2	Central	ST40	pTEF3, pAD1, pEFC1	Entero_phiFL1A
1MPK3	Central	ST40	pTEF3, pAD1, pEFC1	Entero_phiFL1A
1MPK4	Central	ST40	pTEF3, pAD1, pEFC1	Entero_phiFL1A
2MPJ104	Central	ST23-LIKE	pAD1, pEF47	Entero_phiFL3A, Entero_phiFL1A
3MPH1	Central	ST610	pTEF2	-
3MPJ101	Central	ST258	pTEF2	Strept_9871
2UIJ104	Tertiary	ST126	pTEF2, pAD1	Entero_phiFL1A, Lactoc_98201
2UIK2	Tertiary	ST126	pTEF2, pAD1	Entero_phiFL1A, Lactoc_98201, Cronob_vB_CsaM
2UIK3	Tertiary	ST21	pTEF2	Entero_vB_IME197, Lactoc_63301
2UPA3	Tertiary	ST386-LIKE	pTEF3, pEFC1	Entero_phiEf11
2UPC4	Tertiary	ST386-LIKE	pTEF3, pEF47	Entero_phiEf11
2UPF4	Tertiary	ST314	-	-
2UPJ202	Tertiary	ST386-LIKE	pTEF3, pEFC1	Entero_phiEf11
3UIA2	Tertiary	ST16	-	Entero_EF62phi, Strept_phiARI0460_1
3UIC1	Tertiary	ST16	pTEF2, pCF10	Entero_phiEf11
3UIE2	Tertiary	ST268	-	Entero_phiFL1A
3UIJ202	Tertiary	ST282	pTEF2, pAD1	-
3UPF3	Tertiary	ST16	-	Entero_EFC_1, Strept_phiARI0131_1, Strept_phiARI0460_1
3UPF4	Tertiary	ST16	-	Entero_phiFL1A, Entero_EFC_1, Strept_phiARI0460_1, Lactoc_PLgT_1
3UPH1	Tertiary	ST23	pEFC1	Entero_phiFL3A
3UIC2	Tertiary	ST16	pTEF2, pCF10	Entero_phiFL1A, Entero_phiEf11
1CIB1	Regional	ST21	pTEF2	Entero_vB_IME197, Lactoc_63301
1CID1	Regional	ST21	pTEF2	Entero_vB_IME197, Lactob_PLE2
1CIH3	Regional	ST21	pTEF2	Entero_vB_IME197, Lactoc_63301, Stx2_c_1717
1CPK2	Regional	ST21	pTEF2	Entero_vB_IME197, Lactob_PLE2
1CPK3	Regional	ST126	pTEF2, pAD1	Lactoc_98201
2CPF3	Regional	ST41	pTEF3	Entero_vB_IME197, Lactob_PLE2
2CPH2	Regional	ST16-LIKE	-	Entero_phiFL1A
3CPH1	Regional	ST23	-	Entero_phiFL3A, Entero_phiFL1A
2SIL2	District	ST922	pk214, pAD1, pEFR	Entero_phiFL3A, Paenib_Xenia
2SPJ101	District	ST6	pTEF3, pPD1, pRE25, pUB110, pKH7	Entero_SANTOR1
2SPL2	District	ST314	pTEF2	Strept_9872

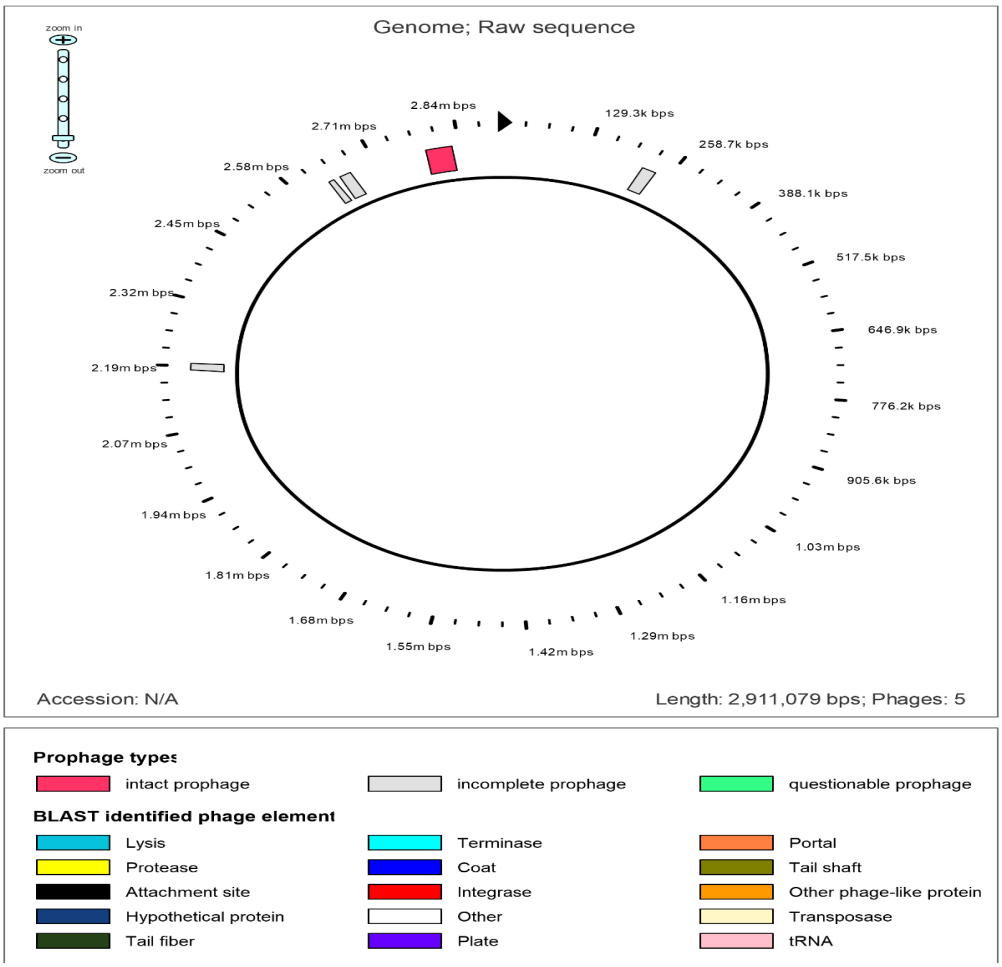


Figure 1: An illustration of the circular genomic structure of the phage Entero_phiFL1A in the *E. faecalis* ST40 (IMPA3). Putative genes are coloured according to the predicted functions of their products. The Entero_phiFL1A is the most predominant phage in the isolates.

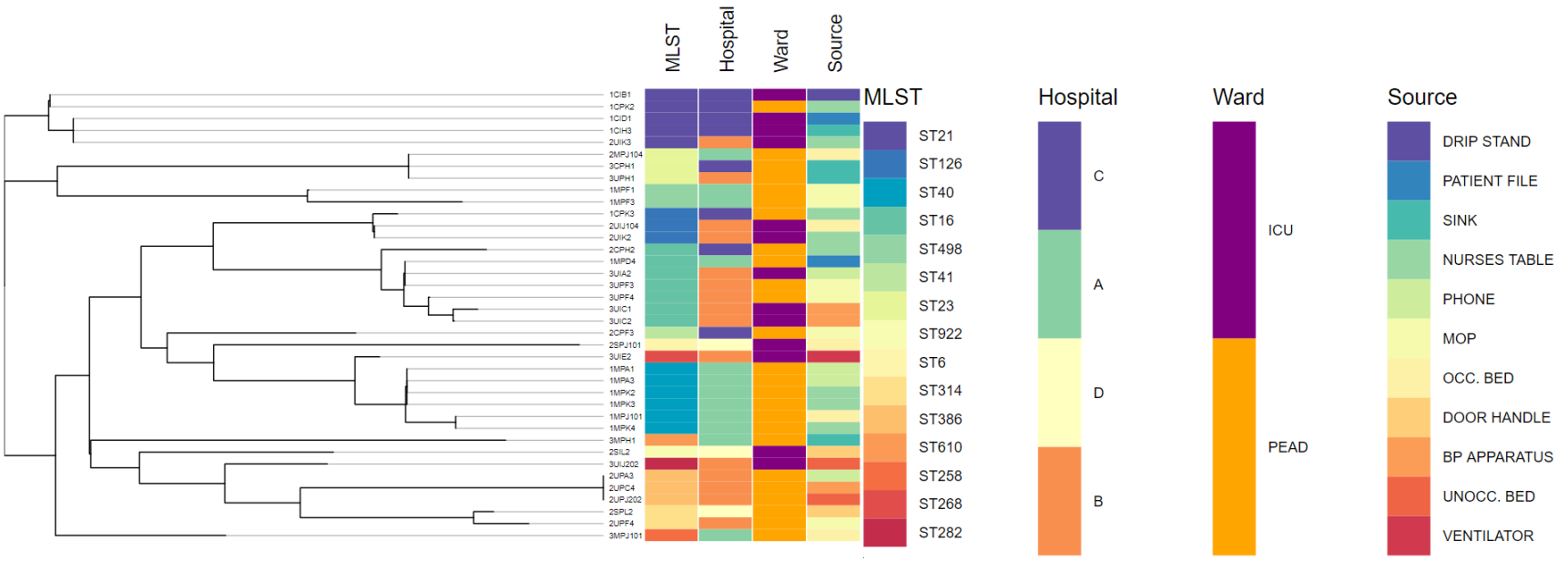


Figure 2: The whole-genome MLST phylogenomic branch and metadata of isolate information (including isolate name, hospital, source, ward) and WGS *in-silico typing* (sequence type and antibiotic resistome) coupled using Phandango *E. faecalis* isolates at different level of care in KZN, South Africa. The linking lines in the phylogenetic tree differentiate between the different clades. Metadata annotations show that there were generally distinct major sequence types between the four hospital levels; however, there was spread of these major clones between different sources in the wards with each hospital.

4.0. Discussion

E. faecalis isolates (n=38), with intermediate susceptibility to vancomycin, from frequent touch sites by patient and healthcare workers, in two different wards and four levels of healthcare (represented as A, B, C and D) in eThekwini district, South Africa were subjected to WGS to gain insights into antibiotic resistance, mobile genetic support, clonal and phylogenomic relationships.

In line with the global trend, reports on bacterial contamination in hospital environments is increasing in Africa across all sectors (Osei Sekyere and Mensah, 2019), and *E. faecalis* is one of the most common enterococcal species isolated from the hospital environment. This is evident from our results where *E. faecalis* 83% (n=245), was the most prevalent organisms compared to *E. faecium* 13%(n= 38). *E. faecalis* is recognised as an important hospital-associated pathogen responsible for approximately 80 to 90% of cases reported in the hospital settings followed by 5 to 10% *E. faecium* (Farman et al., 2019). Hence *E. faecalis* has been placed in the category of pathogens posing a major threat to healthcare systems (Zarb *et al*., 2012).

Furthermore, *E. faecalis* is involve in major infection prevention attributing to their ability to persist for long periods on hands and remain viable on environmental surfaces (inanimate surfaces) due to their microbial structure thus, can serve as a reservoir for ongoing transmission in the absence of regular decontamination (Kramer *et al*., 2006). Additionally, *E. faecalis* possess the ability to acquire additional resistance through the transfer of mobile genetic support such as plasmids, prophages, and insertion sequences (Mikalsen *et al*., 2015; Partridge *et al*., 2018). The acquisition of resistance and genetic support poses a therapeutic challenge. The WGS results showed that none of the *E. faecalis* harbored vancomycin-resistant genes.

This corroborates the view of Ellington *et al.* on the role of WGS in antimicrobial susceptibility testing of bacteria for the explanation of false positive in phenotypic result for samples (Ellington *et al*., 2017) and further confirms WGS as a more discriminatory tool to infer antibiotic susceptibility as compared to relying entirely on phenotypic testing alone. Even though vancomycin resistance in *E. faecalis* remains uncommon (approximately 2% in 2017) (National Department of Health, 2018), there is still a need for careful prescription of this antibiotic to avoid selective pressure induced resistance to vancomycin to which limited

alternatives exist especially in the public health sector. Majority of the isolate were susceptible to ampicillin, penicillin, teicoplanin, levofloxacin, and nitrofurantoin confirming their use as treatment options in South Africa, particularly ampicilin (the drug of choice for *E. faecalis* infections) (National Department of Health, 2018).

Tetracycline demonstrated reduced susceptibility against *E. faecalis* mediated mostly by the ribosomal protection protein, *tet(M)* (Miller *et al*., 2014; Warburton *et al*., 2016). This was consistent with previous studies that found the *tet(M)* as the dominant gene causing tetracycline resistance in *E. faecalis* isolates across all the one-health sectors (human-animal-environment interface) (Osei Sekyere and Mensah, 2019). For instance, in a 2014 hospital-based study in China by Jia *et al*. (Jia *et al*., 2014), tet*(M)* was found to cause tetracycline-resistant *E. faecalis* isolates. Similarly, *Said et al*. (Said and Abdelmegeed, 2019) also detected tet*(M)* as 96.1% of all tetracycline-resistant *Enterococcus* isolates in Egypt. However, high-level tetracycline resistance exhibited by 2SIL2 isolate was mediated by both ribosomal-protection gene [*tet(M)*] and active-efflux gene [*tet(L)*].

This indicates the significant role played by efflux pumps in mediating antibiotic resistance (Blanco *et al*., 2016). The low prevalence of the tet*(L)* was not unusual and pointed to the fact that ribosomal protection protein is the primary mechanism of tetracycline-resistant *E. faecalis* isolates. The moderate level of erythromycin resistance was mediated by *erm(B)* genes which are the most common mechanism of resistance reported for the macrolide class of antibiotics in Africa (Osei Sekyere and Mensah, 2019) and globally (Miller *et al*., 2014; Tian *et al*., 2019) for *Enterococcus*. There was small number of isolates showing aminoglycoside resistance across the different levels of care, which corresponded to the aminoglycoside-modifying enzymes found. However, these isolates exhibited high-level resistance encoding a set of enzymes (*sat4A, aph3-lll, ant6-la, aac6-aph2*). However, this was not unusual as all *Enterococcus* are recognized to generate low-level resistance to all aminoglycosides by reducing drug uptake, linked with the proteins required in electron transport (Shete *et al*., 2017). More so, the *OptrA* gene implicated in linezolid resistance was found in only one isolate (2SIL2) however, it was unexpressed as the isolate was susceptible to linezolid (Table 1 and S2).

A noticeable polyclonal nature was observed in the *E. faecalis* isolates with 15 distinct STs, including one novel STs, highlighting the diverse nature of the strains in the province. The major STs found such as ST16, ST40 and ST21 were previously reported in Saudi Arabia, China, Tunisia, France, and Spain from human subjects, hospitalized patients, animals and wastewater (Farman *et al*., 2019; Kuch *et al*., 2012; McBride *et al*., 2007; Quiñones *et al*., 2009; Zischka *et al*., 2015). Similarly, other studies have also reported the ST126, ST23 and ST386 in different settings (human, animal and environment), hence not suggesting any kind of host specificity in these major STs reported in this study (Raven *et al*., 2016). However, unlike other countries, the population structure of *E. faecalis* from different settings in South Africa are minimally monitored, if at all, making it difficult to correlate our results with studies in South Africa. This calls for the need for *E. faecalis* to be included in surveillance schemes to enable the monitoring of the molecular epidemiology of isolates collected over larger tempo-spatial scales using high throughput technologies such as WGS (Amoako *et al*., 2019). This will assist researchers, doctors, and microbiologists gain increased awareness into the evolution and dissemination of *E. faecalis*.

Characterizing the isolates' genetic support indicated that the majority of *E. faecalis* in the different hospitals are likely reservoirs for diverse mobile genetic elements and associated ARGs (especially for tetracycline, erythromycin). There was a higher plasmid prevalence rate (seven *rep* families) and the detection of two or more distinct replicons in one strain. Accordingly, this finding agrees with the fact that numerous types of plasmids are often present in enterococci from a clinical setting (Garcia-Migura *et al*., 2011; Song *et al*., 2013; Zhu *et al*., 2010). More so, single isolates of *E. faecalis* has shown by other studies to harbour multiple plasmids (Garcia-Migura *et al*., 2011; Sedgley and Clewell, 2004). A comparison of replicons and MLST sequence types showed a correlation of plasmid replicons, clonality, and antibiotic resistance genes. For example, pTEF2 containing the resistance gene set (*ErmB*, *TetM*) were harboured by isolates belonging to ST21 irrespective of the associated hospital. Similarly, all the ST40 clones harboured replicon set pTEF3, pAD1 and pEFC1 had the same resistome (*TetM*) and did not exhibit erythromycin resistance. The ST16 exhibited the highest antibiotic resistome diversity owing to its varied plasmid replicons (Table 1 and 2).

There was no specific pattern between the acquisition of insertion sequence families or transposable elements with respect to the ward and level of care; however, the presence of

prominent IS families in *E. faecalis* clones imply that these elements are spread by horizontal gene transfer (HGT) (Mikalsen *et al*., 2015). Moreover, the acquisition of these elements can lead to transposition in the genome to aid in the transfer of resistance genes, enabling it to adapt to new environmental challenges to colonize new niches (Vandecraen *et al*., 2017). For instance, IS3 family upstream of the *EmrB* gene has been reported for enhanced erythromycin resistance (Vandecraen *et a*l., 2017). The ability of these clonal lineages to acquire novel genetic features may contribute to their increased persistence and highlights its potential public health threat.

Comparative phylogenomics using WGS SNPs analysis revealed a higher genetic diversity between the strains concerning each specific hospital. This implied that the major clones were mostly hospital-specific, concordant with the *in-silico* MLST typing scheme (Figure 2). Interestingly, a study by Kawalec *et al*. (2007) also found a higher diversity in the clonal structure of *E. faecalis* strains among hospitals in Poland. Visualizing the phylogenomic tree with metadata revealed the major clones in the various hospitals. This further depicted the intra-clonal spread of *E. faecalis* strains between different sources within the same hospital, reiterating the need for phylo/meta-analysis to increase confidence in molecular epidemiological studies. For instance, at the paediatric ward of hospital A, the ST40 clone was isolated from a phone, nurses table, patient file, mop and occupied bed which may be due to hand contamination by patients and/or healthcare workers (nurses, janitor staff, etc.) (Figure 2).

A similar scenario occurred in hospital B, where ST386 was found in the paediatric ward (on the phone, BP apparatus and unoccupied bed) while the ST126 was isolated in the ICU (on nurses table and occupied bed). Reports on enterococci transient carriage on the hands of healthcare workers and patients as well their presence on, medical equipment or environmental surfaces has been documented in several studies (Agudelo and Huycke, 2014; Daniel *et al*., 2015; Evans Patterson *et al*., 1995; Tajeddin *et al*., 2016). Other studies have reported colonised patients' movement among different hospital settings responsible for these transmission patterns (Daniel *et al*., 2015; Jackson *et al*., 2019). Moreover, hospitals B and C observed intra-ward spreads (both ICU and paediatric ward) of ST16 and 21 respectively from different sites with each hospital. The transmission of enterococcal strains has been

documented within medical units, given credence to the study findings (D'Agata *et al*., 2001; Lund *et al*., 2002).

Frequent contact with healthcare providers and colonised patients' movement among different healthcare settings is a possible means for these patterns of transmission in hospitals A, B and C. However, there were limited isolates from a district hospital (Hospital D) due to the number of isolates obtained for any detailed comparative analysis. Even though our study's findings may not be generalized to the country's overall situation, this study improves the understanding of the prevalence, genetic content, and relatedness of *E. faecalis* contamination of hospital environments. It is thus recommended that scheduled periodic identification of transmitting sources in the hospitals' inanimate environment, strict enforcement and adhesions of IPC practices amongst the health workers and isolation of colonized patients should be imposed to reduce the incidence and transmission of *E. faecalis* hospital environments.

5.0. Conclusion

This genomic analysis provided a snapshot of the inanimate hospital environment as a reservoir of resistant *E. faecalis*, its associated mobilome (plasmids, prophages, insertion sequences and transposons). It revealed an elaborate intra-clonal spread of *E. faecalis* major clones between the sites within each specific hospital setting. This study improves our understanding of the dissemination of *E. faecalis* in hospital environments and will optimize infection prevention and control strategies in clinical settings.

Acknowledgement

We are grateful to the Sequencing Core Facility, National Institute for Communicable Diseases, National Health Laboratory Service, Johannesburg, South Africa; the South African Research Chairs Initiative of the Department of Science and Technology and the National Research Foundation of South Africa (Grant number: **98342**); and the College of Health Young Researcher Grant, University of KwaZulu-Natal, for sponsoring this research.

Competing interests

Professor Essack is chairperson of the Global Respiratory Infection Partnership and member of the Global Hygiene Council, both sponsored by unrestricted educational grants from Reckitt and Benckiser, Ltd. UK. The other authors have no competing interests to declare.

Author Contributions

C.S co-conceptualized the study, undertook sample collection, microbiological laboratory and bioinformatic analyses, interpreted results, and drafted the manuscript. **D.G.A.** undertook bioinformatics analyses, data interpretation and a critical revision of the manuscript. **M.A and A.I.** performed whole-genome sequencing analysis and critical revision of the manuscript. **S.Y.E.** co-conceptualized and supervised the study and undertook critical revision of the manuscript. **L.A.B.** is the principle investigator, funded, co-conceptualized the study, supervised the study and undertook the critical revision of the manuscript.

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Supplementary table 1: List of genus-and species-specific primers and control strains used in this study

Control Strain	**Primer**	**Primer sequence 5'-3'**	**Product size (bp)**	**Reference**
E. faecalis ATCC 51299	ENT1	TACTGACAAACCATTCATGATG	112	(Molechan *et al*., 2019)
	ENT2	AACTTCGTCACCAACGCGAAC		
E. faecalis ATCC 51299	FA1	ACTTATGTGACTAACTTAACC	360	(Molechan *et al*., 2019)
	FA2	TAATGGTGAATCTTGGTTTGG		

Supplementary table 2: Antibiotic susceptibility profile of *E. faecalis* collected from the hospital environment

a. Antibiotic susceptibility tests were interpreted according to CLSI resistant breakpoints (v 7.1) for *E. faecalis*.

Antibiotic susceptibility testing [a]

STRAIN ID	VAN	TEC	CIP	LEV	GEN	STP	TET	ERY	CHLO	RIF	F300	LZD	PEN	AMP
1MPA1	I	I	I	S	S	S	R	I	S	I	S	S	S	S
1MPA3	I	S	I	S	S	S	R	I	S	I	S	S	S	S
1MPD4	I	S	I	S	S	R	R	R	R	R	S	R	S	S
1MPF1	I	S	R	S	S	S	S	I	S	R	S	I	S	S
1MPF3	I	S	R	S	S	S	R	I	S	I	S	R	S	S
1MPJ101	I	S	S	S	S	S	R	I	S	I	S	R	S	S
1MPK2	I	I	I	S	S	S	R	I	S	I	S	S	S	S
1MPK3	I	S	S	S	S	S	I	I	S	I	S	S	S	S
1MPK4	I	S	I	S	S	S	R	I	S	I	S	S	S	S
2MPJ104	I	S	I	S	S	S	R	I	S	I	S	I	S	S
3MPH1	I	S	R	S	S	S	R	R	S	I	S	S	S	S
3MPJ101	I	S	I	S	S	S	S	I	I	R	S	R	S	S
2UIJ104	I	S	R	S	S	S	R	I	S	R	S	R	S	S
2UIK2	I	S	I	S	S	S	R	I	S	R	S	R	S	R
2UIK3	I	S	I	I	S	S	R	R	S	I	S	S	S	S
2UPA3	I	S	R	I	S	S	I	R	R	S	S	I	S	S
2UPC4	I	S	I	I	S	S	I	I	S	S	S	S	S	S
2UPF4	I	S	I	S	S	S	I	I	I	R	I	R	S	S
2UPJ202	I	S	I	S	S	S	S	I	S	I	S	S	S	S
3UIA2	I	S	I	S	S	S	R	I	R	R	S	S	S	S
3UIC1	I	S	I	S	R	R	R	R	R	R	S	S	S	S
3UIE2	I	S	I	S	S	S	R	I	I	R	S	R	S	S
3UIJ202	I	S	I	S	R	R	S	R	R	R	S	R	S	S
3UPF3	I	S	S	S	S	S	R	R	S	R	S	R	S	S
3UPF4	I	S	R	I	S	S	R	R	S	R	S	R	R	S
3UPH1	I	S	I	I	S	S	R	I	I	R	S	R	S	S
3UIC2	I	S	I	I	R	R	R	R	R	R	S	I	S	S
1CIB1	I	S	I	R	S	S	R	R	I	R	S	S	S	S
1CID1	I	S	S	S	S	S	R	R	S	R	S	R	S	S
1CIH3	I	S	R	S	S	S	R	R	S	R	S	S	S	S
1CPK2	I	S	I	S	S	S	R	R	I	I	S	S	S	S
1CPK3	I	S	S	S	S	S	R	R	I	I	S	S	S	S
2CPF3	I	S	I	S	S	S	R	I	I	I	S	R	S	S
2CPH2	I	S	I	S	S	S	R	R	I	R	S	R	S	S
3CPH1	I	S	I	S	S	S	R	I	I	R	S	S	S	S
2SPJ101	I	S	I	S	S	S	R	R	R	I	S	R	S	S
2SPL2	I	S	I	S	S	S	R	R	R	I	S	R	S	S
2SIL2	I	S	S	S	R	R	R	R	R	R	S	S	S	S

Glycopeptides: VAN = Vancomycin, TEC = Teicoplanin; **Quinolones**: CIP =ciprofloxacin, LEV = levofloxacin; **Aminoglycosides**: STP = streptomycin, GEN = gentamycin; **Tetracyclines**: TET = tetracycline; **Macrolides**: ERY = erythromycin; **Chloromycetin**: CHLO = chloramphenicol; **Rifamycin**: RIF = rifampicin; **Nitrofurans**: F300 = nitrofurantoin; **Oxazolidinones**: LZD = linezolid; **Penicillin**: PEN = penicillin G; AMP = ampicillin

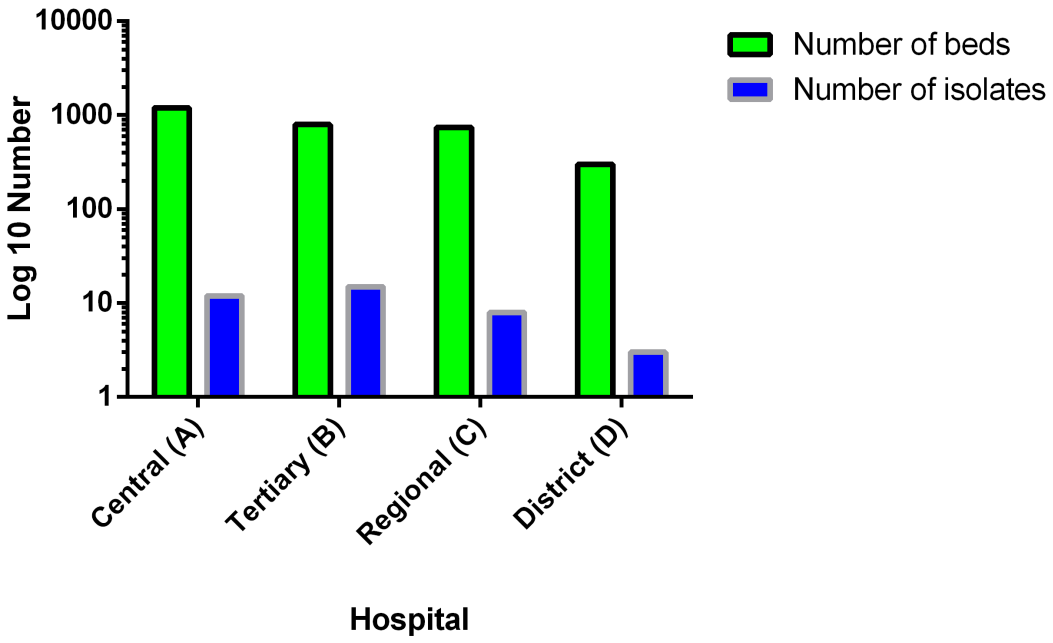
Supplementary table 3: Association between the different levels of care (hospital size/number of beds) and the number of *Enterococcus* isolates collected.

Correlations			
		Number of beds	Number of isolates
Number of beds	Pearson Correlation	1	.755
	Sig. (2-tailed)		.245
	Sum of Squares and Cross-products	407086.750	4335.500
	Covariance	135695.583	1445.167
	N	4	4
Number of isolates	Pearson Correlation	.755	1
	Sig. (2-tailed)	.245	
	Sum of Squares and Cross-products	4335.500	81.000
	Covariance	1445.167	27.000
	N	4	4

A positive correlation (r^2=0.755) was found between the different levels of care (hospital size/number of beds) and the number of isolates obtained however this was not statistically significant (p-value = 0.245).

Supplementary Table 4: Distribution of six major insertion sequence (IS)/transposase families and their predicted sources among *E. faecalis* isolates via the ISFinder database.

IS Family	Number of occurrences	Predicted Sources
IS3	*21*	*Enterococcus faecium* *Streptococcus agalactiae*
IS5	*16*	*Cyanotheca sp.*
IS1595	*15*	*Bacillus subtilis*
ISL3	*9*	*Streptococcus mutans* *Streptococcus thermophilus*
IS607	*9*	*Campylobacter sp.* *Virus NY2A*
Tn3	*7*	*Bacillus thuringiensis*



Supplementary figure 1: A bar chart showing the positive correlation between the number of beds and isolates obtained from the different level of care in KZN

5.0 Chapter 5 – Conclusions, Limitations, and Recommendation

Studies of WGS have few or no studies on hospital environments. This creates a significant data gap, especially from the hospital inanimate environmental sectors, since they play a significant role in bacterial transmission and may be a potential mode of antibiotic resistance transmission. Therefore, resistance data from environmental sectors must be assessed to obtain a holistic view of antibiotic resistance's actual state. This study investigated environmental samples from different public hospitals as reservoirs of antibiotic-resistant bacteria in their epidemiology, phylogenies, clonality, and antibiotic resistance as an indicator of infection, prevention, and control.

5.1. Conclusions

The following were the main findings from the study according to the study objectives:

The following concluded from objective 1:

The functional profile and bacterial community were studied using the 16S rRNA with a total of 150 samples collected in three public hospitals [District, Regional, and Central] from intensive care and a pediatric ward. A total of 7,996,346 reads were found, of which 7,319,569 were quality-filtered reads. The estimated average number of reads, the length of reads (nt), and the number of OTUs across all samples were 285, 583, and 364, respectively. 462, 658, and 325 reads shared OTUs across different hospital levels, locations, and sample sources. A total of 11 phyla, 29 classes, 50 orders, 105 families, 190 genera, and 288 known species were identified. The primary phyla identified were Proteobacteria, Firmicutes, and Actinobacteria. The dominant class identified was Gamma-proteobacteria, followed by Bacilli and Actinobacteria. At the same time, the highest numbers of total and unique Operational Taxonomic Units (OTU) across the samples obtained from the different hospital locations were recorded in the intensive care unit (ICU).

Overcrowding of hospital wards which is often caused by lack of resources and shortage of nursing staff i.e. two or more patients sharing the same bed or having an insufficient spacing between individual patient beds may result in the transfer of different micro-organism in the healthcare setting. In such healthcare facilities, family caregivers will be relied on to provide a large proportion of patient care. A large number of unique OTUs among all the hospital sample sources was found on beds and drip stands. In hospital C, the pediatric unit and patient files

contained the lowest total and unique OTU numbers. The bacterial community structure distinctly differed between the hospital samples. *Acinetobacter* (16%), *Citrobacter* (14%), *Staphylococcus* (10%), and Corynebacterium (6%) were the predominant genera. A total of 279 functional groups at level three Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology were found.

The functional profile analysis identified the citrate cycle (TCA), signal transduction mechanisms, bisphenol degradation, tyrosine metabolism, and transcription-factors as the dominant pathways. Human disease functional classes, including involvement in antibiotic resistance, were significantly identified. The drip stands, patient files, and ward telephones in all the wards of hospitals A and C contained a higher number of human diseases functional classes. Metagenomics tool was shown to be very useful in environmental monitoring; it suggested that the different hospitals have a specific makeup of organisms, and attention is given to that during referrals. Regular checking of the environment should be done using metagenomic tools to see if any shifts arise.

The following concluded from objective 2 and 3:

Here, *Enterococcus* spp. was isolated from a total of 620 swab samples collected over three months from the four healthcare facilities (district, regional, tertiary, and central hospital). A total of 120 isolates were collected from the central hospital (A), 174 from the tertiary hospital (B), 231 from the regional hospital (C), and 95 from the district hospital (D). Overall, 295 *Enterococcus* spp. were identified and confirmed, 54 samples were from the central hospital (A), 111 from the tertiary hospital (B), 101 from the regional hospital (C), and 29 from the district hospital (D) with 64% recovered in the paediatric wards, and 36% from the intensive care units (ICU).

The tertiary hospital had the highest isolation rate with paediatric and ICU at 52% (n=58) and 48% (n=53), respectively, followed by the regional hospital with 60% (n=61) and 40% (n=40) isolated from the paediatric and ICU, respectively. The central, regional, and district hospitals showed a significant p-value < 0.00001, 0.02144, and 0.01314 respectively in the paediatric ward compared to ICU. The site with the highest isolation rate was the occupied beds and the mops, with 15% (n=44) isolate each. In the central hospital (A), the site with the highest isolation rate was the occupied bed 22% (n=12). For the tertiary hospital (B), the sites with the

highest isolation rates were the ward phones and mops, with 14% (n=16) each. In the regional hospital (C), the mops and the occupied beds harboured the most significant enterococci with 14% (n=14) each. For the district hospital (D), the larger part of the samples identified were from door handles 28% (n=8).

Manual routine cleaning techniques are often not perfect (cleaning and disinfecting) and personnel dependent due to the different nature of surfaces being cleaned. Some parts maybe left without proper cleaning. The disinfectants used can also be inadequate for a certain kind of microorganism, overdiluted and contaminated. This results in a quick return to elevated levels of pathogens replication after a few hours of cleaning. This also suggests that mops should be disinfected before being re-used and that each ward should be provided its own labelled mop to avoid cross-contamination. Additionally, more disinfectant stations should be strategically located for easy access.

The highest non-susceptible percentiles of all the species were to macrolides (91%), followed by tetracyclines (83%), rifampicin (75%), and quinolones (72% to ciprofloxacin but a lesser value of 14% to levofloxacin). All isolates of *E. faecium* and *E. faecalis* were completely susceptible to vancomycin. Non-susceptibility rates of >50% to tetracycline, erythromycin, ciprofloxacin and rifampicin (>50%) were recorded for *E. faecium* and *E. faecalis* in the ICU and paediatric units in all the hospitals. Collectively, 82% of all the enterococci isolates were multidrug-resistant (resistant to one or more antibiotics from three or more distinct antibiotic classes). A total of 80 antibiograms were observed. The district, regional, tertiary and central hospitals showed different antibiograms with a total of 90% (n=26), 73% (n=74), 84% (n=93), and 89% (n=48), respectively. The most prominent antibiogram discovered in all the hospitals was the CIP-TET-ERY. *Enterococcus* spp. was isolated from all sites and equipment in the two different wards and at the four different healthcare levels. A large number of isolates sampled were resistant to more than three different antibiotic classes. The hospital environment may serve as a source of MDRO; thus, stringent measures to train and educate HCWs, including janitorial staff, on the risks associated with improper cleaning and IPC failures.

Concerning objectives 4, 5, and 6, the following was concluded:

A total of 38 *Enterococcus faecalis* were selected for genotypic characterization using WGS and bioinformatics analysis. Pearson's correlation coefficient (PCC) analysis revealed a

positive correlation (r^2=0.755) between the different levels of care (hospital size/number of beds) and the number of isolates obtained. The highest number of isolates were recovered from tertiary (B) (n=15) and the lowest number from the district hospital (D) (n=3)). The paediatric ward concealed the highest number of isolates (n=25), followed by the ICU (n=13), while the site with the highest isolation rate within all the hospitals was the nurses' table (n=8). MLST-analyses (ST) revealed that the *E. faecalis* in the provincial public healthcare facilities were multiclonal belonging to 15 different STs with the six major STs being to ST16 (n =7), ST40 (n = 6), ST21 (n =5), ST126 (n = 3), ST23 (n =3) and ST386 (n=3). Novel sequence type (ST922) was isolated from the door handle in the district hospital. Each hospital was generally associated with specific dominant clones i.e., ST40 and ST498 were mostly found in hospital A; ST16, ST126, and ST386 were found in hospital B; and ST21 was predominately found in hospital C. Intra-clonal spread of *E. faecalis* strains was found between different sources within the same hospitals.

In total, 14 resistome and variants were identified, although there were no specific differences in the resistome regarding the hospital level and wards. The occurrence of resistant genes was found to be in the range of two to three genes, whereas 15 isolates predominantly carried three resistance genes. All the isolates harboured acquired antibiotic-resistant genes to tetracycline [*tet(M)* and *tet(L)*], macrolide-lincosamide-streptogramin B (MLS_B) [*erm(B)* and *MphD*], aminoglycosides (*Sat4A, Aph3-lll, Ant6-la, Aac6-Aph2*), trimethoprim-sulfamethoxazole (*dfrG* and *dfrK*) and phenicol (*CatA* and *OptrA*). The *tet(M)* and *erm(B)* genes were found in 82% and 42% of the isolates, respectively. In trimethoprim-sulfamethoxazole, the *dfrG* gene predominately caused resistance.

Eleven different plasmid replicons from seven *rep* families appeared in separate sequences in the *E. faecalis* isolates. The most predominant replicon types were pTEF2 (37%), pTEF3 (34%), pAD1 (34%) and pEFC1 (24%). Two isolates 2SIL2 and 2SPJ101 from hospital D harboured unique plasmid replicons (pk214, pEFR, pPD1, pRE25, pUB110, and pKH7) that were not present in the other isolates. The major complete bacteriophages found were the Entero_phiFL1A 42% (n=16), Entero_phiFL3A 16% (n=6), Entero_vB_IME197 16% (n=6), and Entero_phiEf11 13% (n=5). There is a need for the reporting and surveillance structure of *Enterococcus* spp. HAI and its antibiotics profiles in SA for easy tracking of outbreaks related

to this species. Therefore, it is essential to improve decontamination approaches in clinical surroundings.

5.2. Limitations and Recommendations

The following limitations and recommendations are presented from the findings of the study

Limitation 1

The molecular epidemiology of *Enterococcus faecalis* could be underestimated or overestimated, considering that staff were notified before sample collection according to management gatekeepers' request.

Recommendation

The continuous surveillance of *E. faecalis* is recommended to monitor the trends in the molecular epidemiology of resistance to this organism and isolates collected over larger tempo-spatial scales in both hospital environment, healthcare workers, including janitors using high throughput technologies such as whole-genome sequencing (WGS). Such surveillance would help IPC practitioners gain better insights into the problem and focusing areas. In addition, outcomes will be more accurate if done without prior warning to the staff.

Limitation 2

Given the complex interplay and cross-contamination between the hospitals, wards, and sites, interactions predicted and annotated genomic information concerning mutations, pathogenicity, and possession of other genomic signatures should be interpreted cautiously since the sample size was different from the level of healthcare.

Recommendation

Further studies ascertaining on a broader scale with experimental assays should be undertaken to confirm the pathogenic potentials, genomic signatures, and mechanisms of transmission in South African hospitals to improve the knowledge and help strengthen the existing IPC programs, which could help curb these pathogens.

5.3 Significance of the research

This study provides evidence-based knowledge on the extent of contamination of the hospital environment by enterococci to inform the design and implementation of effective prevention and

control measures to tackle all forms of hospital-acquired infections. Antibiotic resistance and the prevalence of drug-resistant *Enterococcus* spp. are increasing worldwide, as evidenced by the WHO's inclusion of VRE on the high priority list of pathogens that require urgent intervention (among the ESKAPE pathogen). Thus, it is imperative to identify, track, profile, and understand enterococci's diversity from different environments.

This study highlights HAI's sources and enterococci in the environment of public hospitals in SA. It provides valuable insights to the South African health professionals to take extreme care adhering to the IPC safety practices. In particular, sanitization of patients' environments with antimicrobial material for high touch points like door handles. The proper handling of personal and surrounding equipment would breach these pathogens' transmission chains. This calls for strengthening and enforcing the existing infection prevention with strict follow-up strategies, tracking training and education of HCW's to minimize bacterial contamination of medical equipment and inanimate surfaces. The latter encompasses the use of disinfection UV robots rather than the traditional mops and other cleaning equipment. In addition to the potential contribution to knowledge towards epidemiology, the revelation of drivers of antibiotic resistance and bacterial dissemination in hospitals contribute to this study`s strength.

Appendix 1: BREC approval

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

30 May 2017

Dr LA Bester
Biomedical Resource Unit
School of Laboratory Medicine and Medical Sciences
besterl@ukzn.ac.za

Dear Dr Bester

Protocol: To ascertain the nature and extent of infection, prevention and control (IPC) programs at different levels of care in eThekwini district, KwaZulu-Natal. **Degree:** Non-degree
BREC reference number: BE606/16

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application received on 11 November 2016.

The study was provisionally approved pending appropriate responses to queries raised. Your response received on 23 May 2017 to BREC letter dated 18 January 2017 have been noted by a sub-committee of the Biomedical Research Ethics Committee. The conditions have now been met and the study is given **full ethics approval** and may begin as from 30 May 2017.

This approval is valid for one year from **30 May 2017.** To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will **be RATIFIED** by a full Committee at its next meeting taking place on **13 June 2017.**

We wish you well with this study. We would appreciate receiving copies of all publications arising out of this study.

Yours sincerely

[redacted]

Professor J Tsoka-Gwegweni
Chair: Biomedical Research Ethics Committee

cc postgraduate administrator: duchraihp@ukzn.ac.za

Appendix 2: KwaZulu-Natal Department of Health Approval

health
Department:
Health
PROVINCE OF KWAZULU-NATAL

DIRECTORATE:
Health Research & Knowledge Management (HKRM)

330 Langalibalele street,
Private Bag X9051 PMB, 3200
Tel: 033 395 2805/3189/3123 Fax: 033 394 3782
Email: hrkm@kznhealth.gov.za
www.kznhealth.gov.za

Reference: HRKM098/17
KZ_2017RP24_630

23 March 2017

Dear Dr L A Bester
(University of KwaZulu-Natal)

Subject: Approval of a Research Proposal

1. The research proposal titled **'To ascertain the nature and extent of Infection, Prevention and Control (IPC) programs at different levels of care in hospitals in eThekwini district, KwaZulu-Natal'** was reviewed by the KwaZulu-Natal Department of Health (KZN-DoH).

   The proposal is hereby **approved** for research to be undertaken at Addington, RK Khan, Inkosi Albert Luthuli Central and King Edward VIII Hospitals.

2. You are requested to take note of the following:
   a. Make the necessary arrangement with the identified facility before commencing with your research project.
   b. Provide an interim progress report and final report (electronic and hard copies) when your research is complete.

3. Your final report must be posted to **HEALTH RESEARCH AND KNOWLEDGE MANAGEMENT, 10-102, PRIVATE BAG X9051, PIETERMARITZBURG, 3200** and e-mail an electronic copy to hrkm@kznhealth.gov.za

For any additional information please contact Ms G Khumalo on 033-395 3189.

Yours Sincerely

[redacted]

Dr E Lutge
Chairperson, Health Research Committee
Date: 27/03/17

Fighting Disease, Fighting Poverty, Giving Hope

Appendix 3: TRREE Training Certificate

Module 1

TRREE

Zertifikat
Certificat
Certificado
Certificate

Promouvoir les plus hauts standards éthiques dans la protection des participants à la recherche biomédicale
Promoting the highest ethical standards in the protection of biomedical research participants

Clinical Trials Centre
The University of Hong Kong

Certificat de formation - Training Certificate

Ce document atteste que - this document certifies that

Christiana Shobo

a complété avec succès - has successfully completed

Introduction to Research Ethics

du programme de formation TRREE en évaluation éthique de la recherche
of the TRREE training programme in research ethics evaluation

October 25, 2016

Professeur Dominique Sprumont
Coordinateur TRREE Coordinator

FMH Continuing Education Program (5 Credits)
Programme de Formation continue (5 Crédits)

Foederatio Pharmaceutica Helvetiae FPH Programmes de formation continue
Continuing Education Programme
Programme de formation continue

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Module 2

TRREE

Zertifikat

Certificat

Certificado

Certificate

Promouvoir les plus hauts standards éthiques dans la protection des participants à la recherche biomédicale
Promoting the highest ethical standards in the protection of biomedical research participants

Clinical Trials Centre
The University of Hong Kong

Certificat de formation - Training Certificate

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Research Ethics Evaluation

du programme de formation TRREE en évaluation éthique de la recherche
of the TRREE training programme in research ethics evaluation

[redacted]

November 9, 2016
CID : hTmuc2dLJD

Professeur Dominique Sprumont
Coordinateur TRREE Coordinator

FMH Continuing Education Program (5 Credits)
Programme de Formation continue (5 Crédits)

Foederatio Pharmaceutica Helvetiae FPH Programmes de formation continue
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[REV : 20170310]

Module 3.1

Zertifikat
Certificat
Certificado
Certificate

TRREE

Promouvoir les plus hauts standards éthiques dans la protection des participants à la recherche biomédicale
Promoting the highest ethical standards in the protection of biomedical research participants

Clinical Trials Centre
The University of Hong Kong

Certificat de formation - Training Certificate

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a complété avec succès - has successfully completed

Informed Consent

du programme de formation TRREE en évaluation éthique de la recherche
of the TRREE training programme in research ethics evaluation

[redacted]

November 9, 2016
CID : Xwk1kVD9fV

Professeur Dominique Sprumont
Coordinateur TRREE Coordinator

FMH Continuing Education Program (5 Credits)
Programme de Formation continue (5 Crédits)

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[REV : 20170310]

Module 3.2

Zertifikat
Certificat
Certificado
Certificate

TRREE

Promouvoir les plus hauts standards éthiques dans la protection des participants à la recherche biomédicale
Promoting the highest ethical standards in the protection of biomedical research participants

Clinical Trials Centre
The University of Hong Kong

Certificat de formation - Training Certificate

Ce document atteste que - this document certifies that

Christiana Shobo

a complété avec succès - has successfully completed

Good Clinical Practice (GCP)

du programme de formation TRREE en évaluation éthique de la recherche
of the TRREE training programme in research ethics evaluation

April 26th, 2017

Professeur Dominique Sprumont
Coordinateur TRREE Coordinator

FMH Continuing Education Program (5 Credits) Programme de Formation continue (5 Crédits)

Foederatio Pharmaceutica Helvetiae FPH Programmes de formation continue Continuing Education Programme Programmes de formation continue

Ce programme est soutenu par - This program is supported by :

European and Developing Countries Clinical Trials Partnership (EDCTP) (www.edctp.org) - Swiss National Science Foundation (www.snf.ch) - Canadian Institutes of Health Research (http://www.cihr-irsc.gc.ca/e/2891.html) - Swiss Academy of Medical Science (SAMS/ASSM/SAMW) (www.samw.ch) - Commission for Research Partnerships with Developing Countries (www.kfpe.ch)

[REV : 20170310]

Appendix 4: Letter of submission of manuscript 4 to Scientific Report

Gmail **Daniel Gyamfi Amoako <amoakodg@gmail.com>**

Scientific Reports - Receipt of Manuscript 'COMPARATIVE GENOMICS REVEALS...'

Scientific Reports <srep@nature.com> Sun, Oct 18, 2020 at 10:41 AM
To: amoakodg@gmail.com

Ref: Submission ID 70b91509-318f-4b9e-bacb-fbf53e8d11d1

Dear Dr Amoako,

Thank you for submitting your manuscript to Scientific Reports.

Your manuscript is now at our initial Quality Check stage, where we look for adherence to the journal's submission guidelines, including any relevant editorial and publishing policies. If there are any points that need to be addressed prior to progressing we will send you a detailed email. Otherwise, your manuscript will proceed into peer review.

Kind regards,

Peer Review Advisors
Scientific Reports

Springer Nature offers an open access support service to make it easier for our authors to discover and apply for APC funding. For further information please visit http://www.springernature.com/gp/open-research/funding

Our flexible approach during the COVID-19 pandemic

If you need more time at any stage of the peer-review process, please do let us know. While our systems will continue to remind you of the original timelines, we aim to be as flexible as possible during the current pandemic.