COLLEGE OF HEALTH SCIENCES

SCHOOL OF LABORATORY MEDICINE AND MEDICAL SCIENCES

DEPARTMENT OF MEDICAL MICROBIOLOGY

Molecular Epidemiology of Antibiotic Resistant ESKAPE Pathogens Isolated from Public Sector Hospitals in uMgungundlovu District, KwaZulu-Natal, South Africa

Raspail Carrel Founou Zangue

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A

Thesis

Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy (PhD) in Medicine (Medical Microbiology)

School of Laboratory Medicine and Medical Sciences

By

Raspail Carrel Founou Zangue

BSc.; MSc., in Clinical Microbiology

Student number: 216 076 503

Supervisor:

Professor Sabiha Yusuf Essack

Molecular Epidemiology of Antibiotic Resistant ESKAPE Pathogens Isolated from Public Sector Hospitals in uMgungundlovu District, Kwazulu-Natal, South Africa

Raspail Carrel Founou Zangue

216 076 503

A thesis submitted to the School of Laboratory Medicine and Medical Sciences, College of Health Science, University of KwaZulu-Natal, 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 publications, with an overall introduction and final summary.

This is to certify that the content of this thesis is the original research work of Mr Raspail Carrel Founou Zangue.

As the candidate's supervisor, I have approved this thesis for submission.

Supervisor:

Signed: ---------- Name: **Prof. Sabiha Y. Essack** Date :----------

DECLARATION

I, **Raspail Carrel Founou Zangue** declare that:

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DEDICATION

This study is dedicated to all the persons and loved ones who have always encouraged and supported me throughout my life.

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May God bless and protect you all!!!

LIST OF ABBREVIATIONS

ABR	Antibiotic resistance
AMR	Antimicrobial resistance
BSI	Bloodstream infection
CA-MRSA	Community acquired-methicillin resistant *Staphylococcus aureus*
CLSI	Clinical and Laboratory Standards Institute
CTX-M	Cefotaximase-München
ECDC	European Centre for Disease Prevention and Control (ECDC)
EMEA	European Medicines Agency
ERIC-PCR	Enterobacterial repetitive intergenic consensus-polymerase chain reaction
ESBL	Extended-spectrum β-lactamase
EU	European Union
HA	Hospital acquired
HA-MRSA	Hospital acquired-methicillin resistant *Staphylococcus aureus*
HIV	Human immunodeficiency virus
ICU	Intensive care unit
IMP	Imipenem metallo-β-lactamase
KPC	*Klebsiella pneumoniae* carbapenemase
LA-MRSA	Livestock associated-methicillin resistant *Staphylococcus aureus*
LOS	Length of (hospital) stay
MBL	Metallo beta-lactamase
MDR	Multidrug resistant
MIC	Minimum inhibitory concentration
MLST	Multi-locus sequence typing
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-susceptible *Staphylococcus aureus*
NHSN	National Healthcare Safety Network
PBP	Penicillin binding protein

PCR	Polymerase chain reaction
PVL	Panton-valentine leukocidin
REP-PCR	Repetitive Palindromic-Polymerase chain reaction
SHV	Sulfhydryl variable
ST	Sequence type
TEM	Temoneira
UTI	Urinary Tract Infection
VIM	Verona integron-encoded metallo-β-lactamase
VISA	Vancomycin-intermediate *Staphylococcus aureus*
VRE	Vancomycin-resistant *Enterococci*
VRSA	Vancomycin-resistant *Staphylococcus aureus*
WHO	World Health Organization

ABSTRACT

Multi-drug resistant *Enterococcus faecium*, *staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter spp* termed ESKAPE pathogens are commonly implicated in difficult-to-treat infectious diseases in developed and developing countries. The prevalence, risk factors, phenotypic and genotypic profiles including but not limited to clonal relatedness, genetic diversity, resistance and virulence associated with ESKAPE bacteria were investigated in carriage and clinical isolates from patients in a rural, district and an urban tertiary hospital in the public health sector in uMgungundlovu District, Kwazulu-Natal, South Africa.

The overall carriage of MDR ESKAPE Gram-negative bacteria in both hospitals was 37.21%, 42.31% and 57.14% at admission, after 48 hours and at discharge, respectively. The prevalence of MDR ESKAPE Gram-negative bacteria in faecal carriage (46%) was higher than clinical samples (28%) and colonization was mainly associated with referral from the district to the tertiary hospital with high statistical significance (OR: 14.40, 95% CI 0.98-210.84). $bla_{CTX\text{-}M\text{-}group\text{-}9}$, $bla_{CTX\text{-}M\text{-}group\text{-}1}$ and bla_{SHV} were the main resistance genes identified. Similarly, the overall prevalence of faecal VRE carriage was 53% with patients at the district hospital being more likely to be colonized by VRE at admission (44%), after 48 hours (64%) and discharge (100%) than those of the tertiary level. Fifteen (39%) *E. faecium* and 23 (61%) *E. faecalis*, were detected and displayed high level of antibiotic resistance. Extensive genetic diversity of *E. faecalis* and *E. faecium* and clonal dissemination of various lineages were observed across wards and within hospitals. The high levels of resistance in *S. aureus* were attributed to the multi-drug resistant efflux pumps mepA, mexE, AcrB, MATE, qac and qacA. Whole genome analysis revealed that the circulating *S. aureus* isolates belonged to the extremely virulent ST121 clone that harboured a total of 18 virulence genes.

The high prevalence, genetic diversity and virulence of antibiotic-resistant ESKAPE bacteria elucidated in this study necessitates routine screening and surveillance in communities and hospitals, stringent infection prevention and control measures and antibiotic stewardship to monitor epidemiological changes, to contain their spread and inform appropriate antibiotic treatment options respectively.

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CHAPTER I. INTRODUCTION

I. Preamble

Bacteria are microorganisms which are found in humans, animals, and the environment. They are present as commensal or pathogenic organisms depending on the immune system and site of colonization or infection in humans and animals (Hart, 2004). Several actions have been undertaken to combat pathogenic microorganisms, the most important of which has been the discovery and use of potent and safe antibiotics (Rice, 2008). Health care advances could not be possible without the availability of these effective antibiotic medicines (Rice, 2008). These substances have rapidly contributed to the reduction of morbidity and mortality associated with formerly fatal infectious diseases as well as other diseases such as serious heart diseases, cancers, and organ failures requiring prophylactic antibiotics for surgery and transplants respectively (Rice, 2008). However, the selective pressure exerted by the use —appropriate and/or inappropriate— of the antibiotics, has led to the emergence of antibiotic resistance (ABR) (Omulo et al., 2015). ABR is the ability of bacteria to grow despite the presence of antibiotics normally active against them via a range of resistance mechanisms (modification of target, enzyme production, efflux pumps, etc.) and resistance genes (World Health Organization, 2014). All classes of antibiotics and all types of bacteria are affected by the ABR. ABR has been identified as one of the greatest public health threats to the human health by the World Health Organization (WHO) (World Health Organization, 2014).

A small group of pathogens namely *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter spp.* termed "ESKAPE pathogens" have been identified as amongst the main causes of difficult-to-treat infectious diseases in both developed and developing countries (World Health Organization, 2014). These bacteria are also increasingly involved in multi-drug resistant (MDR) infections globally (World Health Organization., 2014). Several reports have confirmed a rapid increase in rates of infections due to methicillin-resistant *S. aureus* (MRSA), extended-spectrum β-lactamase (ESBL) and carbapenemase-producing *K. pneumoniae*, metallo-beta-lactamase-producing *A. baumannii*, metallo-beta-lactamase-producing *P. aeruginosa* (MBL-PA), and extended-spectrum beta-lactamase (ESBL) producing *Enterobacter spp.* (Slavcovici et al., 2015; Rajagunalan et al., 2013). ESKAPE pathogens are simultaneously able to "escape" the activity of other antibiotic

classes frequently used in medicine, notably aminoglycosides and fluoroquinolones via a diversity of resistance mechanisms (Ramirez et al., 2014; Rice., 2008).

II. Background and rationale

The widespread availability and indiscriminate consumption of antibiotics favours the emergence of resistant bacteria around the world. Drug resistant ESKAPE pathogens have all been detected in the developed and developing world, but the threats posed by these bacteria are worse in the latter. In fact, given the high rate of infectious diseases, the lack of surveillance, infection, prevention and control measures and antimicrobial stewardship programmes in healthcare settings and communities in Africa, it is extremely probable that the prevalence and transmission rates of ESKAPE pathogens and MDR strains are under-estimated within populations in the region (Tansarli et al., 2014; Ndihokubwayo et al., 2013) and strategies to contain their emergence and spread are limited (Joloba et al., 2010). Heightening awareness of policy-makers, health and veterinary professionals, laboratory scientists, and the general population about the consequences of antibiotic use, and the risk associated with antibiotic resistance is essential to preserve potent antibiotics for health of future generations (Tansarli et al., 2014; Ndihokubwayo et al., 2013).

In South Africa, the problems posed by ABR in general and MDR ESKAPE pathogens in particular, are among others (i) limited measures for the prevention and control of infectious diseases leading to increases spread of infections caused by MDR ESKAPE pathogens (ii) lack of knowledge and poor communication between health care workers and different sectors (iii) limited implementation of antibiotic stewardship measures to prevent and/or contain ABR and (iv) high levels of antibiotic use in agriculture and veterinary health. Accordingly, drug resistant ESKAPE pathogens are no longer an exclusive HA-problem which can be solved by infection, prevention and containment measures in the hospitals, but requires an all- inclusive approach involving all role-players of the animal-human-environment continuum (Stefani et al., 2012). Studies are for that reason required to raise awareness concerning (i) the consequences posed by ABR and ESKAPE pathogens, (ii) the need to establish and/or enforce surveillance and infection prevention and control policies/programs, (iii) the need to implement appropriate strategies for better use of antibiotics, and (iv) the importance of developing and implementing antibiotic stewardship programs in healthcare settings and communities (Carlet et al., 2012). The study generated evidence for the development and/or implementation of national strategies and programmes for

the prevention and containment of ABR, as well as for strengthening policies promoting the rational use of antibiotics and monitoring ABR in the country, according to the World Health Organization's (WHO) Global Action Plan (GAP) and One Health Concept.

III. Literature review

1. Global situation of antimicrobial drug resistance

Antibiotics which were initially effective for the treatment of bacterial infectious diseases have lost their activity following the development of resistance by many microorganisms. The emergence and spread of resistant bacteria constrains the clinical use of antibiotics and impacts negatively on patient outcome as well as national and global health, leading to an urgent requirement for monitoring systems and the routine surveillance of antibiotic use and antibiotic resistance (Joloba et al., 2010). The sections below discuss the emergence, risk factors and transmission routes as well as prevention and control measures related to ABR.

1.1.Emergence of antimicrobial drug resistance

The most significant advancement in health care has been the discovery of potent and safe antimicrobial agents. These agents have saved countless lives and rapidly reduced the morbidity and mortality associated with a group of previously fatal diseases (World Health Organization, 2014). Since their discovery and the assurance that they could prevent and treat many infections, they have been considered as seminal to the improvement of public health by optimizing the management of infectious diseases (World Health Organization, 2014). The extensive use, misuse and inappropriate use of these agents has however resulted in the emergence of antimicrobial resistance in several pathogens (World Health Organization, 2014). Antimicrobial resistance (AMR) is the ability of microorganisms to counteract the antimicrobial drugs and grow despite their presence in the organism's environment (World Health Organization, 2014). AMR can affect viruses, parasites, fungi and bacteria in humans, animals and the environment. It impedes the control of infections, increases the mortality and costs, deteriorates economies and threatens global health security (World Health Organization., 2014). By 2050, about 10 million deaths will be caused by these infections and the Gross Domestic Product (GDP) of the world will decrease by 2 to 3.5% if nothing is done to contain AMR (O'neill., 2015).

Several reports have showed the worldwide concern posed by AMR in general and ABR, in both community and health care settings (World Health Organization., 2014). Alexander Fleming who discovered penicillin was the first to reveal during his Nobel Prize elocution in 1945 that resistance would occur if antibiotics were extensively used (World Health Organization, 2014; Rice, 2008). In fact, it is the selective pressure (environmental conditions promoting or inhibiting the growth of bacteria with specific characteristics) exerted by the widespread consumption of these components which has led to the emergence and spread of resistant bacteria around the world (Perovic et al., 2015; World Health Organization., 2014; Zafer et al., 2014; Rice., 2008). Antibiotic use is thus the main driver of ABR. Large quantities of antibiotic medicines are needlessly used across the world contributing to the increasing incidence and subsequent prevalence of bacterial resistance.

1.2.Risk factors and transmission routes of antibiotic resistance

The prevalence of AMR differs between and within continents, countries and amongst different pathogens (Joloba., 2010). Several factors can be associated with variations in the prevalence and spread of drug resistant bacteria around the world. The main risk factors for AMR in community's settings are poor socio-economic conditions, limited access to health care facilities. ineffective law enforcement, inadequate diagnostic and hospital capacity, inappropriate use and counterfeit quality of drugs, limitations in the antimicrobials supply chain and poor infection control contribute to increase AMR in settings (Laxminarayan and Heymann., 2012). In hospitals, these risk factors include previous hospitalisation, antibiotic treatment, stay in the intensive care unit (ICU), location in the hospital, duration of hospital stay, urinary catheterisation, injuries and surgeries, respiratory tract infection (RTI), naso-gastric intubation, mechanical ventilation, and central veno us and peripheral catheters. Other factors such as the gender, age, frequent urinary tract infections, co-morbidities such as, diabetes mellitus, malaria, HIV/AIDS and tuberculosis are also considered as risk factors for the emergence and spread of AMR (Laxminarayan and Heymann, 2012). Furthermore, the risk of developing AMR is particularly elevated in countries where monitoring systems, regulatory authorities, surveillance and legislation concerning the use of drugs are inadequate or non-existent. This is generally evident in low- and middle-income countries (LMIC) and particularly in Africa, where antibiotics are the most commonly purchased and consumed drugs (Joloba and Bwanga, 2010). AMR is accordingly a complex interaction of many factors and can be transmitted via different pathways within and between hospital and community settings.

Once the resistance has emerged, the transmission of AMR is possible via indirect and direct contacts in the population. Direct or person-to-person contact comes from immediate exposure to the pathogens via hands and biological substances. Direct contact rapidly and easily spreads drug resistant bacteria and enhances the risk of potential outbreaks in communities and hospitals across the world (Laxminarayan et al., 2016; World Health Organization., 2015a; World Health Organization, 2014; Laxminarayan et al., 2013). Additionally, there is a great concern regarding the indirect transmission of resistance pathogens. Indirect transfer occurs by exposure to contaminated objects such as medical equipments and the environment including dust, air, water and soil which are often unsuspected reservoirs. In fact, the environment has been established as an important reservoir due to the frequent dissemination of active metabolites of antibacterial drugs via biological substance such as urine, blood, semen, saliva and faeces. In developing countries, MDR bacteria significantly increase mortality while in developed countries the same pathogen increases mainly treatment expenses. Indeed, in the latter, numerous strategies and policies have been implemented for the containment of AMR and rational drug use while in developing world there is inadequate surveillance and prevention measures related to infectious diseases, and a dearth of policies concerning antibiotic consumption thereby exacerbating the problem (Laxminarayan et al., 2013; Okeke and Sosa, 2010). Is thus imperative to develop and implement global policies, guidelines, activities and recommendations addressing the prevention and containment of AMR around the world and mainly in LMICs.

2. Prevention and control of antimicrobial drug resistance

AMR is a global public health issue of great importance and the selective pressure exerted by antibiotic use in human, animal and in the environment, has always been regarded as the key driving factors leading to its emergence and spread. "Antimicrobial stewardship is a key strategy to effectively contain AMR" by a group of systematic coordinated and all-inclusive actions, concomitant interventions and guidelines enhancing the rational use of drugs in all sectors. It advocates prudent, responsible and optimal drug use to: (i) improve patient safety (ii) improve patient outcomes (iii) limit the emergence of resistance and (iv) reduce associated costs (Kimang'a, 2012). Antimicrobial stewardship programs (ASPs) are thus an effort to revolutionize cultural, social and behavioural norms and attitudes to antimicrobial use.

The WHO Global Antimicrobial Surveillance System (GLASS) recommends the following measures to monitor, prevent and contain AMR in all nations (World Health Organization, 2015b):

- Encourage national surveillance systems using uniform global standards.
- Estimate the extent and burden of AMR globally through selected indicators.
- Analyse and report global data on AMR on a regular basis.
- Detect emerging resistance and its international spread.
- Inform and implement targeted prevention and control programmes.
- Evaluate the impact of intervention.

Similarly, the WHO Global Action Plan of Antimicrobial resistance set out five strategic objectives for containment of ABR (World Health Organization, 2015a):

- Heighten awareness and understanding of AMR using effective education, communication, and training.
- Strengthen knowledge and evidence base using surveillance and research.
- Lessen the incidence of infection through effective hygiene and infection prevention measures.
- Optimize rational antibiotic use in human and animal health.
- Ensure sustainable investment in containing AMR.

3. Worldwide emergence of multidrug resistant ESKAPE pathogens

The group of ESKAPE pathogens have been reported to develop multidrug resistance and extensive drug resistance in many developed and developing countries. MDR bacteria are pathogens resistant to one or more antibiotics from three or more antibiotic classes; and those resistant to all antibacterial drugs are identified as extensively drug resistance bacteria (Kallen., 2010; Bassetti et al., 2013). The following sections elaborate on antibiotic resistant ESKAPE pathogens.

3.1. Vancomycin resistant *Enterococcus spp.*

Enterococcus spp. are Gram-positive cocci, known to be common residents of human and animal gastrointestinal tracts. About 16 species have been identified but only 2, namely *E. faecalis* and *E. faecium* have emerged as one of the clinical challenges for physicians, because they are involved in serious life-threatening infections such as endocarditis, urinary tract infections (UTI), bacteraemia, neonatal sepsis, meningitis, surgical wound infections and intra-abdominal and

pelvic infections (Abebe et al., 2014). Amongst all enterococcal species, *E. faecium* and *faecalis* represent the most clinically important. *E. faecium* exhibits more resistance to antibiotics than *E. faecalis* whereas the latter is generally more virulent (Abebe et al., 2014). The emergence and spread of resistant enterococci is attributed to several features including their efficient ability to express resistance to antibiotics, their diversity of virulence factors, their capability of biofilm formation, their noteworthy genome plasticity with the carriage of plasmids, transposons, and insertion sequences and their ability to horizontally transfer virulence and resistance genes to other bacteria. Enterococci are considered clinically importance because of the: (i) emergence of vancomycin-resistant enterococci (VRE), (ii) high-level of resistance to multiple antibiotics, (iii) transfer of resistance genes from VRE to *S. aureus*, (iv) presence of different selective pressures that increase the rapid proliferation of VRE, (v) few therapeutic options for disease management, and (vi) limited success of VRE containment measures (Abebe et al., 2014; Gozalan et al., 2015).

VRE become resistant to the glycopeptide family following production of new precursors D-Ala-D-Lac or D-Ala-D-Ser encoding for low-glycopeptide affinity. VREs express eight different phenotypes VanA, VanB, VanD, VanE, VanG, VanL, VanM, and VanN depending on the level of resistance to teicoplanin and vancomycin, as well as the origin and transferability of the resistance genes (Iweriebor et al., 2015; Sujatha and Praharaj, 2012). The operon vanA responsible for the VanA cluster is carried by the highly heterogeneous element Tn1546 generally located on chromosomes or plasmids and transferred among Enterococci (Sujatha and Praharaj., 2012; Gozalan et al., 2015 ; Iweriebor et al., 2015). The phenotype VanA demonstrates an elevated resistance to both teicoplanin and vancomycin, while strains harbouring the vanB phenotype express resistance to vancomycin but are susceptible to teicoplanin. The cluster vanD is characterized by its inducible resistance nature, and by low-level teicoplanin resistance and moderate- to high-level vancomycin resistance. The other phenotypes vanG and VanE express inducible resistance and low-level of resistance to vancomycin (Corso et al., 2007; Gozalan et al., 2015 ; Iweriebor et al., 2015). Among these, the vancomycin-resistance genes vanA and vanB are most commonly involved in VRE infections (Sujatha and Praharaj., 2012; Gozalan et al., 2015 ; Iweriebor et al., 2015).

E. faecium isolates with the VanA phenotype were first detected from clinical cases of VRE infections in Europe in 1986; and were associated with outbreaks in hospitals, particularly in

patients with severe underlying disease or immunocompromised status (Abebe et al., 2014; Corso et al., 2007). In Africa, the first cases of VRE infection were described in South Africa with a 10.9% prevalence of colonized patients in hospitals in 1997 (Gottberg., 2010). In early 2000, VRE colonization and infection became a serious threat when the first case of vancomycin-resistant *S. aureus* harbouring the vanA resistance gene of VRE was reported in clinical isolates in England and France by O'Driscoll et al. (2015) (Crank., 2015). The overuse of glycopeptides and extended-spectrum cephalosporins in hospital settings has probably contributed to the global increase and spread of VRE strains (Shafiyabi et al., 2013). The therapeutic options available for VRE infections are limited.

VRE infections are associated with some risk factors including length of stay (≥7 days), previous antibiotic treatment, prolonged antimicrobial regimens and duration of vancomycin use (≥7 days), stay in intensive care units (ICU) or surgical units, the presence of indwelling urinary or central venous catheter, intra-abdominal or cardio-thoracic surgical procedures, HIV/AIDS, higher severity-of-illness scores and co-morbidities such as renal failure and diabetes (Abebe et al., 2014). VRE have been significantly involved in nosocomial infections and have spread globally (Abebe et al., 2014). In 2013, a high prevalence of VRE has been reported from various clinical samples —these include respiratory samples (70%), blood culture (97%), pus (100%) and urine (100%) — in a tertiary hospital in India. Among these VREs, a 23% rate of high level aminoglycoside resistance (HLAR) and 100% resistance to linezolid were observed (De et al., 2015). *E. faecium* has been shown to express HLAR in combination with ampicillin-resistance (53%) in children with systemic infections in Tanzania (Aamodt et al., 2015). The majority of these infections were hospital-acquired, i.e. infection occurring ≥48 after hospital admission (Aamodt et al., 2015).

However, the foremost clinical relevance of VRE is its colonization in the gastrointestinal tract of humans and animals, representing the reservoirs and main route of exposure for VRE transmission (Corso et al., 2007). The enterococcal colonization among HIV positive patients in a study in Ethiopia was seen to be 91%, with 8 and 90% of VRE and MDR isolates respectively. A report from Argentina showed a 77% prevalence of VRE from rectal swabs of hospitalized patients, with the ICU (47%) and general medicine wards (36%) being the main affected units. A 25% prevalence of VRE infections from paediatric patients and 10% VRE colonization rate of healthcare workers was reported in Egypt (Abebe et al., 2014). Despite a low level of *E. faecium* resistance to linezolid,

vancomycin and teicoplanin from burn patients in China, more than 50% of the enterococcal isolates were multi-drug resistant (Jia et al., 2014). There is insufficient data available on the epidemiology and risk factors of VRE in Africa.

The main concern posed by the VRE is the transmission of the vanA resistance gene to *S. aureus* and the consequent emergence of vancomycin-resistant *S. aureus* described in early 2000 (Crank., 2015). Despite the fact that VRE is considered as a serious pathogen around the world, in South Africa, the threat caused by the VRE is relatively unknown in South Africa as there is a scarcity of information concerning colonization and infections with VRE in both communities and hospitals. Studies are thus needed to provide epidemiological data and improve the understanding of these drug resistant bacteria.

3.2. Methicillin Resistant *Staphylococcus aureus* (MRSA)

The Gram-positive bacterium *S. aureus* is a ubiquitous colonizer of the skin micro-biota and mucosa —anterior nares and axillae. About 20-30% of the human population is generally colonized by *S. aureus* and they are known as asymptomatic carriers. *S. aureus* is also the foremost wound pathogen responsible of both acute and chronic infections, as well as minor to life-threatening infections —including boils, pimples, abscesses, septicemia, meningitis, pneumonia, toxicosis— due to the formation of a biofilm and an arsenal of virulence factors (Kejela et al., 2013). The majority of *S. aureus* strains produce proteases, hemolysins, hyaluronidase, biofilm, collagenase and various toxins such as toxic shock syndrome toxin (*tsst-1*), staphylococcal enterotoxin (*sea, seb, sec, sed, see, sef, seg, seh*), the Panton-Valentine Leukocidin (*PVL*) and staphyloxanthin (Pendleton et al., 2013). Most of these features are located on mobile genetic elements (MGEs) like plasmids, transposons, integrons, genetic islands, insertion sequence and phage-related elements (Stefani et al., 2012).

S. aureus has also an exceptional capacity to acquire and develop resistance to antibiotics (Kejela & Bacha., 2013). Penicillin became ineffective for *S. aureus* infections within 10 years of its introduction due to the acquisition of a plasmid-borne beta-lactamase (Kejela & Bacha., 2013). The beta-lactam antibiotic methicillin, developed in response to penicillin resistance to palliate the failure of the penicillin, lost its activity against *S. aureus* two years after its introduction into clinical practice in 1961. These resistant isolates have been termed methicillin-resistant *S. aureus* (MRSA) or oxacillin-resistant *S. aureus* (ORSA). MRSA is responsible for a number of serious

difficult-to-treat and multidrug resistant infections in humans and generally confer resistance to the whole β-lactam antibiotics family including penicillins, cephalosporins (active against *S. aureus*), carbapenems and beta-lactam inhibitors, following the insertion of a naked DNA fragment called *Staphylococcal Chromosome Cassette mec* (*SCCmec*). The *SCCmec* harbours the mec A gene encoding for a PBP 2a or PBP' with low β-lactam affinity and therefore responsible of methicillin resistance (Stefani S et al., 2012). MRSA can also colonize the skin, anterior nares, nasopharynx, and the rectum without any infections, and disseminate among people directly by person-to-person contact via contaminated hands, skin lesions, nasal discharge or other biological fluids or indirectly via contaminated air, inanimate objects, dust, and food (Kejela & Bacha., 2013). MRSA was first associated with hospital-acquired infections (HA-MRSA) involving several MDR clones like the Brazilian, Iberian, Hungarian, pediatric and New York/Japan clones, which represent the main HA-MRSA (Gordon et al., 2008). Community-acquired MRSA (CA-MRSA) infections were subsequently reported in Australia and America in people without previous risk factors for MRSA colonization and/or infection (Gordon et al., 2008). These infections have progressively emerged and spread around the world and even in health care settings, changing the equilibrium between HA-and CA-MRSA clones (Gordon et al., 2008).

HA-MRSA is generally more resistant than CA-MRSA while the latter is more virulent than HA-MRSA due to the enterotoxin Panton-Valentin leukocidin (PVL) (Gordon et al., 2008). Both strains are present in developed and developing countries with some variations among different regions. In Latin America, a prevalence of 43.8% and 37% of MRSA has been reported in Paraguay and Argentina, respectively (Cabrera et al., 2011). In Africa, data on MRSA infections are relatively limited, with few reports showing high isolation rate in Ethiopia (42.8%), Nigeria (29.6%), Kenya (27.7%), Ivory Cost (16.8%), Morocco (14.4%), Cameroon (72%) and South Africa (52%) (Kejela & Bacha., 2013; Njoungang et al., 2015; World Health Organization., 2014).

The global emergence and/or dissemination of HA- and CA-MRSA, VISA and VRSA has led MRSA being ranked among the principal ESKAPE pathogens involved in human infections worldwide with some variations between regions (Gordon et al., 2008; Monecke et al., 2012). MRSA claims over 25% of *S. aureus* infections in more than a third of EU countries, with prevalence greater than 50% reported from Portugal, Northern Ireland, Great Britain, Greece, France and Spain (Pendleton et al., 2013; Stefani et al., 2012). In other parts of Europe like in

Netherlands, Norway, Denmark and Scandinavia, where strategies for screening and control of MRSA in humans have been successfully implemented, the MRSA prevalence is very low <2% (Pendleton et al., 2013; Stefani et al., 2012). In USA, MRSA alone claims more deaths than HIV and tuberculosis (Klevens., 2007). Human MRSA prevalence varies from 5-10% in Canada to 25-50% in the USA (Klevens., 2007). In low- and middle-income countries (LMIC) where poverty, malnutrition, poor hygienic measures facilitate the emergence and spread of these resistant bacteria, the MRSA prevalence is difficult to determine but appears to be clearly higher than in high-income countries. Reports from Asia showed a prevalence of more than 50% in Malaysia, Taiwan, Nepal, Singapore, Japan and Hong-Kong, a rate of 40% in India, and 24% in Pakistan (Mukhiya et al., 2013; Ray et al., 2011). In China, 82.9% prevalence of MRSA was detected in ICU-acquired pneumonia (ICU-AP) and ventilator-associated pneumonia (VAP) (Zhang et al., 2014).

The glycopeptides are the common first-line treatment for MRSA infections worldwide. However, the selective pressure exerted by the extensive use of these drugs has led to the emergence of strains with reduce susceptibility for vancomycin and are either called vancomycin-intermediate-resistant *S. aureus* (VISA) and vancomycin-resistant *S. aureus* (VRSA). VISA has emerged following fundamental changes in cell wall composition and thickness, “trapping” vancomycin and reducing its permeability in the bacteria. VISA strains are generally resistant to teicoplanin and virtually resistant to all antibacterial drugs (Pendleton et al., 2013). They were first identified in Japan in 1990s and have spread across the world with isolates detected in Europe, Asia and USA (Pendleton et al., 2013). VRSA emerged instead through interspecies transfer of resistance genes from VRE. MDR VRSA strains exhibit both mecA and vanA genes with resistance transferred via the same mechanisms as MRSA and VRE, respectively (Pendleton et al., 2013).

In addition to its implication in human pathogenesis, *S. aureus*, including MRSA are involved in the infections of livestock, wildlife, and domestic animals including bovine mastitis among dairy cattle, lameness in poultry and rabbits. Many studies have identified MRSA and livestock-associated *S. aureus* (LA-MRSA) isolates in humans and animals. Tracy et al., (2013) and Chen (2013) has observed the transfer of LA-MRSA isolates from animal to human due to contact and persistent nasal colonization. LA-MRSA is frequently detected in individuals with direct animal exposure (Chen., 2013; Nicholson et al., 2013; Van et al., 2011). Accordingly, MRSA it is no

longer an exclusive HA-problem which can be solved by infection prevention and containment measures only in hospitals, but requires an all-inclusive approach involving all players of health care settings (Stefani et al., 2012).

In South Africa, the epidemiology of MRSA is quite complex, and the prevalence depends on the geographical location, population and medical practices. Mortality associated with MRSA infections varies from 29% to 63%. A 36% prevalence was recently identified from hospital patients in Guateng during 2012-2013. A laboratory based surveillance mapping of AMR further demonstrated a 46% prevalence of MRSA with rates declining significantly from 53% in 2010 to 40% in 2012, but with more resistance to other classes of antibiotics such as aminoglycosides and fluoroquinolones which may be linked to the mobile genetic elements (Perovic et al., 2015). The study showed the presence of various HA-MRSA clones including ST612/CC8, ST36/CC30, ST239/CC8, ST5/CC5 (Perovic et al., 2015). It is therefore, it is important to determine risk factors that are associated with MRSA infection to reduce MRSA infection and to assist clinicians in choosing appropriate antibiotics.

3.3.Multidrug resistant Gram-negative ESKAPE bacteria

Of the 6 infamous "ESKAPE pathogens, 4 are Gram negative bacteria —*K. pneumoniae, A. baumannii, P. aeruginosa*, and *Enterobacter spp*. They have been associated with three major types of multi-drug resistance namely ESBL-producing *K. pneumonia* and *Enterobacter spp.*, carbapenemase-producing *A. baumannii* and *P. aeruginosa,* and MDR *A. baumannii* and *P. aeruginosa* (Wang et al., 2017; Pendleton et al., 2013; Rice., 2008).

3.3.1. Resistance mechanisms involved in the MDR of Gram-negative ESKAPE bacteria

Diverse biochemical and physiological mechanisms have been associated with the emergence of MDR Gram-negative ESKAPE bacteria. The bacterial production of enzymes hydrolysing antibiotics, particularly β-lactam antibiotics, is the most important mechanism of resistance in ESKAPE Gram-negative bacteria. The β-lactamase enzymes have emerged following chromosomal mutation and acquisition of resistance genes harboured by MGEs like plasmids, integrons, insertion sequences, transposons, genomic islands and bacteriophages (Dantas & Sommer., 2014). These MGEs carrying resistance genes can be transferred vertically within the same population or horizontally among bacteria of the microbial flora through conjugation,

transformation and transduction increasing the complexity and diversity (Dantas & Sommer., 2014).

Two systems have been described for the β-lactamase classification: (i) The Ambler scheme based on the amino-acid sequence (molecular classification) and (ii) The Bush-Medeiros-Jacoby system based on the substrate and inhibitor profiles (functional classification). The molecular classification is commonly used and divides the β-lactamases into 4 different classes (A, B, C and D). The classes A, C and D require serine in their active site to hydrolyse the beta-lactam antibiotics while the class B metallo-enzymes utilize divalent zinc ions for substrate hydrolysis. ESBLs are Ambler class A enzyme hydrolyzing several β-lactam drugs including penicillin, aminopenicillin, 1^{st}, 2^{nd}, 3^{rd} and 4^{th} generation cephalosporins. They are generally inhibited by beta-lactamase inhibitors and inactive against cephamycins and carbapenems (Curcio., 2014).

Numerous ESBL variants have been identified globally and grouped into 3 different ESBL types: Temoneira (TEM), Sulfhyldryl-variable (SHV) and Cefotaximase-München (CTX-M) (Jacoby and Bush, 2010). *K. pneumoniae* has been identified as the foremost ESBL-producer. Other clinically relevant enzymes are Amp-C beta-lactamases and metallo beta-lactamases (MBLs). Class C Amp-C beta-lactamases including FOX and MOX, differ from the ESBLs in their ability to inactivate cephamycins and are unaffected by currently available β-lactam inhibitors. The MBLs are Ambler's class B, enzymes possessing a metal ion on their active site to catalyse the β-lactams. They can inactivate the entire β-lactam family except for aztreonam. The most common MBLs involved in clinical Gram-negative infections are *Klebsiella pneumoniae* carbapenemase (KPC), imipenemase (IMP), Veronera integron encoded metallo-beta-lactamase (VIM), Serratia marcescens enzyme (SME), New-Delhi metallo-beta-lactamase (NDM) and oxacillin-hydrolyzing carbapenemases (OXA-types). They have been identified mainly in *K. pneumonia*, *P. aeruginosa*, *A. baumannii*, *Enterobacter spp*. and *E. coli* (Curcio., 2014).

3.3.2. ESBL-producing *K. pneumoniae*

The rates of ESBL-producing *K. pneumoniae* in various regions of the world suggests a lower resistance level in USA, Canada, Northwest Europe, and increasing resistance in the developing countries of Southeast Europe, Latin America, Asia and Africa (Curcio., 2014). The 2014's WHO report on AMR presented a 45% prevalence of deaths in both Africa and South-East Asia related

to MDR infections. It further confirmed that these diseases were associated with 77%, 50%, 81% and 72% approximately of *K. pneumonia*e resistant to third generation cephalosporins in Africa, the Eastern Mediterranean region, South East Asia and the Western Pacific region respectively, with 4%, 54%, 8%, 8%prevalence of *K. pneumonia*e resistant to carbapenems respectively identified in the same regions (World Health Organization., 2014).

Several authors have highlighted hospital settings as a potential reservoir and source of nosocomial infections in their studies, ESBL-producing *K. pneumoniae* increased from 11.7 to 77.8% in two hospitals in Tunisia from 1999-2005 and in 2010 (Mahrouki et al., 2012). In some Algerian hospitals, Amp-C β-lactamases, mainly DHA-1 and CMY-2, were most commonly observed in *Enterobacter spp.* The National Healthcare Safety Network (NHSN) has recently indicated the implication of drug resistant Gram-negative bacteria in more than 30% of hospital acquired infections (HAI), with a predominance in cases of ventilator-associated pneumonia (47%) and urinary tract infections (45%) in USA in 2010 (Kanerva et al., 2012). The European Centre for Disease Prevention and Control (ECDC) and European Medicines Agency (EMEA), reported 25 000 deaths associated with 7% being MDR *K. pneumoniae*.

Furthermore, Kanerva et al., (2010) reported 6% prevalence of HAI resulting in 3.2% of deaths due to *K. pneumoniae, Acinetobacter spp., P. aeruginosa* and *Enterobacter spp.* in Finland (Kanerva et al., 2012). Reports from Egypt showed a 76% prevalence of ESBL-producing *K. pneumoniae* in bloodstream infections (BSIs) (Saied & Elkholy ., 2011). In addition, carbapenem resistance has been identified in nosocomial ESBL-producing *K. Pneumoniae.* A 21.6% prevalence of ESBL-producing-*K. Pneumonia* and 9.3% carbapenemase-producing-*K. pneumoniae* have been reported from various clinical samples in Nigeria (Motayo et al., 2013).

A national sentinel surveillance study investigating *K. pneumoniae* in 13 academic centres serving the public health sector in five South African provinces from mid-2010 to mid-2012 revealed that the prevalence of ESBL-producing *K. pneumoniae* among bacteraemic patients was 68.7% with isolates showing high resistance to third and fourth generation cephalosporins, aminoglycosides and fluoroquinolones (Perovic et al., 2014). Likewise, another South African study revealed that 60% of referral *Enterobacteriaceae* were *K. pneumoniae* during a four-year period (2012-2015) (Perovic et al., 2016). The study showed that among these, 68% of *K. pneumoniae* were carbapenemase-producers with bla_{NDM} being the main carbapenemase gene identified (Perovic et

al., 2016). These reports thus point to rapid and global dissemination of resistant *K. pneumoniae* and require the urgent implementation of infection prevention and control measures in South African settings.

3.3.3. Multi-drug resistant *A. baumannii*

A. baumannii is another worldwide public health threat which hampers the control of infectious diseases. The latter epitomizes the successful ability of pathogens to escape antibiotics and deserves close attention. It exhibits resistance mechanisms to all available antibiotic families as well as an exceptional ability to acquire new resistance determinants (Andriamanantena et al., 2010; Chan et al., 2014; Perez et al., 2007). There is evidence of the increasing emergence of MDR *A. baumannii* (MDRAB) and extensively drug-resistant *A. baumannii* (XDRAB) resistant to the entire β-lactam family including broad-spectrum cephalosporins and carbapenems, aminoglycosides, fluoroquinolones and, oxazolidinones (Andriamanantena et al., 2010). β-lactam resistance is of great concern. Several strains harboring TEM-1 beta-lactamase, a variety of ESBLs, PER-1, IMP-1, IMP-2, IMP-4, IMP-5, IMP-6, IMP-11, OXA-23, OXA-24, OXA-58, and OXA-51, and chromosomally encoded AmpC genes have been detected in *A. baumannii* across the world (Chan et al., 2014; Perez et al., 2007).

A report from India revealed a 25% prevalence of MBL-producing *A. baumannii*, 16% of which co-produced AmpC and 9% of which co-produced ESBL in combination with MBL in burn patients (Gupta et al., 2013). The prevalence of XDR-AB from clinical samples escalated from 15% in 2005 to 41% in 2010 in Taiwan (Chan et al., 2014). A resistance rate of 100% MDRAB was reported in clinical samples in Pakistan where the main units affected were the neonatal intensive care unit (NICU) (42.85%), medical ICU (19.78%) and the out-patient department (9.8%) (Begum et al., 2013). Carbapenem-resistant *A. baumannii* (CRAB) was involved in 44% of infections in surgical units in Madagascar and 21.05 % of imipenem resistant *A. baumannii* was identified in intensive care unit in Cameroon (Andriamanantena et al., 2010; Ebongue et al., 2015). Class D ESBLs, specifically OXA-1 predominated in Tunisia and ranged from 3.3 to 93.3%, OXA-48 has been described by Andriamanantema et al., (2010) in the rectal swabs from hospitalized patients in Libya. In South Africa, a study reported a 10.9% prevalence of *A. baumannii* although none were carbapenem-resistant, in bloodstream infections in neonates hospitalized in a tertiary care hospital (Ballot et al., 2012). However, Ramsamy et al. (2013) reported that *A. baumannii*

was detected in 46% of lower respiratory, 23% of surgical site and 30.7% of bloodstream infections among critically injured patients over a 12-month period in KwaZulu-Natal, South Africa. Of these, 10% were carbapenem-resistant and 27% were multi-drug resistant (Ramsamy, Muckart, & Han, 2013) confirming the seriousness of infections caused by these isolates.

3.3.4. Metallo-beta-lactamase producing *P. aeruginosa*

P. aeruginosa is one of the most frequent Gram-negative ESKAPE bacteria isolated in hospital settings. It has become resistant to carbapenems because of the production of MBL enzymes. MBLs are broad–spectrum enzymes hydrolysing most β-lactam antibiotics, except monobactams, and are not in-activated by commonly used β-lactamase inhibitors such as tazobactam, sulbactam and clavulanic acid (Curcio., 2014). MBL-producing *P. aeruginosa* in ICUs is of concern. Their emergence is associated with the misuse and overuse of carbapenems while its persistence is due to sub-optimal infection prevention and control measures (Kali et al., 2013; Uddin et al., 2013). MDR *P. aeruginosa* (MDR-PA) is a major cause of nosocomial infections particularly ventilator-associated pneumonia (VAP) in ICUs (Rizek, Fu et al., 2014; Sawa et al., 2014). The treatment options are limited constrained to a small number of antibiotics such as the fluoroquinolones and colistin. *P. aeruginosa* producing MBL was reported in surgical tertiary care hospitals in Saudi Arabia where the rate of carbapenems-producing *P. aeruginosa* was 33%, with 27% in young and middle-aged adults (19-65 years) with wound infections (Chan et al., 2014). The prevalence of MBL producing *P. aeruginosa* was 27% in Egypt in 2009, with 41.7% and 10.4% of these isolates carrying OXA-10 and VEB-1 genes respectively. In 2013, 37.03% carbapenemase- producing *P. aeruginosa* and 32.3% MBL-producing *P. aeruginosa* was described (Zafer et al., 2014). In India, a prevalence of 22.4% *P. aeruginosa* producing MBLs was reported in tertiary care hospitals (Kali et al., 2013; Slavcovici A et al., 2015).

In South Africa, 57% of *P. aeruginosa* involved bacteraemia in an academic hospital were nosocomially-acquired and among these, 15% were MDR isolates (Perovic et al., 2008). Similarly, an outbreak due to MDR *P. aeruginosa* occurred in immunocompromised patients in a haematology intensive care unit of a South African tertiary academic hospital with an 80% case fatality rate (Mudau et al., 2013). The study revealed that all isolates were resistant to all antibiotics except colistin, and concluded that poor infection prevention and control measures may have contributed to the transmission to these resistant strains (Mudau et al., 2013).

3.3.5. ESBL-producing Enterobacter spp.

Enterobacter spp. is a Gram-negative bacillus frequently observed in normal flora of the gastrointestinal tract of 40 to 80% of humans. It is also one of the main agents causing several resistant hospital-acquired infections and has been identified in high risk wards such as the ICU and neonatal unit (Qama et al., 2014). It is frequently isolated in different site infections including blood stream, abdomen, lower respiratory tract, urinary tract, meningeal, eye, bone, and surgical infections. It has been involved in the dissemination of MDR due to mobile genetic elements (transposons, plasmids, integrons) and has become a major threat of public health around the world (Khajuria et al., 2014) due to high level of resistance to various antibiotics among the family of β-lactams particularly. The principal resistance genes described in *Enterobacter spp.* were *bla*CTX-M, *bla*NDM, *bla*IMP and *bla*VIM and have been reported worldwide. For example, a 68.57% prevalence of carbapenemase-producing *Enterobacter spp.* was reported in India (Khajuria et al., 2014). Singh et al. (2015), revealed a high prevalence (72%) of carbapenemase-producing *E. cloacae* harbouring $bla_{\text{OXA-48}}$, bla_{VIM}, and bla_{IMP} in Eastern Cape hospitals in South Africa. The authors concluded that MBL-producing *Enterobacter spp.* are prevalent in South Africa and that horizontal transmission of resistant bacteria in healthcare settings is an important contributing factor (Singh et al., 2015). Similarly, over the 2012-2015 period, a study investigating suspected carbapenemase-producing *Enterobacteriaceae* (CREs) revealed that 12% were *Enterobacter spp.* and blaIMP was the predominant carbapenemase gene (Singh-Moodley & Perovic, 2016). Controlling resistant bacteria in South Africa require intensive efforts.

4. Concluding remarks

Antimicrobial resistance is a major problem and MDR infections are a serious clinical challenge globally. MDR infections considerably increase therapeutic cost as well as morbidity and mortality in both developed and developing countries (World Health Organization., 2014). The treatment options are limited to a small number of antibiotics due to the diverse mechanisms involved in the resistance. The situation is exacerbated by the emergence of MDR bacteria from animals and the environment with their subsequent spread to humans in communities and hospitals (Kali et al., 2013).

Containing ABR in general and ABR in ESKAPE bacteria in particular, is an urgent priority in South Africa. Understanding the resistance mechanisms, resistance genes, virulence factors, risk factors and transmission routes of drug resistant ESKAPE pathogens in South Africa, may serve

as evidence for policy-makers to implement strategies to contain ABR in the country (Basseti et al. 2013).

IV. Research aims and objectives of the study

1. Overarching aim

The aim of this study was to determine the risk factors, prevalence, phenotypic and genotypic profiles including but not limited to clonal relatedness, genetic diversity and resistance mechanisms associated with ESKAPE bacteria from carriage and clinical isolates from patients in a rural, district and tertiary urban hospital within the public health sector in uMgungundlovu District, Kwazulu-Natal, South Africa.

2. Specific objectives

More specifically, the study objectives are:

1. To isolate resistant ESKAPE bacteria from carriage samples including nasal and rectal swabs of in-patients, using selective media and relevant biochemical tests.
2. To collect resistant ESKAPE bacteria from different clinical samples routinely processed by the National Health Laboratories affiliated with the hospitals in question identified by the Vitek 2 system.
3. To establish the risk factors associated with colonization and infection by resistant ESKAPE bacteria including but not limited to socio-demographic and health system factors such as age, gender, profession, date of admission, date of discharge, duration of hospitalization, ward type, specimens source, diagnosis, co-morbidity and invasive procedures using a structured questionnaire.
4. To determine the antibiotics resistance profiles of ESKAPE bacteria by performing antibiotic susceptibility testing through by broth micro-dilution and the Vitek 2 automated method in carriage and clinical isolates respectively.
5. To undertake conventional, multiplex and real-time PCR as well as whole genome sequencing of isolates to delineate:
    - Antibiotic resistance genes
    - Virulence genes
    - Mobile genetic elements such as plasmids, transposons and integrons associated with resistance and virulence

- MLST profiles

6. To additionally undertake a systematic review and meta-analysis to comprehensively analyze the published literature on the clinical and economic implications of ABR in developing countries.

3. Study design and methodology

This was an observational study that identified ESKAPE bacteria from carriage (nasal and rectal swabs) and clinical specimens of hospitalized patients between May and July 2017, in a rural, district, and an urban tertiary hospital in uMgungundlovu, South Africa. Risk factors for the carriage of ESKAPE bacteria were ascertained using chi-square test and univariate and multivariate logistic regression.

Minimum inhibitory concentration (MICs) were determined via micro-broth dilution, and the ROSCO DIAGNOSTICA (Taastrup, Denmark) kits was used to identify ESBL, MBL, AmpC, VRE, MRSA and GISA isolates. Conventional, multiplex and real-time PCR were used to determine the presence of antibiotic resistance genes including *bla*$_{\text{CTX-M}}$ (group 1, 2, 9, 8/25), *bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{OXA-1-like}}$, *bla*$_{\text{GES}}$, *bla*$_{\text{IMP}}$, *bla*$_{\text{OXA-48}}$, *bla*$_{\text{Amp-C}}$, VanA, VanA-1, VanB, VanC-1/2, associated with the relevant ESKAPE bacteria. Representative isolates underwent genomic fingerprinting via ERIC and REP-PCR and whole genome sequencing using an Illumina Miseq machine with multiplex paired-end libraries (2×300 bp) prepared from the genomic DNA. Assembling of the raw read was performed with the QIAGEN CLC Genomic Workbench and SPAdes, annotation was performed with NCBI PGAP, RAST, ARG-ANNOT and ResFinder to identify antibiotic resistance genes. VirulenceFinder and PlasmidFinder were used to determine virulence factors and plasmid elements, respectively.

4. Thesis outline

The thesis is in the form of published journal articles and unpublished manuscripts in six chapters as follows:

- **Chapter 2. Article I:** Extended Spectrum β-lactamase Mediated Resistance in Carriage and Clinical Gram-Negative ESKAPE Bacteria Isolated in uMgungundlovu District, South Africa: A Comparative Study Between an Urban Tertiary and a Rural, District Hospital in uMgungundlovu District, South Africa. This original research paper has been submitted to PloS One and reports on the risk factors, prevalence, phenotypic profiles, genetic diversity and resistance mechanisms associated with ESBLs in Gram-negative ESKAPE bacteria from

faecal carriage and clinical samples obtained from hospitalized patients in the public health sector in KwaZulu-Natal, South Africa. It addresses objectives One, Two, Three, Four and Five.

- **Chapter 3. Article II:** Faecal Carriage of VanC-1-Mediated Vancomycin-Resistant *Enterococcus faecium* and *Enterococcus faecalis* in Hospitalized Patients in uMgungundlovu District, KwaZulu-Natal, South Africa. This original research paper has been submitted to Frontiers in Microbiology and reports on the risk factors, phenotypic profiles, genetic diversity and resistance mechanisms associated with VRE carriage among hospitalized patients in the public health sector in KwaZulu-Natal, South Africa. It addresses objective One, Two, Three, Four, and Five.
- **Chapter 4. Article III:** Whole Genome Sequencing of Methicillin-susceptible *Staphylococcus aureus* (MSSA) and methicillin resistant *Staphylococcus haemolyticus* Isolated from Hospitalized Patients in the uMgungundlovu District, South Africa. This original research paper will be submitted to Frontiers in Microbiology and reports on the resistance and virulence mechanisms as well as clonal relatedness of circulating *S. aureus* and *S. haemolyticus* isolates from hospitalized patients in the uMgungundlovu District, South Africa using whole genome sequencing. It addresses objective One, Four and Five.
- **Chapter 5. Article IV:** Clinical and economic impact of antimicrobial resistance in developing countries: A systematic review and meta-analysis. This paper has been accepted for publication, pending corrections by PloSOne. This systematic review analysed the published literature on the clinical and economic implications of AMR in developing countries by highlighting the main bacteria incriminated and comparing the current prevalence of AMR among World Health Organization (WHO) regions and within the World Bank classification of countries and addresses objective Six.
- **Chapter 6. Conclusion:** This chapter presents the extent to which the overarching aim was accomplished by highlighting the findings of each objective. It also delineates the limitations, recommendations and the significance of the study.

CHAPTER II.

ARTICLE 1. Extended Spectrum Beta-lactamase Mediated Resistance in Carriage and Clinical Gram-Negative ESKAPE Bacteria: A Comparative Study of a Tertiary, Urban and a Rural, District Hospital in uMgungundlovu District, South Africa[1]

Author contributions:

- Raspail Carrel Founou, as the principal investigator, co-conceptualized the study, undertook sample collection, laboratory and statistical analyses, prepared tables and figures, and drafted the manuscript.
- Luria Leslie Founou, undertook sample collection, laboratory analysis, vetting of the results, and reviewed the manuscript.
- Sabiha Yusuf Essack, as the principal supervisor, co-conceptualized the study, contributed to vetting of the results and undertook critical revision of the manuscript

Objectives met: This paper addresses objectives One, Two, Three, Four and Five.

[1] This paper has been submitted to PloS One and is currently under review.

Extended Spectrum Beta-lactamase Mediated Resistance in Carriage and Clinical Gram-Negative ESKAPE Bacteria: A Comparative Study of a Tertiary, Urban and a Rural, District Hospital in uMgungundlovu District, South Africa

Raspail Carrel Founou[1,2*], Luria Leslie Founou[1,3], Sabiha Yusuf Essack[1]

[1]Antimicrobial Research Unit, School of Health Sciences, University of KwaZulu-Natal, Durban, South Africa

[2]Department of Clinical Microbiology, Centre of Expertise and Biological Diagnostic of Cameroon, Yaoundé, Cameroon (CEDBCAM)

[3]Department of Food safety and Environmental Microbiology, Centre of Expertise and Biological Diagnostic of Cameroon, Yaoundé, Cameroon (CEDBCAM)

***Corresponding author**

Raspail Carrel Founou

Antimicrobial Research Unit

College of Health Sciences

University of KwaZulu-Natal

Durban, 4000

South Africa

Email: czangue@yahoo.fr

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Abstract

Background: Gram-negative ESKAPE bacteria are increasingly implicated in several difficult-to-treat infections in developed and developing countries. They are listed by the World Health Organization as resistant bacteria of critical priority in research.

Objectives: To determine the risk factors, prevalence, phenotypic profiles and genetic diversity of extended-spectrum β-lactamase (ESBL)-producing multi-drug resistant (MDR) Gram-negative ESKAPE bacteria in faecal carriage and clinical samples from patients in an urban, tertiary and a rural, district hospital in uMgungundlovu District, KwaZulu-Natal, South Africa.

Methods: Gram-negative ESKAPE bacteria were screened for ESBL production on MacConkey agar supplemented with 2µl of cefotaxime and confirmed using ROSCO kits. Minimum inhibitory concentrations were determined, and real time and multiplex PCR were used to ascertain the presence of $bla_{CTX\text{-}M}$ group-1-2-9, $bla_{CTX\text{-}M}$ group 8/25, bla_{SHV}, bla_{TEM}, $bla_{OXA\text{-}1\text{-}like}$, bla_{KPC}, bla_{VIM}, bla_{IMP}, bla_{GES} and AmpC genes. Genomic fingerprinting was also performed by ERIC-PCR.

Results: Overall prevalence of carriage was 37.21% (16/43), 42.31% (11/26) and 57.14% (4/7) at admission, after 48 h and at discharge respectively. The prevalence of MDR ESKAPE bacteria in faecal carriage (46%) was higher than clinical samples (28%). Colonization was mainly associated with referral from district to tertiary hospital with high statistical significance (OR: 14.40, 95% CI 0.98-210.84). $bla_{CTX\text{-}M\text{-}group\text{-}9}$, $bla_{CTX\text{-}M\text{-}group\text{-}1}$ and bla_{SHV} were the main resistance genes identified. Several patients carried more than two different isolates. A *K. pneumoniae* (K1) clone was circulating within wards and between hospitals.

Conclusions: The wide dissemination of ESBL-producing MDR Gram-negative ESKAPE bacteria in hospitals necessitates improvements in routine screening and reinforcement of infection, prevention and control measures.

Introduction

The selective pressure exerted by the use of antibiotics and aggravated by the dearth of new active substances in the current therapeutic pipeline has led to a considerable increase in antibiotic resistance (ABR) worldwide [1,2]. A small group of bacteria, i.e., *Enterococcus spp.*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter spp*., termed "ESKAPE" due to their ability to escape the activity of and develop high levels of resistance to multiple antibiotics, have recently gained global attention [3-5]. Of the six infamous ESKAPE pathogens, the four Gram-negative bacteria, ie., *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *Enterobacter spp*., have been associated with four major types of multi-drug resistance (MDR), namely extended-spectrum β-lactamase (ESBL)-producing *K. Pneumoniae* and *Enterobacter spp.*, carbapenemase-producing *A. baumannii* and metallo-β-lactamase producing *P. aeruginosa* (MBL-PA) which limit therapeutic options and negatively affect clinical outcome [3-6]. Gram-negative ESKAPE bacteria are increasingly implicated in several difficult-to-treat infections in both developed and developing countries and were recently listed by the World Health Organization (WHO) as resistant bacteria of critical priority in research [3, 7-10].

Several resistance genes have been associated with the emergence of MDR Gram-negative ESKAPE bacteria globally. The bacterial production of enzyme hydrolysing antibiotics, particularly beta-lactam antibiotics, is the most common mechanism of resistance in Gram-negative ESKAPE. Beta-lactamase enzymes have emerged following chromosomal mutation and acquisition of resistance genes carried on diverse mobile genetic elements (MGEs) such as plasmids, integrons, insertion sequences, transposons, genomic islands and bacteriophages [10].

The aim of this study was to compare the prevalence, risk factors, phenotypic and genotypic profiles of ESBL-producing MDR Gram-negative ESKAPE bacteria in faecal carriage and clinical samples obtained from patients in an urban, tertiary and a rural, district hospital in uMgungundlovu District, South Africa.

Materials and methods

Ethical approval

Ethical approval was obtained from the Biomedical Research Ethics committee (BREC) (**No. BF512/16**, **sub-study of BCA444/16**). Permission to conduct the research was also granted from the Department of Health, uMgungundlovu District and hospital managers.

Study population and settings

This study was conducted in a rural, district and urban, tertiary hospital during two months from May 2017 to June 2017 in uMgungundlovu district, South Africa, encoded for ethical reasons as H1 and H2, respectively. The district hospital (H1) represents the smallest level of hospital and provides four services including obstetrics and gynaecology, paediatrics and child health, general surgery and general medicine with 141 beds. In contrast, the tertiary hospital (H2) offers several specialties, receives referral patients according to a nationally agreed referral plan and has approximately 505 beds. These two public hospitals belong to the Department of Health of South Africa.

Patient enrolment and questionnaire survey

Oral and written informed consent were obtained from all study participants after explanation of the procedure and purpose of the study. Patient information was gleaned from questionnaires completed by patients and data from patient records. Information was codified prior to analysis to maintain confidentiality.

Sample collection

The sample collection took place in both surgical and general medical wards during a two-month period, one month at each of the hospitals. Rectal swabs that were collected aseptically with Amies swabs from all admitted in-patients >18 years old, at three-time points, at admission, after 48h and at discharge (whenever possible) formed the carriage sample. Isolates routinely processed in the microbiological laboratory during the sampling period formed the clinical sample.

Labor atory analysis

Identification of Gram-negative ESKAPE bacteria

During the sample collection, 76 rectal swabs were collected and directly cultured onto MacConkey agar with and without cefotaxime (2 mg/L). After incubation for 18-24h at 37°C, each morphotype growing on MacConkey with cefotaxime (MCA+CTX) was subjected to Gram

staining, catalase and oxidase tests, followed by biochemical identification with API 20E (bioMerieux, Marcy l'Etoile, France) according to the manufacturer's instructions. Pure colonies were stored into Tryptone Soya Broth supplemented with 30% glycerol at −20°C for future use. Clinical isolates were identified via Vitek® 2 System (bioMérieux, Marcy l'Etoile, France) using the GN card according to the manufacturer's instructions.

Phenotypic screening

All growing colonies were phenotypically screened for ESBL, AmpC, KPC, MBL, and OXA-48 production using ROSCO DIAGNOSTICA (Taastrup, Denmark) using 0.5 McFarland on Mueller-Hinton agar according to the manufacturer's instructions.

Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) were determined via micro-broth dilution for all confirmed ESBLs and/or AmpCs, and/or MBL producers. Ampicillin, cefoxitin, cefuroxime, cefotaxime, ceftriaxone, imipenem, meropenem, amikacin, gentamicin, trimethoprim, ciprofloxacin, moxifloxacin, nitrofurantoin, tetracycline, tigecycline and colistin constituted the antibiotic panel for carriage isolates. The Vitek® 2 System and Vitek® 2 Gram negative Susceptibility card (AST-N255) were used to determine the MICs of clinical isolates. The results of MIC tests were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints [11]. *E. coli* ATCC 25922, *K. pneumoniae* ATCC 700603 and *K. pneumoniae* ATCC 51503 were used as controls.

Genomic characterization

Genomic extraction

Genomic DNA of selected strains were extracted using GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. Genomic DNA was stored at -20°C for future use.

Multiplex polymerase chain reaction (M-PCR)

The isolates were subjected to molecular testing using conventional and M-PCR assays to identify $bla_{CTX\text{-}M}$ group 8/25 ($bla_{CTX\text{-}M\text{-}gp8/25}$), bla_{SHV}, bla_{TEM}, $bla_{OXA\text{-}1\text{-}like}$, $bla_{OXA\text{-}48}$, bla_{KPC}, bla_{VIM}, bla_{IMP} and bla_{GES} genes as previously described by Dallenne et al. (2010) [12] (S1 Table).

Real-time polymerase chain reaction (RT-PCR)

RT-PCR was performed to ascertain bla_{AmpC}, $bla_{CTX\text{-}M\text{-}group\text{-}1}$ ($bla_{CTX\text{-}M\text{-}gp1}$), $bla_{CTX\text{-}M\text{-}group\text{-}2}$ ($bla_{CTX\text{-}M\text{-}gp2}$) and $bla_{CTX\text{-}M\text{-}group\text{-}9}$ ($bla_{CTX\text{-}M\text{-}gp9}$) resistance genes. Results were analysed on a programmable automate QuantStudio5™ (Applied Biosystems, CA, USA) using the Taqman Universal Master Mix 2× (Applied Biosystems, CA, USA) and ready-made assays (Thermo Scientific, CA, USA). Thermal temperature running conditions were as follows: UNG activation at 50 °C for 2 min, initial denaturation at 95 °C for 10 min, 30 cycles of denaturation at 95 °C for 10 s, annealing/extension at 60 °C for 1 min and a final extension at 60°C for 30 s. The results were interpreted with QuantStudio™ design and analysis software version 1.4 (Applied Biosystems, CA, USA).

Genomic fingerprinting

Enterobacterial Repetitive Intergenic Consensus-Polymerase Chain Reaction (ERIC-PCR) was used to establish the link of different strains within and between hospitals, wards, carriage and clinical samples as well as across sampling points. The primers ERIC1 5'ATGTAAGCTCCTGGGGATTCAC3' and ERIC2 5'AAGTAAGTGACTGGGGTGAGCG3'[13] were used and PCR reactions were carried out in a 10 µl volume containing 5 µl of Dream*Taq* Green Polymerase Master Mix 2X (Thermo Fisher Scientific, Johannesburg, South Africa), 2.8 µl of nuclease free water, 0.1 µl of each primer (100 µM), and 2 µl of DNA template. The reactions were carried out with the following cycling conditions: initial denaturation at 94°C for 3 min, 30 cycles consisting of a denaturation step at 94°C for 30 s, annealing at 50°C for 1 min, extension at 65°C for 8 min, a final extension step at 65°C for 16 min and final storage at 4°C. The generated amplicons were resolved by horizontal electrophoresis on 1.5% (wt/vol) Tris-Borate-EDTA (Merck, Germany) agarose gels together with the Quick-load®1-kb (Biolabs, New England) and run in an electric field of 110 V for 2 h 30 min. Electrophoresis gels were visualized by a UV light trans-illuminator, images were captured using a Gel Doc™ XR+ system (BioRad Laboratories, CA, Foster City, USA) and analysed by Image Lab™ Software (version 4.0, BioRad Laboratories, CA, Foster City, USA).

ERIC-PCR profiles were normalized using the Quick-load®1-kb (Biolabs, New England) DNA molecular weight marker as external standard. For cluster analysis, data were exported to Bionumerics software (version 7.6, Applied Maths, TX, USA). Strains were allocated to different clusters by calculating the similarity coefficient from the homology matrix using the Jaccard

method. Dendrograms were constructed based on the average linkages of the matrix and using the Unweighted Pair-Group Method (UPGMA). Optimization and band tolerance were set at 1% (version 7.6, Applied Maths, TX, USA) and 80% similarity cut-off was used to define clusters.

Data analysis

Data was coded and entered on an Excel spreadsheet (Microsoft Office 2016) and analysed using STATA (version 14.0, STATA Corporation, TX, USA). Risk factors for ESBL-producing, MDR Gram-negative ESKAPE colonization were ascertained by univariate and multivariate logistic regression analyses. Prevalence of MDR carriage was compared between categories (viz. hospital, ward and time-point) using the chi-square and Fisher's exact test as appropriate. A p-value < 0.05 was regarded as statistically significant.

Results

Population characteristics

A total of 75 hospitalized patients were recruited, amongst whom, 45 (60%) agreed to participate, answered the questionnaire and provided samples. Overall, males were more colonized than females at all time-points as were patients referred from another hospital (Table 1). Patients in tertiary hospital were more likely to be colonized by MDR ESKAPE bacteria at admission (50%) and discharge (67%) than those of the district hospital (Table 1). Further, patients admitted in the general medical ward were more colonized in the district hospital at all time-points whereas in the tertiary hospital, the prevalence in the surgical ward was higher at admission and discharge.

Table 1. Fecal carriage of resistant Gram-negative ESKAPE bacteria isolated from hospitalized patients in a rural district and a tertiary urban hospital. Out of the 45 patients enrolled, some refused rectal sampling, some were discharged or transferred after 48 hours, while other could not be sampled due to their condition, leading to variability in number.

Variables	District Rural Hospital n=27						Tertiary Urban Hospital n=18					
	Admission, n (%)	p	After 48 hours, n (%)	p	At discharge, n (%)	p	Admission, n (%)	p	After 48 hours, n (%)	p	At discharge, n (%)	P
Socio-demographic factors												
Gender												
Female	3/14 (21.4)	0.333	3/9 (33)	0.229	2/4 (50)	….	2/5 (40)	0.590	1/2 (50)	0.571	1/2 (50)	0.386
Male	5/13 (38.4)		5/8 (63)		/		6/11 (55)		2/7 (29)		1/1 (100)	
Clinical history												
Previous hospitalization (within one year)												
Yes	1/8 (13)	0.206	0/5 (0)	**0.012**	0/1	0.248	1/5 (20)	0.106	1/4 (25)	0.635	1/1 (100)	0.386
No	7/19 (37)		8/12 (67)		2/3 (66.67)		7/11 (64)		2/5 (40)		1/2 (50)	
Antibiotic use (during hospital stay)												
Yes	3/18 (27.78)	0.766	2/6 (33.33)	0.402	1/2 (50)	1.000	1/3 (33)	0.522	1/3 (33)	1.000	1/1 (100)	0.386
No	3/9 (33.33)		6/11 (54.55)		1/2 (50)		7/13 (54)		2/6 (33)		1/2 (50)	
Underlying disease (co-morbidity)												
None	5/22 (23)	0.132	7/15 (47)	0.365	2/4 (50)	…	8/15 (53)	0.302	3/8 (38)	0.453	2/2 (100)	0.083
Respiratory disease	1/1 (100)		/		/		/		/		/	
HIV	1/1 (100)		/		/		0/1 (0)		0/1 (0)		0/1	
HIV/Tuberculosis	0/1		0/1 (0)		/		/		/		/	
Hypoglycaemia	0/1 (0)		/		/		/		/		/	
Diabetes	1/1 (100)		1/1 (100)		/		/		/		/	
Transferred from another hospital												
Yes	1/1 (100)	0.116	/	…	/	….	6/8 (75)	**0.046**	2/4 (50)	0.343	1/1 (100)	0.386
No	7/26 (27)		8/17 (47)		2/4 (50)		2/8 (25)		1/5 (20)		1/2 (50)	
Diagnostic at admission												
Acute indisposition	2/3 (67)	0.273	2/3 (67)	0.759	1/2 (50)	0.368	1/1 (100)	0.370	/	0.441		0.083
Acute psychosis	1/1 (100)		/		/		/		/			
Malignancy	1/5 (20)		/		1/1 (100)		0/2 (0)		0/2 (0)			
Diabetes	0/1 (0)		/		/		/		/			
Heart disease	/		/		/		0/1 (0)		0/1 (0)			
Respiratory disease	1/2 (50)		1/1 (100)		/		1/2 (50)		1/2 (50)			
Gastro-intestinal disease	1/1 (100)		/		/		/		/			
Chronic disorders	0/4 (0)		1/3 (33)		/		0/1 (0)		0/1 (0)		0/1	
Wound	1/5 (20)		1/2 (50)		/		1/1 (100)		/			
Invasive surgery	1/5 (20)		1/4 (25)		0/1 (0)		5/8 (63)		2/3 (67)		2/2 (100)	
Hospital ward												
Medicine	6/15 (40)	0.187	5/9 (56)	0.457	1/2 (50)	1.000	2/5 (40)	0.590	2/5 (40)	0.635	1/2 (50)	0.386
Surgery	2/12 (17)		3/8 (38)		1/2 (50)		6/11 (55)		1/4 (25)		1/1 (100)	

Risk factors for MDR Gram-negative ESKAPE bacteria carriage

Patients at the tertiary hospital had an increased risk of being colonized by resistant bacteria at admission (OR=9, 95% CI 0.93-86.52) and discharge (OR=4; 95% CI 0.21-75.67, Table 2). Similarly, the gender (male) and Type of hospital wards (Surgical ward) increase the odds of being colonized at admission and after 48h in both district and tertiary hospitals (Table 2).

The multivariate analysis further confirmed that referral from district to tertiary hospital was significantly associated with MDR Gram-negative ESKAPE bacteria at admission (OR=14.40, 95% CI 1. 0.98-210.84) and after 48h (OR=5.72, 95% CI 0.17-189.00) as was the gender for these two time-points in the district hospital (Table 3).

Table 2. Risk factors associated with fecal carriage of ESBL-producing Gram-negative ESKAPE bacteria (Univariate Logistic regression)

Variables	District hospital		Tertiary hospital	
	Admission	After 48 hours	Admission	After 48 hours
	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)
Gender (F or M)	**2.29** (0.42-12.50)	**3.33** (0.45-24.44)	**1.8** (0.21-15.40)	**0.4** (0.16-10.02)
Antibiotic use (Yes or No)	1.3 (0.23-7.32)	0.42 (0.05-3.31)	0.43 (0.03-5.98)	1
Co-morbidity	1.05 (0.61-1.83)	1.03 (0.48-2.24)	1.05 (0.61-1.83)	1.03 (0.48-2.24)
Previous hospitalization	0.24 (0.02-2.40)	1	0.14 (0.01-1.76)	0.5 (0.03-8.95)
Transferred from another hospital	**1**	1	**9** (0.93-86.52)	**4** (0.21-75.67)
Ward (Medicine or Surgery)	**0.3** (0.05-1.88)	**0.48** (0.07-3.35)	**1.8** (0.21-15.40)	**0.5** (0.03-8.95)

Table 3. Predictive risk factors associated with fecal carriage of MDR Gram-negative ESKAPE bacteria in a district and tertiary hospital (Multivariate Logistic regression)

Variables	District Hospital		Tertiary Hospital	
	Admission; OR (95% CI)	After 48 hours; OR (95% CI)	Admission; OR (95% CI)	After 48 hours; OR (95% CI)
Gender (F or M)	**7.12** (0.54-93.75)	**3.61** (0.34-37.83)	**1.21** (0.09-15.61)	0.29 (0.005-16.27)
Antibiotic use (Yes or No)	**4.73** (0.28-80.57)	0.93 (0.08-11.40)	0.26 **(**0.007-9.01)	0.41 (0.009-17.46)
Transferred from another hospital	1	1	**14.40** (0.98-210.84)	**5.72** (0.17-189.00)
Hospital Ward (Medicine or Surgery)	0.08 (0.004-1.39)	0.42 (0.05-3.81)	**2.09** (0.10-42.29)	**1.14** (0.03-49.14)

Prevalence of MDR Gram-negative ESKAPE bacteria

Out of 159 non-duplicates resistant Gram-negative bacteria isolated, 31 (19.50%) were MDR Gram-negative ESKAPE bacteria of which 21. (67.74%) were clinical isolates (11 tissue, 2 bloods, 3 urines, 3 intravenous catheters, 2 sputum) obtained from hospitalized patients (15 males and 6 females) with symptomatic infections in different departments (medicine, surgery, intensive care units). Ten (32.26%) MDR Gram-negative ESKAPE bacteria were isolated from rectal swab of in-patients (6 females, 4 males). In the district hospital, seven isolates were identified, five (71.43%) in carriage and two (28.57%) in clinical samples. The main pathogens identified were *K. pneumoniae* and *E. cloacae* in carriage and clinical isolates respectively. In contrast, in the tertiary hospital, five (20.83%) isolates were identified in carriage and 19 (79.16%) in clinical samples. The main pathogen identified in carriage was *E. aerogenes* while *P. aeruginosa* and *A. baumannii* were the main clinical isolates.

Antimicrobial resistance profiles

In the tertiary hospital, especially in the medical ward, isolates expressed high resistance to ampicillin (100%), cefuroxime (100%), cefotaxime (100%), ceftazidime (100%), gentamicin (100%), amikacin (100%), ciprofloxacin (100%) and nitrofurantoin (100%) in both carriage and clinical samples (Table 4). In contrast, in the surgical ward in the same hospital, clinical samples showed lower resistance to ceftazidime (50%), gentamicin (50%), ciprofloxacin (50%), imipenem (33%), amikacin (33%), tigecycline (33 %) and ertapenem (0%).

In the district hospital, the isolates identified in carriage samples in the surgical ward displayed maximum resistance (100%) to all antibiotics except colistin while those detected in medical ward exhibited high level of resistance to ampicillin (100%), cefuroxime (100%), cefotaxime (100%), ceftazidime (100%), amikacin (100%), gentamicin (100%), nitrofurantoin (100%) and tigecycline (100%) (Table 4).

Table 4. Resistance to selected antibiotics in ESBL-producing Gram-negative ESKAPE bacteria isolated from carriage and clinical samples in a district and tertiary hospital

Antibiotics	Tertiary hospital								District hospital					
	Medical ward				Surgical ward				Medical ward				Surgical ward	
	Carriage		Clinical		Carriage		Clinical		Carriage		Clinical		Carriage	
	MIC (µg/ml) range	No. resistant isolates (%)	MIC (µg/ml) range	No. resistant isolates (%)	MIC (µg/ml) range	No. resistant isolates (%)	MIC (µg/ml) range	No. resistant isolates (%)	MIC (µg/ml) range	No. resistant isolates (%)	MIC (µg/ml) range	No. resistant isolates (%)	MIC (µg/ml) range	No. resistant isolates (%)
Ampicillin	≥512	4 (100)	≥32	2 (100)	≥512	1 (100)	16-32	6 (100)	≥512	4 (100)	16-≥32	4 (100)	≥512	1 (100)
Cefoxitin	8-512	3 (75)	4	0 (0)	16	1 (100)	8-≥64	5 (83.3)	128≥512	4 (100)	≥64	4 (100)	≥512	1 (100)
Cefuroxime	≥512	4 (100)	≥64	2 (100)	128	1 (100)	8-64	6 (100)	256≥512	4 (100)	16-≥64	4 (100)	≥512	1 (100)
Cefotaxime	≥512	4 (100)	≥64	2 (100)	128	1 (100)	1-≥64	5 (83.3)	32-≥512	4 (100)	<1-32	3 (75)	≥512	1 (100)
Ceftazidime	512	4 (100)	≥64	2 (100)	256	1 (100)	1-≥64	3 (50)	32-≥512	4 (100)	≤1-16	3 (75)	≥512	1 (100)
Meropenem	0.5-2	2(50)	0.25	0 (0)	2	1 (100)	0.25≥16	4 (66.6)	0.25-16	2 (50)	0.25	0 (0)	16	1 (100)
Imipenem	4-8	4 (100)	0.25	0 (0)	16	1 (100)	0.5-≥16	2 (33.3)	2-32	2 (50)	0.25-1	0 (0)	64	1 (100)
Ertapenem	1-2	4 (100)	0.5	0 (0)	16	1 (100)	≤0.5	0 (0)	0.25-8	3 (75)	0.5	0 (0)	64	1 (100)
Amikacin	8-128	4 (100)	8	2 (100)	64	1 (100)	2-≥64	2 (33.3)	8-128	2 (100)	2	0 (0)	≥512	1 (100)
Gentamicin	128	4 (100)	≥16	2 (100)	8	1 (100)	1-≥16	3 (50)	4-16	4 (100)	1	0 (0)	≥512	1 (100)
Ciprofloxacin	64-512	4 (100)	≥4	2 (100)	32	1 (100)	0.25-≥4	3 (50)	0.5-64	3 (75)	≤0.25	0 (0)	32	1 (100)
Tigecycline	16-64	4 (100)	1	0 (0)	16	1 (100)	0.5-≥8	2 (33.3)	2-64	4 (100)	1	0 (0)	8	1 (100)
Nitrofurantoin	≥512	4 (100)	128	2 (100)	≥512	1 (100)	≥512	6 (100)	≥512	4 (100)	16-≥512	1 (25)	≥512	1 (100)
Colistin	8-512	4 (100)	0.5	0 (0)	8	1 (100)	≤0.5	0(0)	0(0)	0(0)	≤0.5	0 (0)	≤0.5	0

Genetic diversity of isolated MDR strains

Overall, the predominant ESBL genes were $bla_{CTX\text{-}M\text{-}gp9}$ (90%), bla_{SHV} (60%), $bla_{CTX\text{-}M\text{-}gp1}$ (50%), bla_{TEM} (40%) and $bla_{OXA\text{-}1\text{-}Like}$ (40%) for both carriage and clinical samples. In the tertiary hospital, $bla_{CTX\text{-}M\text{-}gp9}$ (100%), $bla_{CTX\text{-}M\text{-}gp1}$ (87.5%), bla_{KPC} (75%) and bla_{VIM} (50%) were the main resistance genes detected in *A. baumannii* while *K. pneumoniae* strains harboured mainly $bla_{CTX\text{-}M\text{-}gp8/25}$ (66.6%), bla_{TEM} (66.6%), bla_{SHV} (66.6%), $bla_{CTX\text{-}M\text{-}gp9}$ (50%) and $bla_{CTX\text{-}M\text{-}gp1}$ (50%) (Table 5). It is noteworthy that all isolates harboured at least two resistance genes and a maximum of seven genes were detected in one *E. aerogenes* (G702R2B5) isolate (Figure 1B). In the district hospital, $bla_{CTX\text{-}M\text{-}gp9}$ (100%), bla_{SHV} (100%), and bla_{TEM} (100%) were the predominant genes in *K. pneumoniae* whereas $bla_{CTX\text{-}M\text{-}gp9}$ (100%), $bla_{OXA\text{-}1\text{-}Like}$ (50%), $bla_{CTX\text{-}M\text{-}gp1}$ (50%) and $bla_{CTX\text{-}M\text{-}gp8/25}$ (50%) were the main genes identified in *E. cloacae* (Table 5).

Table 5. Resistance genes in ESBL-producing Gram-negative ESKAPE bacteria

Bacteria	No. of strains, n=31 (%)	Resistance genes, n (%)									
		AmpC	TEM	SHV	CTX-M group-1	CTX-M group-9	CTX-M Group 8/25	IMP	VIM	KPC	OXA-1-like
Tertiary hospital (n=24)											
K. pneumoniae	6 (25)	2 (33.3)	4 (66.6)	4 (66.6)	3 (50)	3 (50)	4 (66.6)	1 (16.6)	1 (16.6)	1 (16.6)	2 (33.33)
A. baumannii	8 (33.3)	3 (37.5)	/	3 (37.5)	7 (87.5)	8 (100)	/	/	4 (50)	6 (75)	2 (25)
P. aeruginosa	7 (29.5)	1 (14.28)	/	/	7 (100)	7 (100)	/	/	/	/	/
E. aerogenes	2 (8.33)	2 (100)	/	2 (100)	/	2 (100)	2 (100)	/	/	/	2 (100)
E. cloacae	1 (4.16)	/	/	/	1 (100)	1 (100)	1 (100)	1 (100)	/	1 (100)	/
District hospital (n=7)											
K. pneumoniae	2 (28.57)	/	2 (100)	2 (100)	1 (50)	2 (100)	1 (50)	/	/	/	2 (33.33)
P. aeruginosa	1 (14.28)	/	/	/	1 (100)	1 (100)	/	/	/	/	/
E. aerogenes	2 (28.57)	1 (50)	1 (50)	1 (50)	1 (50)	2 (100)	2 (100)	/	/	/	/
E. cloacae	2 (28.57)	1 (50)	/	1 (50)	1 (50)	2 (100)	1 (50)	/	/	/	1 (50)

Genomic fingerprint

ERIC-profiles revealed a high similarity within species and transmission across patients, wards and hospitals. For *K. pneumoniae*, one main cluster showing high genetic similarities was observed (Fig 1A). *K. pneumoniae* strains A111R1B2 and A105R2B2 detected among two patients at admission for the former and after 48 h for the latter, in the medical ward of the district hospital showed 100% of similarity and shared common ancestors with one carriage and three clinical strains isolated in the tertiary hospital (Fig 1A). Similarly, one pair of *E. aerogenes*, A105R1B5 and G702R1B5 isolated from two patients in the medical ward of the district and tertiary hospital, both at admission, also exhibited 100% of similarity and shared a common ancestor with another strain G702R2B5 collected after 48 h (Fig 1B). Although, *A. baumannii* (Fig 1C) and *P. aeruginosa* (Fig 1D) were more genetically diverse, some isolates shared a common ancestor within and between carriage and clinical samples.

Figure 1A-1D: Dendrograms of ESBL-producing of Gram-negative ESKAPE bacteria isolated from carriage and clinical samples from hospitalized patients. A: *K. pneumoniae*, **B:** *E. aerogenes* and *cloacae*, **C:** *A. baumannii*, **D:** *P. aeruginosa*.

Discussion

Patient follow-up rates were 96%, 58% and 16 % of rectal swabs collected at admission, after 48 h and at discharge, respectively. The overall prevalence of carriage at admission was 37.21% (16/43) and we found that 42.31% (11/26) and 57.14% (4/7) were still MDR ESKAPE carriers after 48 h and at discharge. Notwithstanding the small sample size, our results showed that the carriage of MDR Gram-negative ESKPAPE bacteria increased with the hospital length of stay. Our results are consistent with a Norwegian prospective cohort study carried out from 2009-2011 investigating the risk factors for and duration of prolonged faecal carriage of ESBL-producing *K. pneumoniae* amongst patients with community acquired urinary tract infections which revealed high prevalence of ESBL faecal carriage (ranging from 15%-61%) at six different time points[14].

At hospital level, the rate of carriage at admission in the district (30%) hospital compared with the tertiary hospital (50%) suggests that patients admitted to the tertiary hospital are likely to be more colonized by MDR Gram-negative ESKAPE bacteria than those of the district healthcare facility (Table 1). Our findings could be explained by the fact that all patients admitted in this level of hospital are generally transferred from lower level healthcare facilities of the South African health

system. This is further confirmed by the increased odds of being colonized in the univariate (OR=**9**; %CI 0.93-86.52; Table 2) and multivariate analysis (OR=**14.40;** %CI 0.98-210.84; Table 3). Similarly, at discharge, patients of the tertiary hospital (67%) were more colonized than those of the district hospital (50%). This could be explained by the complexity of cases with associated invasive medical procedures and greater antibiotic use in the tertiary hospital. However, after 48 h, the prevalence of carriage was higher in patients in the district hospital (47%) compared with the tertiary hospital (33%) intimating. This contrast could point out sub-optimal infection prevention and control measures.

Besides, tertiary hospital with its more complicated cases and subsequent higher antibiotic use would have likely created greater selection pressure for resistance, but an anomalously greater resistance was observed in carriage samples in the district hospital. The small sample numbers preclude nonetheless definitive conclusions about carriage rates and resistance patterns.

The prevalence of MDR Gram-negative ESKAPE bacteria in faecal carriage (46%) was higher than that of clinical samples (28%) during the study period. Faecal carriage of resistant bacteria has been demonstrated to precede infections and consequently such high prevalence of asymptomatic fecal carriage is of critical significance. Our results concur with a study from France where the prevalence of MDR Gram-negative bacilli isolated from stool samples was higher than that of clinical samples during a non-outbreak situation in a French Hospital [15]. They are however higher than a report from Mahomed and Coovadia (2014) which demonstrated 4.7% of faecal carriage of ESBL producing Enterobacteriaceae amongst children from community in KwaZulu-Natal, South Africa [16]. Our findings may be an under-estimation because of different diagnostic, stewardship practices, preference for empirical treatment and budget constraints such that not every infection generates a microbiological sample.

During the two-months period, 21 clinically relevant MDR Gram-negative ESKAPE bacteria out of 74 isolates were identified in both hospitals. Moreover, the prevalence of MDR *A. baumannii* and *P. aeruginosa* were 41.61% (10 out of 21 MDR Gram-negative ESKAPE bacteria) and 33.33% (7 out of 21 MDR Gram-negative ESKAPE bacteria) in clinical samples, respectively. The isolation of three *A. baumannii* strains, cluster A1, from tissue of three different patients (ED01498924, ED01498793, ED01498924) in surgery indicates, consolidate the likely dissemination of this cluster within this ward in the tertiary hospital (Table 6 and Fig 1C).

Table 6. Antibiotic Resistance Profiles and Resistance Genes of Isolates from Single Patients

Patient ID	Isolate (ID code)	Hospital	Ward	Source	MIC values (µg/ml)										Beta-lactamase resistance genes								ERIC cluster
					Ampicillin	Cefoxitin	Cefotaxime	Ceftazidime	Imipenem	Meropenem	Gentamicin	Amikacin	Ciprofloxacin	Tigecycline	AmpC	TEM	SHV	CTX-M gp1	CTX-M gp9	CTX-M gp8/25	OXA-1-like	Carbapenemases	
Carriage samples																							
A105	*E. aerogenes* (A105R1B5)	District	Medicine	Admission	≥512	≥512	≥512	≥512	16	16	16	32	64	16	-	-	-	+	+	+	+	KPC	**E2**
	K. pneumoniae (A105R2B2)			After 48h	≥512	≥512	≥512	≥512	32	16	8	8	64	64	-	+	+	+	+	+	+	-	**K1**
A109	*P. aeruginosa* (A109R1B4)	District	Medicine	Admission	≥512	128	32	32	2	1	8	128	0.5	2	-	-	-	+	+	-	-	-	P2
A111	*K. pneumoniae* (A111R1B2)	District	Medicine	Admission	≥512	≥512	≥512	32	2	0.25	4	8	32	16	-	+	+	+	-	+	+	-	**K1**
A202	*E. aerogenes* (A202R2B5)	District	Surgery	After 48h	≥512	≥512	≥512	≥512	64	16	≥512	≥512	32	8	-	+	+	+	+	+	-	-	**E1**
G702	*K. pneumoniae* (G702R1B2)	Tertiary	Medicine	Admission	≥512	≥512	≥512	≥512	4	2	≥512	128	≥512	16	+	-	-	+	+	-	-	IMP	**K3**
	E. aerogenes (G702R1B5)			Admission	≥512	64	≥512	≥512	8	0.5	128	32	≥512	32	+	-	+	+	+	+	+	-	**E2**
	E. aerogenes (G702R2B5)			After 48h	≥512	≥512	≥512	≥512	4	2	≥512	128	≥512	16	+	-	-	+	+	+	+	GES	**E2**
	K. pneumoniae (G702R3B2)			Discharge	≥512	8	≥512	512	64	0.5	512	8	64	64	-	+	+	+	+	+	+	-	**K1**
G804	*A. baumannii* (G804R1B3)	Tertiary	Surgery	Admission	≥512	16	128	256	16	2	8	64	32	16	+	-	-	+	+	-	-	-	A3
Clinical samples																							
ED01498924	*A. baumannii*	Tertiary	Surgery	Tissue	≥32	≥64	≥64	≥64	≥16	≥16	≥16	≥64	≥4	≤0.5	-	-	+	+	+	-	-	VIM, KPC	**A1**
EA00306601	*E. cloacae*	District	Medicine	Blood	≥32	≥64	32	16	1	≤0.25	≤1	≤2	≤0.25	1	-	-	+	-	+	+	+	-	E4
EA00306600	*E. cloacae*	District	Medicine	Blood	≥32	≥64	32	16	0.5	≤0.25	≤0.25	≤2	≤0.25	1	+	-	-	+	+	-	-	-	E3
ED01499889-2	*P. aeruginosa*	Tertiary	Surgery	Suppuration	≥32	32	32	2	0.5	≤0.25	2	8	≥4	4	-	-	-	+	+	-	-	-	P3
ED01501266-2	*E. cloacae*	Tertiary	Surgery	Suppuration	16	≥64	≤1	≤1	0.5	≥16	≤2	≤1	≤0.25	1	-	-	-	+	+	+	-	-	**E1**
ED01500733	*K. pneumoniae*	Tertiary	ICU	Urine	≥32	≤4	≥64	16	≤0.25	≤0.25	≥16	≤2	2	≤0.5	-	+	+	+	+	+	-	-	**K1**

ED01498793	*A. baumannii*	Tertiary	Surgery	Tissue	≥32	≥64	≥64	≥64	≥16	≥16	≥16	≥64	≥4	1	-	-	+	+	+	-	-	VIM, KPC	**A1**
ED01498924	*A. baumannii*	Tertiary	Surgery	Tissue	≥32	≥64	≥64	≥64	≥16	≥16	≥16	≥64	≥4	≤0.5	-	-	+	+	+	-	-	VIM, KPC	**A1**
ED01502268	*K. pneumoniae*	Tertiary	Medicine	Sputum	≥32	≤4	≥64	≥64	≤0.25	≤0.25	≥16	8	≥4	1	+	+	+	+	+		+	-	**K1**
ED01503757	*K. pneumoniae*	Tertiary	Medicine	Sputum	≥32	≤4	≥64	≥64	≤0.25	≤0.25	≥16	8	≥4	1	+	+	-	+	+	+	-	-	K2
ED01501066	*P. aeruginosa*	Tertiary	Urology clinic	Urine	≥32	≥64	8	2	≤0.25	1	2	4	≤0.25	≥8	-	-	-	+	+	-	-	-	P2
ED01504366	*P. aeruginosa*	Tertiary	Surgery	Suppuration	≥32	≥64	32	4	≤0.25	2	≤2	2	≤0.25	≥8	-	-	-	+	+	-	-	-	/
ED01504363	*K. pneumoniae*	Tertiary	Medicine	Suppuration	≥32	8	≥64	32	8	≤0.25	≥16	4	≥4	≤0.5	-	-	+	+	+	-	-	VIM, KPC	**K3**
ED01507915	*P. aeruginosa*	Tertiary	ICU	Suppuration	≥32	≥64	≥64	≥64	1	1	≤1	≤2	≤0.25	≥8	-	-	-	+	+	-	-	-	P1
ED01507331	*P. aeruginosa*	Tertiary	Surgery	Suppuration	≥32	≥64	32	4	1	≤0.25	≤1	≤2	≤0.25	≥8	-	-	-	+	+	-	-	-	/
ED01507028-1	*P. aeruginosa*	Tertiary	ICU	Tracheal aspirate	≥32	≥64	32	4	1	1	≥16	16	≤0.25	≥8	-	-	-	+	+	-	-	-	P1
ED01506083	*A. baumannii*	Tertiary	Obstetrics and gynecology	Catheter tip	≥32	≥64	≥64	≥64	≥16	≥16	≥16	≤2	≥4	4	-	-	-	+	+	-	-	KPC	A1
ED01506443	*A. baumannii*	Tertiary	Surgery	Catheter tip	≥32	≥64	≥64	≥64	≥16	≥16	≥16	≥64	≥4	2	-	-	-	+	+	-	-	KPC	A1
ED01506571	*A. baumannii*	Tertiary	ICU	Suppuration	≥32	≥64	≥64	16	≥16	≥16	≥16	≤2	≥4	1	-	-	-	+	+	-	-	VIM, KPC	A2
ED01507028-2	*A. baumannii*	Tertiary	ICU	Tracheal aspirate	≥32	≥64	≥64	16	≥16	≥16	≥16	≥64	≥4	4	+	-	-	+	+	-	-	-	A2
ED01506433	*P. aeruginosa*	Tertiary	ICU	Wound	≥32	≥64	≥64	4	2	≤0.25	≤1	≤2	≤0.25	≥8	-	-	-	+	+	-	-	-	/

In carriage samples, MDR *K. pneumoniae* and *Enterobacter spp.* were the predominant bacteria in both hospitals. This is consistent with a South African study where *K. pneumoniae* was the main pathogen identified in stool samples of children from the community of KwaZulu-Natal, South Africa [16]. Similarly, a 68% prevalence of ESBL-producing *Enterobacteriaceae* faecal carriage was shown amongst Egyptian patients with community-acquired gastrointestinal complaints [17]. An interesting finding was the inter-hospital and inter-patient spread of *K. pneumoniae* (cluster K1) in carriage, which were isolated from two patients (A105R2B2 and A111R1B2) hospitalized in general medicine in district hospital, sharing common ancestor with a patient (G702R3B2) from tertiary hospital (Table 6). It is noteworthy that the isolated strains were identified in the medical ward and at different time-points, confirming the dissemination of this cluster across hospitals. In addition, *K. pneumoniae* strains from the same cluster (K1) were detected in urine (ED01500733) and sputum (ED01502268) of clinically ill patients hospitalized in intensive care unit (ICU) and medical ward in the tertiary hospital, respectively. This suggests that the *K. pneumoniae* K1 strains is circulating within wards and hospitals, and consequently could probably be source of nosocomial infections in hospitals.

Two patients, A105R1B5 and G702R1B5 also carried *Enterobacter spp.* (cluster E2) at admission in both district and tertiary hospitals, specifically in the medical wards (Table 6) intimating the emergence of these strains in the community with subsequent entry into the district hospital, as the first level of care, and followed by spread to the tertiary hospital through referral (OR=14.40 %CI 0.98-210.84). This result is consistent with our analyses which demonstrated that in the district hospital, the main risk factors were antibiotic use and gender while the referral and hospital ward were the principal risk factors at tertiary level (Tables 2 and 3).

Overall, the predominant ESBLs detected in carriage were $bla_{CTX\text{-}M\text{-}gp9}$ (90%), bla_{SHV} (60%), $bla_{CTX\text{-}M\text{-}gp1}$(50%), bla_{TEM} (40%) and $bla_{OXA\text{-}1\text{-}like}$ (40%). CTX-M is predominantly reported in community-acquired infections which would be more prevalent in the district hospital as the first level of care. These results are consistent with global reports. For instance, $bla_{CTX\text{-}M\text{-}group}$ were recently observed in adults in a community in Netherlands and ambulatory patients in Egypt with both gastrointestinal complaints [17,18]. Similarly, studies from Guinee-Bissau (2012), Niger (2011), Gabon (2013) and Tanzania (2016), reported high prevalence of ESBL faecal carriage with $bla_{CTX\text{-}M}$, bla_{TEM} and bla_{SHV} being the main genes identified [18-21]. The prevalence of AmpC was also higher in carriage (40%) compared to clinical samples (23.80%). Finally, carbapenemases

were identified in clinical samples from these hospitals, specifically, KPC and VIM in clinical *A. baumannii* isolates as well as IMP in a carriage *K. pneumoniae* isolate. An *E. aerogenes* isolate further showed bla_{GES} along with $bla_{CTX\text{-}M\text{-}gp1}$, $bla_{CTX\text{-}M\text{-}gp9}$, $bla_{CTX\text{-}M\text{-}gp8/25}$, bla_{SHV} and $bla_{OXA\text{-}1\text{-}like}$ in a carriage sample. The faecal carriage of MDR Gram-negative ESKAPE bacteria appears to be a source of cross-transmission between patients. The substantial genetic similarity within and between carriage and clinical isolates as well as wards and hospital settings reveals their potential implications in future outbreak situations that may occur either in hospitals or in communities. Efforts should thus be made amongst communities and asymptomatic patients for better containment of antibiotic resistance dissemination.

Gender, antibiotic use, type of healthcare settings and referral from another hospital were the main risk factors identified. These results suggest that routine screening for MDR Gram-negative ESKAPE bacteria at admission should be implemented, and infection, prevention and control measures reinforced to prevent potential outbreaks by these resistant pathogens [22].

This study highlights the high prevalence of ESBL-producing MDR Gram-negative ESKAPE bacteria in carriage and clinical samples among hospitalized patients in uMgungundlovu. It is imperative to implement regular screening and surveillance of MDR Gram-negative ESKAPE bacteria in communities and hospitals, to monitor epidemiological changes, ascertain socio-economic impact and inform antibiotic treatment. These screening and surveillance measures coupled with strict infection prevention and control programmes and antimicrobial stewardship programmes (ASP) are essential to address antibiotic resistance in these settings.

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Conflict of interest

Professor Essack is a member of the Global Respiratory Infection Partnership sponsored by an unrestricted educational grant from Reckitt and Benckiser. All other authors declare that there is no competing financial interest.

Author contributions

Conceptualization: RCF and SYE

Data curation: RCF

Formal analysis: RCF and LLF

Funding acquisition: SYE

Investigation: RCF and LLF

Methodology: RCF and SYE

Project administration: SYE

Resources: SYE

Supervision: SYE

Validation: RCF, LLF and SYE

Visualisation: RCF and LLF

Writing original draft: RCF

Review and editing: SYE

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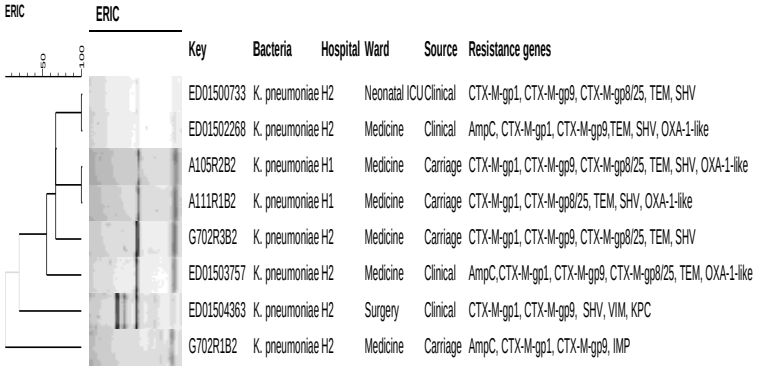

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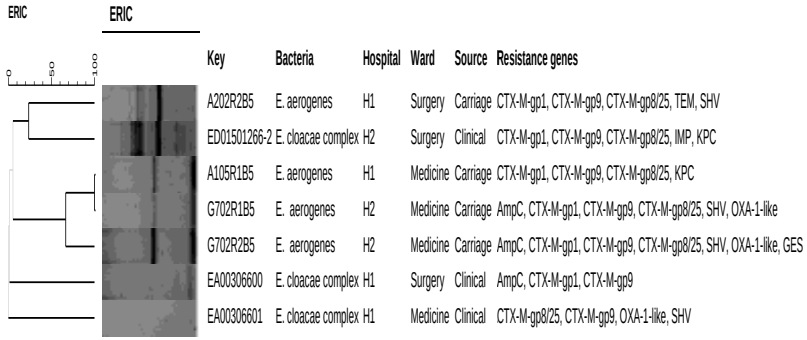

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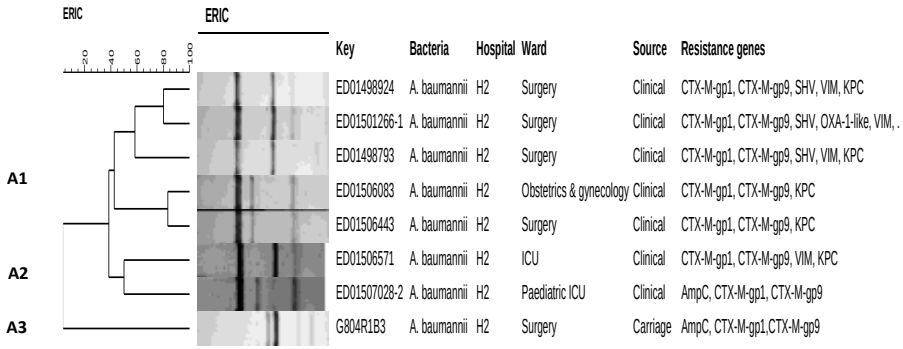

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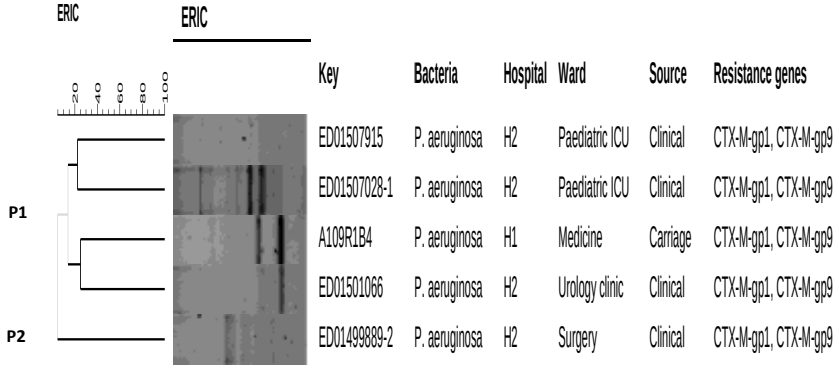

D

Figure 1: Dendrograms of ESBL-producing of Gram-negative ESKAPE bacteria isolated from carriage and clinical samples from hospitalized patients. A: *K. pneumoniae*, **B:** *E. aerogenes* and *cloacae*, **C:** *A. baumannii*, **D:** *P. aeruginosa*

Supplementary Table 1. Oligonucleotide sequences for ESBL and carbapenemase resistance genes included in multiplex PCR assays

PCR name	Target gene	Primer name	Sequence (5'-3')	Amplicon size (bp)	Ref.
Multiplex I TEM, SHV, OXA-1-like	TEM -1 and TEM-2	MultiTSO-T_for MultiTSO-T_rev	CATTTCCGTGTCGCCCTTATTC CGTTCATCCATAGTTGCCTGAC	800	13
	SHV-1	MultiTSO-S_for MultiTSO-S_rev	AGCCGCTTGAGCAAATTAAAC ATCCCGCAGATAAATCACCAC	713	
	OXA-1, OXA-4 and OXA-40	MultiTSO-O_for MultiTSO-O_rev	GGCACCAGATTCAACTTTCAAG GACCCCAAGTTTCCTGTAAGTG	564	
Multiplex II CTX-M group 8/25	CTX-M-8, CTX-M-25, CTX-M-26 and CTX-M-39 to CTX-M-41	CTX-Mg8/25_for CTX-Mg8/25_rev	AACRCRCAGACGCTCTAC[a] TCGAGCCGGAASGTGTYAT[a]	326	
Multiplex III GES and OXA-48-like	GES-1 to GES-9 and GES-11	MultiGES_for MultiGES_rev	AGTCGGCTAGACCGGAAAG TTTGTCCGTGCTCAGGAT	399	
	OXA-48-like	MultiOXA-48_for MultiOXA-48_rev	GCTTGATCGCCCTCGATT GATTTGCTCCGTGGCCGAAA	281	
Multiplex IV IMP, VIM and KPC	IMP variants except IMP-9, IMP-16, IMP-18, IMP-22 and IMP-25	MultiIMP_for MultiIMP_rev	TTGACACTCCATTTACDG[a] GATYGAGAATTAAGCCACYCT[a]	139	
	VIM variants including VIM-1 and VIM-2	MultiVIM_for MultiVIM_revc	GATGGTGTTTGGTCGCATA CGAATGCGCAGCACCAG	390	
	KPC-1 to KPC-5	MultiKPC_for MultiKPC_rev	CATTCAAGGGCTTTCTTGCTGC ACGACGGCATAGTCATTTGC	538	

[a]Y=T or C; R=A or G; S=G or C; D=A or G or T.

CHAPTER III.

ARTICLE 2. Faecal Carriage of VanC-1-Mediated Vancomycin-Resistant *Enterococcus faecium* and *Enterococcus faecalis* in Hospitalized Patients in uMgungundlovu District, KwaZulu-Natal, South Africa[2]

Author contributions:

- Raspail Carrel Founou, as the principal investigator, co-conceptualized the study, undertook sample collection, laboratory and statistical analyses, prepared tables and figures, and drafted the manuscript.
- Luria Leslie Founou, undertook sample collection, laboratory analysis, vetting of the results, and reviewed the manuscript.
- Mushal Allam, undertook bioinformatics analyses.
- Arshad Ismail, performed whole genome sequencing.
- Sabiha Yusuf Essack, as the principal supervisor, co-conceptualized the study, contributed to vetting of the results and undertook critical revision of the manuscript

Objectives met: This paper addresses objectives Two, Three, Four, Five.

[2] This paper has been submitted to Frontiers in Microbiology and is currently under review.

Faecal Carriage of VanC-1-Mediated Vancomycin-Resistant *Enterococcus faecium* and *Enterococcus faecalis* in Hospitalized Patients in uMgungundlovu District, KwaZulu-Natal, South Africa

Raspail Carrel Founou[1,2*], Luria Leslie Founou[1,3], Mushal Allam[4], Arshad Ismail[4], Sabiha Yusuf Essack[1]

[1]Antimicrobial Research Unit, School of Health Sciences, University of KwaZulu-Natal, Durban, South Africa
[2]Department of Clinical Microbiology, Centre of Expertise and Biological Diagnostic of Cameroon, Yaoundé, Cameroon (CEDBCAM)
[3]Department of Food safety and Environmental Microbiology, Centre of Expertise and Biological Diagnostic of Cameroon, Yaoundé, Cameroon
[4]Sequencing Core Facility, National Health Laboratory Service, Johannesburg, South Africa

***Corresponding author**

Raspail Carrel Founou Zangue

Antimicrobial Research Unit

College of Health Sciences

University of KwaZulu-Natal

Durban, 4000

South Africa

Email: czangue@yahoo.fr

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Abstract

Background: Vancomycin resistant enterococci (VRE) are categorized as high priority bacteria in the Global Priority List of Antibiotic-Resistant Bacteria to Guide Research, Discovery, and Development of New Antibiotics published by the World Health Organization. VRE are members of the ESKAPE group of pathogens and are implicated in serious difficult-to-treat infections.

Objectives: This study assessed the carriage, phenotypic and genotypic profiles, and clonal lineages of vancomycin-resistant *E. faecium* and *E. faecalis* isolated from hospitalized patients.
Methods: From May to June 2017, rectal swabs were collected from patients admitted to medical and surgical wards of an urban, tertiary and a rural, district hospital, in uMgungundlovu, South Africa. Enterococci were screened for vancomycin resistance on bile esculin azide agar supplemented with 6 mg/L of vancomycin and vancomycin resistance was confirmed using ROSCO kits. Conventional and real-time PCR assays were used to confirm the identification to species level and to ascertain the presence of VanA, VanB, VanC-2/3 and VanC-1 genes. REP-PCR was undertaken to ascertain the clonal relatedness and WGS was used to characterize the circulating sequence types (STS), resistance and virulence genes and plasmids.
Results: Fifteen (39%) *E. faecium* and 23 (61%) *E. faecalis*, were detected. High levels of antibiotic-resistance were observed for cefoxitin (100%), erythromycin (100%), clindamycin (100%) and teicoplanin (100%) for isolates from both district and tertiary hospitals. Two *E. faecium* strains, A109R2B0 and A206R1B0, detected in two different patients, at two different time-points (admission and after 48h) and in two different wards (medicine and surgery) in the district hospital (H1), revealed high genetic similarity and suggested potential transmission across patients and wards within this hospital. Four single sequence types (STs) were identified among *E. faecium* in district hospital namely ST822, ST636, ST97 along with a novel ST assigned ST1386, while one lineage (ST29) was detected in the tertiary hospital.
Conclusion: The study reveals the genetic diversity and high pathogenicity of circulating VRE in uMgungundlovu district, South Africa. It underlines the necessity to implement routine screening of admitted patients at all level of care to contain the spread of VRE in this district.

INTRODUCTION

Enterococcus spp. are Gram-positive cocci, frequently isolated in the gastrointestinal tract of both humans and animals (Abebe et al., 2014; Abamecha et al., 2015). This genus encompasses approximately 16 species, but only *E. faecalis* and *E. faecium* have been identified as clinically important due to their implications in serious difficult-to-treat nosocomial infections such as endocarditis, urinary tract infections, peritonitis, bacteraemia, neonatal sepsis, meningitis, surgical wound and intra-abdominal infections in hospitals and communities (Abebe et al., 2014; Abamecha et al., 2015).

Enterococci are clinically relevant because of the (i) emergence of vancomycin-resistant *E. faecium* (VRE), (ii) high-levels of resistance to multiple antibiotics, (iii) transfer of resistance gene from VRE to *Staphylococcus aureus*, (iv) presence of different selective pressures increasing the proliferation and rapid spread of VRE, (v) few therapeutic options for disease management, and (vi) limited success of VRE containment measures (Mundy et al., 2000; Van Schaik et al., 2010; Abebe et al., 2014; Yu et al., 2015). However, the foremost clinical relevance of VRE is its colonization in the gastrointestinal tract of humans and animals, creating reservoirs for VRE transmission. VRE were recently ranked as high priority in the Global Priority List of Antibiotic-Resistant Bacteria to Guide Research, Discovery and Development of New Antibiotics by the World Health Organization (WHO)(World Health Organization, 2017).

Van A phenotype strains of *E. faecium* were first detected from clinical cases of VRE infections in Europe in 1986, where they were associated with outbreaks in hospitals, particularly in patients with severe underlying diseases or an immunocompromised status (Corso et al., 2007; Abebe et al., 2014). In Africa, the first cases of VRE infections were described in South Africa where a 10.9% prevalence of VRE-colonized patients was reported at a hospital in 1997 (von Gottberg et al., 2000). VRE is associated with nosocomial infections in various hospital wards and risk factors include an immunocompromised status, urinary or central venous catheterization, intra-abdominal or cardio-thoracic surgical procedures, and co-morbidities such as HIV/AIDS, diabetes and cancers (Kuo et al., 2014).

Leclerc et al, (1988) described nine operons capable of conferring resistance to glycopeptides (Leclercq et al., 1988). The differentiation of these operons is based on ligase genes encoding D-alanyl-D-lactate ligase (VanA, VanB, VanD, and VanM) or D-alanyl-D-serine ligases (VanC-1, VanC-2, VanC-3, VanE, VanG, VanN and VanL) (Sun et al., 2014). The resistance

to glycopeptides can be acquired except for VanC-type resistance which is chromosomal and intrinsic to *E. gallinarum* and *casseliflavus* (Sun et al., 2014).

The overuse of glycopeptides and extended-spectrum cephalosporins in hospital settings has probably contributed to the increased prevalence and spread of these resistant pathogens (Shafiyabi et al., 2013). The VRE threat is relatively under-investigated in South Africa. This study therefore assessed the carriage, risk factors, resistance and virulence genes, and clonal lineages of vancomycin-resistant *Enterococcus faecium* and *faecalis* isolated from hospitalized patients in the uMgungundlovu District, KwaZulu-Natal, South Africa.

I. Materials and Methods

1. Ethical considerations

Ethical approval was obtained from the Biomedical Research Ethics committee (BREC) (**No. BF512/16**, **sub-study of BCA444/16**). Permission to conduct the research was also granted from the Department of Health, uMgungundlovu District and hospital managers.

2. Study population and settings

This study was conducted during two months from May to June 2017 in a rural, district hospital and an urban tertiary hospital. The district hospitals (H1) covers four services i.e., obstetrics and gynaecology, paediatrics and child health, general surgery and general medicine with 141 beds. The tertiary hospital (H2) offers several specialties, receives referral patients according to a nationally agreed referral plan and has approximately 505 beds.

3. Patient enrolment and questionnaire data collection

After explanation of the study, oral and written informed consent was obtained from all participants. Patients thereafter completed a questionnaire that yielded socio-demographic information while the clinical history was extracted from patient records. Information was codified prior to analysis to ensure confidentiality.

4. Sample collection

Sample collection took place in both surgical and general medical wards. Rectal swabs were aseptically collected with Amie swabs from all admitted in-patients >18 years old, at admission, after 48h and at discharge whenever possible.

5. Laboratory analysis

5.1 Culture and Identification

Rectal swabs (n=76 specimens) were cultured onto Bile-Esculin-Azide agar (Oxoid, Dardilly, France) with and without vancomycin (6 mg/L). After incubation for 18-24h at 37°C, each black colony growing on Bile-Esculin-Azide agar supplemented with vancomycin (BEA+VAN) that further hydrolysed and reduced 0.04% potassium tellurite, was selected for Gram staining, the oxidase and catalase tests and L-pyrrolidonyl-b-naphthylamidase activity. Biochemical identification was confirmed using API Strept (bioMérieux, Marcy l'Etoile, France). Pure colonies of *E. faecium* and *E. faecalis* were stored into tryptone soya broth (TSB) (Merck, Darmstadt, Germany) supplemented with 20% glycerol at −20°C for future use.

5.2. Phenotypic screening

All colonies were phenotypically screened for vancomycin, teicoplanin and daptomycin resistance using the package of MRSA, VISA, GISA, VRE ROSCO DIAGNOSTICA Kit (Taastrup, Denmark) using 0.5 McFarland on Mueller-Hinton agar (Oxoid, Dardilly, France) according to the manufacturer's instructions.

5.3. Antimicrobial susceptibility testing by microbroth dilution

Minimum inhibitory concentrations (MICs) were determined by broth microdilution. Ampicillin, cefoxitin, gentamycin, streptomycin, ciprofloxacin, moxifloxacin, erythromycin, clindamycin, linezolid, teicoplanin, vancomycin, tetracycline, doxycycline, tigecycline, fusidic acid, trimethoprim, nitrofurantoin, and chloramphenicol, were tested and interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2017) breakpoints using *E. faecium* ATCC 29212 as the control strain.

5.4. Genomic characterization

5.4.1. Genomic extraction

Genomic DNA of selected strains were extracted using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. DNA were stored at -20°C.

5.4.2. Molecular identification of species

Two simplex PCR reactions consisting of different primer sets were performed to identify isolates to the species level in a final volume of 10 µl consisting of 5 µl of Dream*Taq* Green Polymerase Master Mix 2X (Thermo Scientific Inc.), 1.8 µl of nuclease free water, 0.1 µl of each primer (100 µM), and 3 µl of DNA template. The oligonucleotide primers were synthesized by Inqaba Biotech (Pretoria, South Africa). Primer sequences were those previously described by Iweriebor et al. (2015) and are listed in Supplementary Table 1. Simplex PCR were performed in 0.2 ml PCR-tube in a programmable Bio-Rad Thermal Cycler

(BioRad Laboratories, CA, Foster City, USA) according to the following conditions: initial denaturation at 95 °C for 4 min, 30 cycles of denaturation at 95 °C for 30 s, annealing at 48 °C (*E. faecium*) or 52 °C (*E. faecalis*) for 1 min, and elongation at 72 °C for 1 min followed by a final extension at 72 °C for 7 min. Four microliters of amplification products were electrophoresed on a 2% (wt/vol) Tris-Borate-EDTA (Merck) agarose gel run at 110V for 45 min along with the Quick-load®100bp molecular weight marker (Biolabs, New England). Electrophoresis gels were stained in a solution containing 0.5 µg/ml ethidium bromide. After a brief destaining in water gels were visualized by a UV light transilluminator images were captured using a Gel Doc™ XR (BioRad Laboratories, CA, Foster City, USA) and analysed by Image Lab™ Software (version 5.2, BioRad Laboratories, CA, Foster City, USA).

5.4.3. Molecular characterization of vancomycin resistance genes

5.4.3.1. Conventional polymerase chain reaction (PCR)

All confirmed VRE were screened by simplex PCR to identify associated vancomycin resistance genes with specific primers for VanA, VanB, and VanC2/3 as previously described (Iweriebor et al., 2015; Supplementary Table 1). The oligonucleotide primers were also synthesized by Inqaba Biotech (Pretoria, South Africa). PCR were performed in 0.2 ml PCR-tube in a programmable BioRad Thermal Cycler (CA, Foster City, USA) with the following conditions: initial denaturation at 95 °C for 4 min, 30 cycles of denaturation at 95 °C for 30 s, annealing at 56.5 °C for 1 min, and elongation at 72 °C for 1 min followed by a final extension at 72 °C for 7 min and an infinite hold a 4 °C. Amplification products were electrophoresed and visualized as described above.

5.4.3.2. Real-time polymerase chain reaction (RT-PCR)

RT- PCR was performed to ascertain specific vancomycin resistance genes on a programmable automate QuantStudio5™ (Applied Biosystems, CA, USA) using the Taqman Universal Master Mix 2× (Applied Biosystems, CA, USA) and ready-made assays (Thermo Scientific, CA, USA). Thermal temperature running conditions were as follows: UNG activation at 50 °C for 2 min, initial denaturation at 95 °C for 10 min, 30 cycles of denaturation 95 °C for 10 s, annealing/extension at 60 °C for 1 min and a final extension at 60°C for 30 s. The results were interpreted with QuantStudio™ design and analysis software version 1.4 (Applied Biosystems, CA, USA).

5.4.4. Molecular fingerprinting

Repetitive Element Palindromic-Polymerase Chain Reaction (REP-PCR) was performed to ascertain the relationship of isolates at each time points, within and between wards and hospitals. The (GTG)5 primer 5'-GTGGTGGTGGTGGTG-3' (Versalovic, 1991) synthetized by Inqaba (South Africa) was used and PCR reactions performed in a final reaction mixture of 10 µl consisting of 5 µl of Dream*Taq* Green Polymerase Master Mix 2X (Thermo Scientific Inc.), 1.8 µl of nuclease free water, 0.2 µl of (GTG)5 primer (100 µM), and 3 µl of DNA template. PCR were performed in 0.2 ml PCR-tube in a programmable Bio-Rad Thermal Cycler (BioRad Laboratories, CA, Foster City, USA) and according to the following protocol: initial denaturation at 94°C for 7 min, 30 cycles consisting of a denaturation step at 94°C for 1 min, primer annealing at 40°C for 1 min, extension at 65°C for 8 min, a final extension step at 65°C for 16 min and final storage at 4°C (Versalovic et al., 1991). The amplicons were resolved in by horizontal electrophoresis in a 1.5% (wt/vol) Tris-Borate-EDTA (Merck) agarose gel together with the Quick-load®1-kb molecular weight marker (Biolabs, New England) and run in an electric field of 110 V for 2 h 30 min. Electrophoresis gels were thereafter stained in a solution containing 0.5 µg/ml ethidium bromide. After a brief destaining in water, gels were visualized by a UV light transilluminator, images were captured using a Gel Doc^{TM} XR+ system (BioRad Laboratories, CA, Foster City, USA) and analysed by Image Lab^{TM} Software (version 5.2, BioRad Laboratories, CA, Foster City, USA).

5.4.5. Computer-Assisted Image Analysis and Cluster Assignment

REP-PCR profiles were normalized using the Quick-load®1-kb DNA molecular weight marker (Biolabs, New England) as standard. Fingerprint were assigned to isolates with Bio-Rad's Image Lab$_{TM}$ software (version 5.2, BioRad). For cluster analysis, data were exported to Bionumerics software (version 7.6, Applied Maths, TX, USA). Isolates were allocated to different clusters by calculating the similarity coefficient from the homology matrix with Dice method. Dendrograms were generated based on the average linkages of the matrix and using the Unweighted Pair-Group Method (UPGMA). Optimization and band tolerance were set at 1% (version 7.6, Applied Maths, TX, USA) and 80% similarity cut-off was used to define clusters.

5.5. Whole genome analysis

The Nextera XT DNA sample preparation kit (Illumina, San Diego, CA, USA) was used for the preparation of multiplex paired-end libraries (2×300 bp). The Illumina MiSeq machine was used for library sequencing with 100× coverage. The generated reads were checked for quality

and trimmed using the CLC Genomics Workbench version 10 (CLC, Bio-QIAGEN, Aarhus, Denmark). *De novo* assembling was subsequently performed with CLC Genomics and SPAdes version 3.5.0 (Bankevich et al., 2012). The assembled reads were uploaded and annotated using the Bacterial Analysis Pipeline of GoSeqIt tools, NCBI PGAP (https://www.ncbi.nlm.nih.gov/genome/annotation_prok/) and ARG-ANNOT (http://en.mediterranee-infection.com). ResFinder (Zankari et al., 2012), VirulenceFinder (Joensen et al., 2014) and PlasmidFinder (Carattoli et al., 2014) were used for the identification of antibiotic resistance genes, virulence factors and plasmids, respectively. The multi-locus sequence type (MLST) was determined from the WGS data. Contigs of *E. faecalis* G702R1B0 were mapped against the finished genome of *E. faecalis* DENG1 (CP004081.1) for visualization of the genomic structure (Figure 1). Phylogenetic analyses were performed to contextualize our strains against a collection from international complete genomes (accession no.: CP004081; NC017316; NC004668; CP003351; NC017960; CP019988) (Figure 1).

5.6. Nucleotide sequence accession number

This whole-genome shotgun project PRJNA417366 of *E. faecalis* and *E. faecium* isolates A113R1B0, G701R2B0C1, G702R1B0, A206R2B0, A201R2B0, A108R2B0, and A209R1B0C1 has been deposited at DDBJ/EMBL/GenBank under accession numbers PGCX00000000, PGCW00000000, PGCV00000000, PGCU00000000, PGCT00000000, PGCS00000000, and PGCR00000000, respectively. The version described in this paper are the version PGCX01000000, PGCW01000000, PGCV01000000, PGCU01000000, PGCT01000000, PGCS01000000, and PGCR0000000, respectively.

5.7.Data analysis

Data was coded and entered in Excel spreadsheet (Microsoft Office 2016) and analysed using STATA (version 14.0, STATA Corporation, TX, USA). Risk factors for VRE colonization were ascertained by univariate and multivariate logistic regression analysis. Prevalence of VRE carriage was compared between categories (viz. hospital, ward and time-point) using the chi square test, and a p-value < 0.05 was regarded as statistically significant.

II. Results

1. Study population and risk factors for VRE in carriage

A total of 75 patients hospitalized were contacted. Of these, 45 (60%) agreed to participate, answered the questionnaire and provided at least one sample at admission. Overall, males were

more colonized than females at admission and at discharge as were patients referred from another hospital (Table 1). The mean age of patient was 50.7 years range (19-70). Out of 45 patients, 31(69%) were treated with an antibiotic during their hospitalization. Patients in the district hospital were more likely to be colonized by VRE bacteria at admission (44%), after 48h (64%) and discharge (100%) than those at the tertiary hospital (Table 1). Gender, antibiotics use, co-morbidity, previous hospitalization, transfer from another hospital were the main risk factors identified at admission in both hospitals while the odds of VRE colonization were higher in surgical wards after 48h (Tables 2 and 3).

Of the 45 participants, 24 (53%) were asymptomatic faecal carriers of VRE with some harbouring multiple strains, yielding a total of 38 non-duplicate VRE in both hospitals. Of these 15 (39%) and 23 (61%) were confirmed *E. faecium* and *E. faecalis*, respectively. More specifically, 14 (54%) *E. faecium* and 12 (46%) *E. faecalis* were isolated in the district hospital while 11 (92%) *E. faecalis* and one (8%) *E. faecium* were identified in the tertiary hospital (Table 4).

2. Antimicrobial susceptibility and resistance genes

High levels of antibiotic resistance were observed among isolates in both the district and tertiary hospitals. In the district hospital, 100% resistance to cefoxitin, erythromycin, clindamycin, teicoplanin was evident in both bacterial species while 100% resistance was observed against all antibiotics except moxifloxacin, gentamicin, erythromycin, and ampicillin in *E. faecium* in the tertiary hospital (Table 4).

VanC1 was the only glycopeptide resistant gene detected in all VRE isolates. In addition, the *E. faecalis* ST6 (G702R1B0) carried VanG together with the vancomycin tolerance locus (vex2, vex3), macrolide, lincosamide, sterptogramines B (ermB, Isa, emeA), tetracycline (TetM), fosfomycine (fosB), fluoroquinolones (ParC, ParE, gyrA, gyrB) resistance genes as well as the multidrug resistant efflux pumps (MATE, MFS, mepA, emeA) encoding for resistance to several antibiotic classes (Table 5 and 6). Similarly, all *E. faecium* harboured Vex2 and Vex3 concurrently with fluoroquinolone resistance genes (ParC, ParE, gyrA, gyrB, Lde) and the multidrug resistant efflux pumps (MATE, MFS, pmrA) (Table 6).

3. Genomic fingerprint

Two *E. faecium* strains, A109R2B0 and A206R1B0, detected in two different patients, at two different time-points (admission and after 48h) and in two different wards (medicine and surgery) in the district hospital (H1), revealed high genetic similarity and suggested potential

transmission across patients and wards within this hospital (Figure 1). In contrast, *E. faecalis* isolates showed similarity across hospitals and wards since two strains, G812R3B0 isolated in surgical ward of the tertiary hospital, and A107R1B0 detected in medical ward of the district hospital were closely related and shared common ancestors with a third isolate (A210R1B0C2) from the surgical ward in the district hospital (Figure 2). This suggests the probable inter-hospital transmission of VRE between the tertiary and district hospitals, as well as inter-ward dissemination across surgery and medical wards. Seven *E. faecalis* isolates identified in tertiary and three in district hospitals were unique.

4. Multi-locus sequence typing (MLST)

MLST-analyses were performed for four *E. faecium* (A206R2B0, A201R2B0, A108R2B0, A209R1B0C1) and three *E. faecalis* (A113R1B0, G701R2B0C1, G702R1B0) strains that were selected based on their relatedness on REP-PCR (Table 5). Four single sequence types (ST) were identified among *E. faecium* in district hospital namely ST822, ST636, ST97 along with a novel ST assigned ST1386 detected in district hospital based on seven house-keeping genes including adk, atpa, ddl, gdh, gyd, psts, purk. Similarly, three different STs were observed in *E. faecalis* based on the variation amongst the seven house-keeping genes (aroe, gdh, gki, gyd, psts, xpt, yqil). Two singletons namely ST563 and ST6 were identified in tertiary hospital while ST21 was also identified in the district hospital.

5. Virulence factors

WGS data revealed that *E. faecalis* strains were more virulent than *E. faecium*, with a total of 16 virulence genes for the former compared to two for the latter. The distribution of virulence genes among these isolates are presented in Table 6. Overall, all *E. faecalis* carried at least 14 virulence genes including ace, cad, camE, cCF10, cOB1, ebpA, ebpB, ebpC, efaAfc, ElrA, gelE, hylA, SrtA, tpx. Furthermore, 25% of these isolates were also biofilm formers (fsrB) and hyaluronidase (hylB) producers. In contrast, all *E. faecium* concomitantly harboured only two virulence genes (efaAfm and acm) and none produced biofilm or hylB.

6. Plasmid detection

Only two *E. faecalis*, ST6 and ST563, isolated in tertiary hospital medical ward harboured multiple plasmids. The *E. faecalis* ST6 hosted CDS16(pTEF3) and repA2(pTEF2) while the *E. faecalis* ST563 carried four plasmid replicon types namely CDS16(pTEF3), repA2(pTEF2), rep(pUB110), rep(pKH7) and a single open reading frame (ORF) in contig 1183. *E. faecium* (ST29) carried two plasmid replication proteins namely repE (pAMbeta1) and, rep(pUB110)

with an additional ORF in contig 287. The remaining strains (ST822, ST636, ST97) harboured only repE (pAMbeta1).

III. Discussion

VRE is an important bacterial species implicated in severe, difficult-to-treat infections globally. To the best of our knowledge, this study is the first report of VRE-harbouring VanC-1 genes in South Africa. Hospitalized patients who were followed-up at three-time points for colonization with VRE showed an overall prevalence of 50%, 57% and 83% VRE at admission, after 48H and at discharge respectively. These findings are higher than a South African prevalence study reported in 2000 which revealed 11% of high-risk patients colonized by VRE (von Gottberg et al., 2000) and generally lower than an Argentinian study that showed a 77% prevalence of VRE from rectal swabs of hospitalized patients, with the ICU (47%) and general medicine wards (36%) being the main affected units.

Gender, antibiotic use, co-morbidity, previous hospitalization, referral from district to tertiary hospital were the main risk factors identified at admission while hospitalization in a surgical ward increased the odds of VRE colonization after 48h (Table 2). Our results are consistent with an Australian hospital-wide point prevalence study which revealed that age, duration of hospitalisation, antibiotic use and ward type were the main risk factors for VRE colonisation in a tertiary hospital in Melbourne (Karki et al., 2012).

None of the isolates tested positive for VanA and VanB genes but exhibited vancomycin-resistance as evident from the MICs (Table 3 and 4). Although the VanC-1 gene is an intrinsic chromosomal gene of *E. gallinarum* and *E. casseliflavus,* its presence in our *E. faecium* could probably be attributed to horizontal gene transfer. The VanC-1 gene was first described in vancomycin susceptible *E. faecalis* isolated from pig manure (De Moura et al., 2013; Sun et al., 2014) intimating that the chromosomal location of intrinsic resistant genes does not preclude horizontal genes tranfer to other species, therby contributing to species diversification (De Moura et al., 2013; Sun et al., 2014). The mobility of the VanC-1 gene may result in laboratory misidentification of *E. gallinarum* and *E. casseliflavus* whose identification is premised on the presence of this gene. The presence of multidrug resistant efflux pumps harboured by all isolates could explain the high level of multi-drug resistance our isolates (Tables 2 and 3).

The most interesting finding of the study, was the inter-ward, inter-patient and intra-hospital spread of *E. faecalis* strains (cluster B1), isolated from two patients (A100R3B0 and

A105R2B0) hospitalized in medical ward in the district hospital, which were closely related and shared a common ancestor with one patient (A200R3B0) from the surgical ward of the same hospital. Of note is the fact that these strains were identified at different time-points (after 48h and discharge), confirming the dissemination of this cluster within this hospital. Similarly, *E. faecalis* strains from another cluster (B2) were detected in two patients (A107R1B0 and A210RB0C2) hospitalized in the medical and surgical wards in this hospital, respectively. This suggests that *E. faecalis* B2 strains are circulating within wards in the district hospital, and could be implicated in future nosocomial infections.

MLST-analyses revealed that *E. faecalis* isolated from patient hospitalized in the district hospital belonged to the ST21. This concurs with other studies where the *E. faecalis* ST21 has been described in community patients in Denmark (Jesper et al., 2011) and in clinical samples in Cuba where it concomitantly harboured four virulence factors (esp, efaAfs, gelE, agg, acc) (Quiñones et al., 2009). In addition, one study in Norway implicated this clonal lineage in peripheral periodontitis in hospitalised patients. The authors suggested that this strain *could* be a potential source of transferable antimicrobial resistance due to carriage of several plasmid elements (Song et al., 2013).

The *E. faecalis* strain (G702R1B0) isolated in the tertiary hospital belonged to the ST6. This ST was described in VRE isolated in small mammals in Spain although it harboured the vanB2 gene (Lozano et al., 2015). It has also been identified in clinical samples in Cuba and carried the esp, efaAfs, acc and virulence genes (Quiñones et al., 2009). *E. faecalis* ST6 maybe an example of the selection of an enterococcal sub-population with increased resistance, virulence, and mobility to easily spread among humans.

The fact that all *E. faecalis* STs detected in this study carried a minimum of 14 virulence genes attests to their high pathogenicity (Table 7). The camE gene encoding for sex pheromone activates the conjugation of the plasmid pAM373 that drives the transfer of virulence and resistance determinants among enterococci (Wardal et al., 2010). Additionally, the *fsr*B gene encoding for biofilm formation was associated with ST6 and ST583 with the former isolated in the tertiary hospital and the latter in the district hospital (Table 7).

The *E. faecium* strains, A109R2B0, A201R2B0, A206R1B0 and A206R2B0 belonging to cluster A1, evidenced intra-hospital and inter-ward dissemination in the district hospital (Table 2). However, the detection of one isolate at admission and the other after 48h intimated that

they probably emerged in the community, entered the district hospital, as the first level of care, where they spread across wards (Tables 2 and 3).

MLST analysis of *E. faecium* isolates confirmed a high level of genetic diversity. An interesting finding of this study was the characterization of a novel lineage *E. faecium* ST1386 (1-4-9-6-1-20-3) isolated in the rural, district hospital. In addition, the ST 822, 636, and 97 were identified in the same hospital but in different wards, suggesting that different clonal lineages of *E. faecium* are circulating in this hospital. The detection of these different STs along with two virulence genes (acm and efaAfm) suggests that various clonal lineages of vancomycin-resistant *E. faecium* are actively disseminating within the communities and could enter hospital settings where they could increasingly be associated with high mortality and morbidity rates. The scarcity of data on the population structure of these *E. faecium* STs in African countries makes it difficult to discuss the regional dissemination of these lineages detected in South Africa. Although these STs have rarely been reported in other countries to date, the variability in their allelic profiles shows high levels of diversity amongst *E. faecium,* suggesting non-human origin. This result is similar to the study of Weng et al. (2013) who demonstrated 27 pulsotypes and four STs (ST17, ST78, ST203, ST601) associated with *E. faecium* isolated from clinical samples in a tertiary teaching hospital in Malaysia (Weng et al., 2013).

Our study established the genetic diversity and clonal dissemination of various *E. faecalis* and *E. faecium* lineages across wards and within hospitals in uMgungundlovu district. The presence of plasmids in two *E. faecalis* and all *E. faecium* further contributed to the phenotypic and genotypic plasticity of these resistant bacteria which could be linked to easy transfer of resistance genes and virulence factors.

Conclusion

We report here the faecal carriage of VRE-harboring VanC-1 gene among hospitalized patients in uMgungundlovu district. VRE isolates were genetically diverse and highly pathogenic. Infection control procedures, antimicrobial stewardship and awareness should be strengthened to prevent and/or contain the carriage and spread of VRE in hospitals and communities in South Africa.

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Author contributions

R.C.F co-conceptualized the study, undertook sample collection, microbiological laboratory analyses, prepared tables and figures, contributed to bioinformatics analysis, interpreted results and drafted the manuscript. **L.L.F** undertook sample collection, microbiological laboratory analyses and reviewed the manuscript. **M.A.** undertook bioinformatics analyses. **A.I.** performed whole genome sequencing analysis. **S.Y.E** co-conceptualized the study, vetted the results and undertook critical revision of the manuscript. All authors read and approve the manuscript.

Conflict of interest

Professor Essack is a member of the Global Respiratory Infection Partnership sponsored by Reckitt and Benckiser. All other authors declare that there is no competing financial interest.

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Table 1. Faecal carriage of vancomycin-resistant *Enterococcus spp.* isolated from hospitalized patients in relation to socio-demographic factors, clinical history and diagnosis at admission in a rural district, and an urban tertiary hospital. Out of the 45 patients enrolled, some refused rectal sampling after 48 h and/or at discharge, while some were discharged or transferred after 48 hours, leading to variability in number throughout the sampling period.

Variables	District Rural Hospital; n=27			Tertiary Urban Hospital; n=18		
	Admission, n (%)	**After 48 hours, n (%)**	**At discharge, n (%)**	**Admission, n (%)**	**After 48 hours, n (%)**	**At discharge, n (%)**
Total	12/27 (44)	9/14 (64)	4/4 (100)	7/18 (39)	3/7 (43)	1/2 (50)
Gender						
Female	5 (36)	6 (67)	4 (100)	2 (50)	0 (0)	0 (0)
Male	7 (64)	3 (60)	0 (0)	5 (56)	3 (60)	1 (100)
Previous hospitalization (within one year)						
Yes	3 (50)	1 (50)	1 (100)	3 (75)	1 (33)	0 (0)
No	9 (47)	8 (67)	3 (100)	4 (44)	2 (50)	1 (50)
Antibiotic use (during hospital stay)						
Yes	4 (50)	2 (50)	2 (100)	2 (67)	0 (0)	0 (0)
No	8 (47)	7 (70)	2 (100)	5 (50)	3 (75)	1 (50)
Referral from another hospital						
Yes	1 (100)	0 (0)	0 (0)	3 (50)	1 (33)	0 (0)
No	11 (46)	9 (64)	4 (100)	4 (57)	2 (40)	1 (50)
Hospital ward						
Medicine	8 (53)	5 (56)	2 (100)	3 (60)	2 (40)	0 (0)
Surgery	4 (40)	4 (80)	2 (100)	4 (50)	1 (50)	1 (100)

Table 2. Univariate logistic regression of potential risk factors for VRE carriage in the district and tertiary hospital

Variables	District hospital		Tertiary Hospital	
	Admission OR (95% CI)	**After 48 hours OR (95% CI)**	**Admission OR (95% CI)**	**After 48 hours OR (95% CI)**
Gender (F or M)	**3.15** (0.61-16.31)	0.75 (0.08-7.21)	**1.25** (0.11-13.24)	1
Antibiotic use (Yes or No)	1.13 (0.20-6.04)	0.43 (0.04-4.64)	**2** (0.13-29.80)	1
Previous hospitalization	1.11 (0.17-6.97)	0.50 (0.02-10.25)	**3.75** (0.27-51.37)	0.5 (0.02-11.08)
Transferred from another hospital (Yes or No)	1	1	0.75 (0.08-6.71)	0.5 (0.02-11.08)
Hospital Ward (Medicine or Surgery)	0.58 (0.11-2.95)	**3.2** (0.25-41.21)	0.66 (0.07-6.40)	**1.25** (0.05-40.63)
Hospital (Rural District or Urban Tertiary)	1.26 (0.33-4.84)	0.42 (0.06-2.66)	0.90 (0.14-5.71)	0.35 (0.37-14.65)

Table 3. Multivariate logistic regression of predictors of VRE carriage at admission in the district and tertiary hospital

Variables	District hospital OR (95% CI)	Tertiary Hospital; OR (95% CI)
Gender (F or M)	**4.44** (0.59-33.21)	1.19 (0.09-14.69)
Previous hospitalization (Yes or No)	**1.87** (0.09-36.58)	**3.41** (0.14-81.94)
Current Antibiotic use (Yes or No)	1.46 (0.07-27.66)	1.05 (0.03-32.62)
Referral from another hospital	1	0.95 (0.07-12.83)
Hospital ward (Medicine or Surgery)	0.4 (0.05-2.97)	0.76 (0.05-10.05)

Table 4. Antibiotic resistance profiles of vancomycin-resistant *E. faecium* and *E. faecalis* isolated from hospitalized patients

Antibiotics	District hospital, n=26				Tertiary hospital, n=12			
	E. faecalis (n=12)		*E. faecium* (n=14)		*E. faecalis* (n=11)		*E. faecium* (n=1)	
	MIC (µg/ml) range	No. resistant isolates (%)	MIC (µg/ml) range	No. resistant isolates (%)	MIC (µg/ml) range	No. resistant isolates (%)	MIC (µg/ml) range	No. resistant isolates (%)
Ampicillin	4-≥512	8 (67)	0.5-256	6 (43)	1-≥512	3 (27)	8	0 (0)
Cefoxitin	32-≥512	12 (100)	128-≥512	14 (100)	16-≥512	11 (100)	≥512	1 (100)
Erythromycin	4-≥512	12 (100)	2-≥512	14 (100)	1-≥512	10 (91)	0.5	0 (0)
Clindamycin	2-≥512	12 (100)	8-≥512	14 (100)	4-≥512	11 (100)	128	1 (100)
Teicoplanin	4-≥512	12 (100)	2-≥512	14 (100)	4-≥512	11 (100)	32	1 (100)
Vancomycin	8-≥512	10 (83)	8-≥512	10 (71)	8-≥512	10 (91)	32	1 (100)
Tigecycline	8-128	7 (58)	2-64	10 (71)	8-≥512	10 (91)	16	1 (100)
Fusidic acid	64-≥512	10 (83)	16-≥512	10 (83)	2-256	9 (82)	256	1 (100)
HLR*-Gentamicin	128-≥512	8 (67)	32-≥512	9 (64)	8-≥512	7 (64)	64	0 (0)
HLR-Streptomycin	256-≥512	8 (67)	64-≥512	9 (64)	16-≥512	10 (91)	256	1 (100)
Chloramphenicol	64-≥512	10 (83)	64-≥512	10 (71)	64-≥512	10 (91)	128	1 (100)
Ciprofloxacin	128-≥512	10 (83)	128-≥512	10 (71)	64-≥512	10 (91)	128	1 (100)
Moxifloxacin	16-≥512	10 (83)	8-≥512	10 (71)	2-≥512	10 (91)	2	0 (0)
Doxycycline	16-256	10 (83)	0.5-256	9 (64)	2-512	9 (82)	4	1 (100)
Tetracycline	32-≥512	9 (75)	4-≥512	10 (71)	4-≥512	10 (91)	16	1 (100)
Nitrofurantoin	32-≥512	9 (75)	64-≥512	10 (71)	64-≥512	10 (91)	128	1 (100)
Trimethoprim	≥512	11 (92)	128-≥512	14 (100)	≥512	11 (100)	≥512	1 (100)

***HLR**: High-Level resistance

Table 5. Resistance to selected antibiotics in vancomycin-resistant *E. faecium* and *E. faecalis* isolated from single patients

Patient ID	Isolate	Hospital	Ward	Time-points	REP Cluster	Antibiotics (MIC µg/ml)											
						VAN	TEI	E	CLI	CN	STR	CIP	MOX	FA	DOX	TET	FT
A100	*E. faecalis* (A100R1B0)	District	Medicine	Admission	/	16	8	8	≥512	256	≥512	≥512	512	256	128	256	256
	E. faecalis (A100R2B0)			After 48h	/	8	8	8	≥512	128	≥512	≥512	≥512	256	128	≥512	128
	E. faecalis (A100R3B0)			Discharge	B1	16	32	≥512	≥512	512	≥512	≥512	≥512	256	128	≥512	128
A101	*E. faecalis* (A101R1B0)	District	Medicine	Admission	B2	32	256	≥512	128	512	≥512	≥512	256	256	128	≥512	256
A105	*E. faecalis* (A105R1B0)	District	Medicine	Admission	B3	16	8	8	256	256	≥512	256	≥512	256	16	32	≥512
	E. faecalis (A105R2B0)			After 48h	B1	16	8	8	256	≥512	≥512	≥512	≥512	64	16	32	≥512
A107	*E. faecalis* A107R1B0	District	Medicine	Admission	B2	≥512	8	16	256	128	≥512	≥512	≥512	128	128	≥512	32
	E. faecalis A107R2B0			After 48h	/	16	4	4	256	128	≥512	≥512	≥512	128	256	512	≥512
A108	*E. faecium* A108R1B0C1	District	Medicine	Admission	A4	16	4	16	≥512	128	256	128	128	128	8	64	128
	E. faecium A108R2B0			After 48h	A2	16	8	2	512	32	512	≥512	256	128	16	16	64
A109	*E. faecium* A109R1B0	District	Medicine	Admission	/	16	8	16	8	128	256	128	128	32	64	512	512
	E. faecium A109R2B0			After 48h	A1	≥512	8	32	32	≥512	≥512	≥512	128	16	4	16	≥512
	E. faecium A109R3B0			Discharge	/	≥512	8	8	32	≥512	≥512	≥512	≥512	16	4	16	≥512
A110	*E. faecium* (A110R1B0)	District	Medicine	Admission	/	16	8	≥512	≥512	≥512	≥512	≥512	≥512	32	256	≥512	256
A113	*E. faecalis* (A113R1B0)	District	Medicine	Admission	Singleton 1	16	8	4	256	128	512	128	128	64	128	512	128
A200	*E. faecalis* (A200R3B0)	District	Surgery	Discharge	B1	≥512	≥512	≥512	2	256	≥512	≥512	16	128	32	64	32
A201	*E. faecium* (A201R2B0)	District	Surgery	After 48h	A1	8	4	16	8	128	256	512	512	32	2	4	512
A202	*E. faecium* (A202R2B0)	District	Surgery	After 48h	/	≥512	≥512	4	128	64	128	≥512	128	≥512	64	128	512
	E. faecium (A202R3B0)			Discharge	/	8	2	≥512	64	128	≥512	≥512	≥512	128	128	≥512	≥512

Table 5. Resistance to selected antibiotics in vancomycin-resistant *E. faecium* and *E. faecalis* isolated from single patients (End)

Patient ID	Isolate	Hospital	Ward	Time-points	REP Cluster	Antibiotics (MIC µg/ml)											
						VAN	TEI	E	CLI	CN	STR	CIP	MOX	FA	DOX	TET	FT
A203	*E. faecalis* (A203R2B0)	District	Surgery	After 48h	Singleton 2	≥512	≥512	4	128	≥512	≥512	≥512	≥512	≥512	128	≥512	≥512
A206	*E. faecium* (A206R1B0)	District	Surgery	Admission	A1	8	32	16	128	64	256	≥512	256	64	2	8	≥512
	E. faecium (A206R2B0)			After 48h		8	8	16	8	64	≥512	≥512	256	32	0.5	4	128
A207	*E. faecalis* (A207R1B0)	District	Surgery	Admission	Singleton 3	16	16	4	256	128	≥512	≥512	≥512	128	64	32	256
A209	*E. faecium* (A209R1B0C1)	District	Surgery	Admission	A3	≥512	4	8	32	64	128	128	8	64	128	32	≥512
A210	*E. faecalis* (A210RB0C2)	District	Surgery	Admission	B2	/	32	16	2	128	256	≥512	≥512	64	256	≥512	512
G700	*E. faecalis* (G700R1B0C2)	Tertiary	Medicine	Admission	Singleton 4	16	4	8	256	128	512	256	≥512	64	32	512	256
	E. faecalis (G700R2B0)			After 48h	/	≥512	≥512	2	256	64	512	≥512	≥512	128	256	≥512	≥512
G701	*E. faecalis* (G701R1B0C1)	Tertiary	Medicine	Admission	Singleton 5	16	64	≥512	≥512	≥512	256	≥512	128	32	512	≥512	512
	E. faecalis (G701R2B0C1)			After 48h	Singleton 6	8	4	≥512	≥512	≥512	512	≥512	128	64	128	256	256
G702	*E. faecalis* (G702R1B0)	Tertiary	Medicine	Admission	Singleton 7	16	256	≥512	≥512	≥512	≥512	≥512	512	64	128	512	256
G802	*E. faecalis* (G802R1B0)	Tertiary	Surgery	Admission	Singleton 8	16	64	≥512	4	8	16	≥512	32	2	64	≥512	128
G803	*E. faecalis* (G803R1B0)	Tertiary	Surgery	Admission	Singleton 9	16	4	2	256	128	≥512	≥512	256	256	8	32	128
G805	*E. faecalis* (G805R1B0)	Tertiary	Surgery	Admission	Singleton 12	8	8	≥512	≥512	≥512	256	128	64	128	128	≥512	64
G809	*E. faecalis* (G809R1B0C2)	Tertiary	Surgery	Admission	/	≥512	≥512	16	64	64	256	256	≥512	16	256	≥512	≥512
G812	*E. faecalis* (G812R2B0C1)	Tertiary	Surgery	After 48h	Singleton 13	32	8	1	64	64	256	64	2	128	2	4	128
G812	*E. faecium* (G812R3B0)	Tertiary	Surgery	Discharge	B2	32	32	0.5	128	64	256	128	2	256	4	16	128

Table 6. Resistance profiles and plasmids associated with vancomycin-resistant *E. faecium* and *E. faecalis* isolated from hospitalized patients

Isolate	MLST	Resistance genes																	Plasmids
		VanC-1	VanG	Vex2	Vex3	emeA	Isa	TetM	fosB	ParC	ParE	gyrA	gyrB	ermB	MATE	MFS	pmrA	mepA	Lde	
E. faecalis																				
A113R1B0	**ST21**	+	-	-	-	-	-	+	-	+	+	+	+	+	+	+	+	-	-	-
G701R2B0C1	**ST563**	+	-	-	+	-	-	+	-	+	+	+	+	+	+	+	+	-	-	rep(pUB110); CDS16(pTEF3); repA2(pTEF2); rep(pKH7)
G702R1B0	**ST6**	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-	+	-	repA2(pTEF2); CDS16(pTEF3)
E. faecium																				
A206R2B0	**ST822**	+	-	+	+	-	-	-	-	+	+	+	+	-	+	+	+	-	+	-
A201R2B0	**ST636**	+	-	+	+	-	-	-	-	+	+	+	+	-	+	+	+	-	+	-
A108R2B0	**ST1386***	+	-	+	+	-	-	-	-	+	+	+	+	-	+	+	+	-	+	-
A209R1B0C1	**ST97**	+	-	+	+	-	-	-	-	+	+	+	+	-	+	+	+	-	+	-

***New ST

Table 7. Virulence profiles and plasmids associated with vancomycin-resistant *E. faecium* and *E. faecalis* isolated from hospitalized patients

Isolate	MLST	Virulence genes																Plasmids
		Ace	camE	cCF10	cOB1	ebpA	ebpB	ebpC	efaAfs	ElrA	gelE	hylA	SrtA	tpx	fsrB	hylB	acm	efaAfm	
E. faecalis																			
A113R1B0	**ST21**	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
G701R2B0C1	**ST563**	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	rep(pUB110); CDS16(pTEF3); repA2(pTEF2); rep(pKH7)
G702R1B0	**ST6**	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	repA2(pTEF2); CDS16(pTEF3)
E. faecium																			
A206R2B0	**ST822**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
A201R2B0	**ST636**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
A108R2B0	**ST1386***	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
A209R1B0C1	**ST97**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-

***New ST

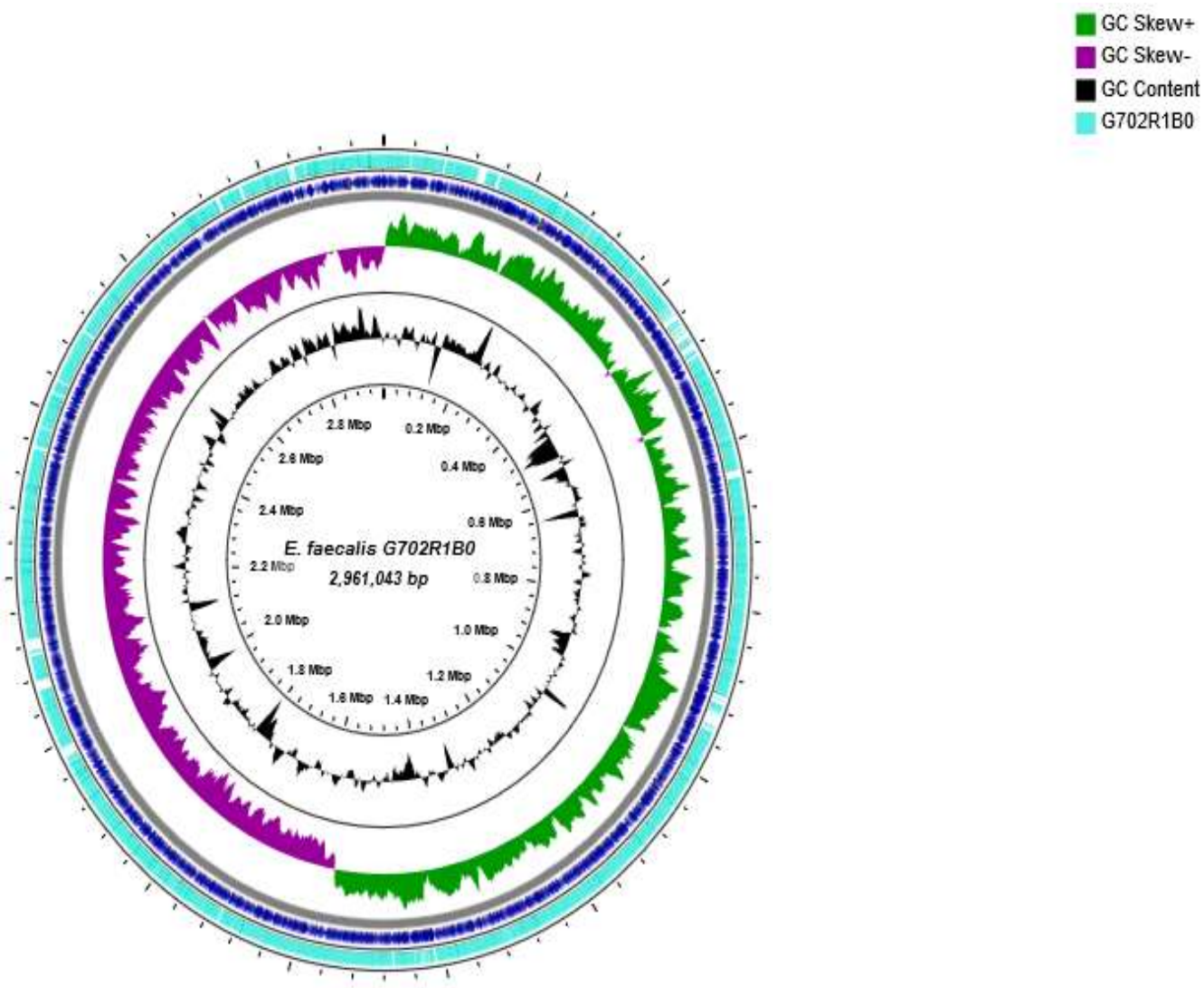
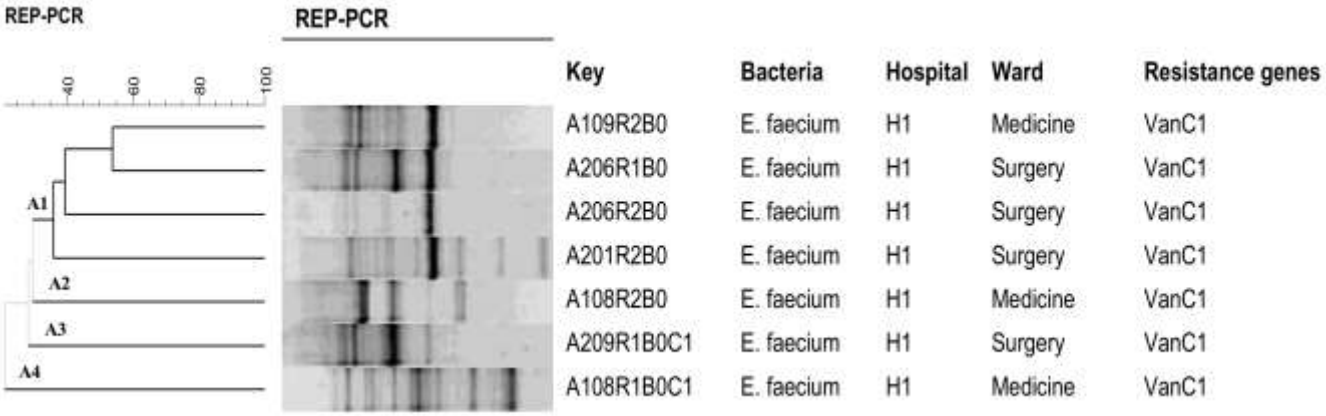
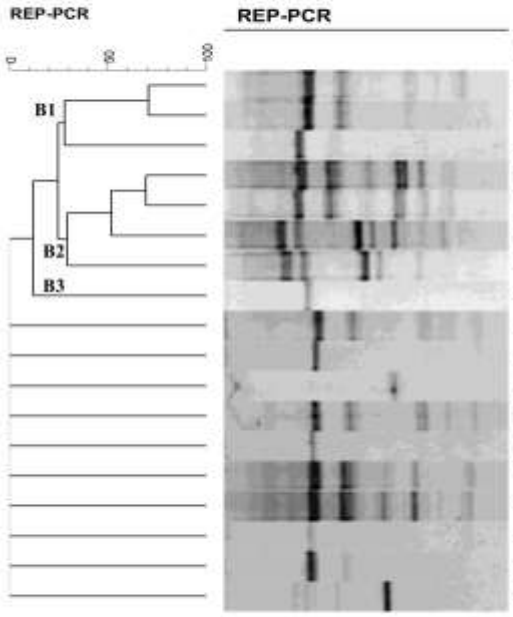


Figure 1. *E. faecalis* G702R1B0 ring representation using CGView Server version 1.0 (Grant et al., 2012). The inner ring shows the percent of identity comparing *E. faecalis* G702R1B0 and the finished genome of *E. faecalis* DENG1 (CP004081.1). The first two inner rings show the GC content and GC skew. The next inner ring, alternating blue and green regions represents the contigs delimitation of G702R1B0. The last outer ring presents the genome of *E. faecalis* G702R1B0.



Key	Bacteria	Hospital	Ward	Resistance genes
A109R2B0	E. faecium	H1	Medicine	VanC1
A206R1B0	E. faecium	H1	Surgery	VanC1
A206R2B0	E. faecium	H1	Surgery	VanC1
A201R2B0	E. faecium	H1	Surgery	VanC1
A108R2B0	E. faecium	H1	Medicine	VanC1
A209R1B0C1	E. faecium	H1	Surgery	VanC1
A108R1B0C1	E. faecium	H1	Medicine	VanC1

A



Key	Bacteria	Hospital	Ward	Resistance genes
A105R2B0	E. faecalis	H1	Medicine	VanC1
A200R3B0	E. faecalis	H1	Surgery	VanC1
A100R3B0	E. faecalis	H1	Medicine	VanC1
G812R3B0	E. faecalis	H2	Surgery	VanC1
A107R1B0	E. faecalis	H1	Medicine	VanC1
A210R1B0C2	E. faecalis	H1	Surgery	VanC1
A101R1B0	E. faecalis	H1	Medicine	VanC1
A105R1B0	E. faecalis	H1	Medicine	VanC1
A113R1B0	E. faecalis	H1	Medicine	VanC1
A203R2B0	E. faecalis	H1	Surgery	VanC1
A207R1B0	E. faecalis	H1	Surgery	VanC1
G700R1B0C2	E. faecalis	H2	Medicine	VanC1
G701R1B0C1	E. faecalis	H2	Medicine	VanC1
G701R2B0C1	E. faecalis	H2	Medicine	VanC1
G702R1B0	E. faecalis	H2	Medicine	VanC1
G802R1B0	E. faecalis	H2	Surgery	VanC1
G803R1B0	E. faecalis	H2	Surgery	VanC1
G805R1B0	E. faecalis	H2	Surgery	VanC1

B

Figure 2. Dendograms of vancomycin-resistant *E. faecium* (A) and *E. faecalis* (B) from faecal carriage of hospitalized patients in South Africa.

CHAPTER IV.

ARTICLE III. Whole Genome Sequence Analysis of Methicillin Susceptible *Staphylococcus aureus* and Methicillin Resistant *Staphylococcus haemolyticus* Isolated from Hospitalized Patients in uMgungundlovu District, South Africa[3]

Author contributions:

- Raspail Carrel Founou, as the principal investigator, co-conceptualized the study, undertook sample collection, laboratory and statistical analyses, prepared tables and figures, and drafted the manuscript.
- Luria Leslie Founou, undertook sample collection, laboratory analysis, vetting of the results, and reviewed the manuscript.
- Mushal Allam, undertook bioinformatics analyses.
- Arshad Ismail, performed whole genome sequencing.
- Sabiha Yusuf Essack, as the principal supervisor, co-conceptualized the study, contributed to vetting of the results and undertook critical revision of the manuscript

Objectives met: This paper addresses objectives Two, Three, Four, Five.

[3] This paper has been submitted to Frontiers in Microbiology and is currently under review.

Whole Genome Sequence Analysis of Methicillin Susceptible *Staphylococcus aureus* and Methicillin Resistant *Staphylococcus haemolyticus* Isolated from Hospitalized Patients in uMgungundlovu District, South Africa

Raspail Carrel Founou[1,2*], Luria Leslie Founou[1,3], Mushal Allam[4], Arshad Ismail[4], Sabiha Yusuf Essack[1]

[1]Antimicrobial Research Unit, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa
[2]Department of Clinical Microbiology, Centre of Expertise and Biological Diagnostic of Cameroon (CEDBCAM), Yaoundé, Cameroon
[3]Department of Food Safety and Environmental Health, Centre of Expertise and Biological Diagnostic of Cameroon (CEDBCAM), Yaoundé, Cameroon
[4]National Institute for Communicable Diseases Sequencing Core Facility, National Institute for Communicable Diseases, Johannesburg, South Africa

Corresponding author:

Raspail Carrel Founou Zangue

Antimicrobial Research Unit

College of Health Sciences

University of KwaZulu-Natal

Durban, 4000

South Africa

czangue@yahoo.fr

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Abstract

Resistance genes, virulence factors and plasmids of *Staphylococcus aureus* and *Staphylococcus haemolyticus* isolated from hospitalized patients in uMgungundlovu District, South Africa, were investigated by whole genome sequencing (WGS).

From May to June 2017, 45 nasal swabs were collected from patients hospitalized in an urban, tertiary and a rural, district hospital in uMgungundlovu District, KwaZulu-Natal, South Africa. Four isolates (two *S. aureus* and two *S. haemolyticus*) were subjected to WGS using an Illumina MiSeq machine with multiplex paired-end libraries prepared from the genomic DNA. The raw reads were assembled using the Qiagen CLC Genomics Workbench and SPAdes. The assembled contigs were annotated with NCBI PGAP, RAST and ARG-ANNOT. ResFinder, VirulenceFinder and PlasmidFinder were used for identification of antibiotic resistance genes, virulence factors and plasmids, respectively.

High resistance to antibiotics attributed to multi-drug resistant efflux pumps mepA, mexE, AcrB, MATE, qac and qacA, were observed. *S. aureus* belonged to the ST121 and *S. haemolyticus* to ST25. There was chromosomal integration of several plasmid replicon types in both species. In addition, *S. aureus* isolates harboured more virulence factors than *S. haemolyticus*, with a total of 18 virulence genes identified whereas the latter was more resistant. Although there was no clear evidence of genetic exchange of resistance or virulence genes from one species to another, there appears to be the potential for horizontal gene transfer that could create a highly resistant and virulent *S. aureus* and/or *S. haemolyticus* strain. Horizontal transfer of antibiotic resistance genes from haemolyticus to S. aureus or virulence factors from *S. aureus* to *S. haemolyticus* should thus be further investigated.

Introduction

Staphylococcaceae is a ubiquitous Gram-positive family that are natural inhabitants of skin, mucosa and anterior nares. Staphylococcal species have been involved in a variety of pathologies including pimples, abscesses, septicaemia, meningitis, pneumonia and toxicosis (Kejela and Bacha, 2013; Njoungang et al., 2015) and are a major cause of infections in hospital and community settings (Cavanagh et al., 2014). The emergence of methicillin resistant staphylococci including *S. aureus* and *S. haemolyticus* are of global public health concerns (Kejela & Bacha, 2013; Njoungang et al., 2015). Methicillin resistant *S. aureus* (MRSA) are one of the most clinically important pathogens in hospital and community settings. In contrast, methicillin resistant *S. haemolyticus* (MRSH) are important nosocomial bacteria in hospitals (Kejela and Bacha, 2013; Cavanagh et al., 2014; Njoungang et al., 2015). Methicillin resistance results from the recombinase-mediated insertion of staphylococcal cassette chromosome mec (*SCC*mec), the mobile genetic element carrying mecA, at the 3' end of a chromosomal open reading frame designated as orfX. The mecA gene encodes for complete resistance to the β-lactam family and various level of co-resistance to other antibiotics. It has been postulated that coagulase-negative staphylococci including *S. haemolyticus* could act as reservoirs of resistance genes harboured on mobile genetic elements (MGEs) including the *SCC*mec, plasmids, prophages, transposons and pathogenicity islands which enable the horizontal transmission of resistance genes within staphylococcal species (Cavanagh et al., 2014; Rossi et al., 2016).

In South Africa, the molecular epidemiology of *S. aureus* and *haemolyticus* is quite complex, and depends on the geographical location, population and medical practices. The objectives of this study were to investigate antibiotic resistance and virulence genes as well as plasmids and clonal lineages of *S. aureus* and *S. haemolyticus* isolated from hospitalized patients in uMgungundlovu District, South Africa using whole genome sequencing (WGS).

Materials and Methods

Ethical considerations

Ethical approval was obtained from the Biomedical Research Ethics committee (BREC) (**No. BF512/16**, **sub-study of BCA444/16**). Permission to conduct the research was also granted from the Department of Health, uMgungundlovu District and hospital managers.

Informed consent was obtained from all participants of the study and a questionnaire survey was undertaken upon verbal and written consent. Data on clinical history were extracted from patient records and data were codified prior to analysis to maintain confidentiality.

Study population and settings

This study conducted over from May to June 2017, at a tertiary and district hospital in uMgungundlovu district, South Africa ascertained the carriage of *Staphylococcus spp.* in patients admitted to surgical and medical wards at admission, after 48h and at discharge as far as possible. Nasal swabs were aseptically collected using Amies medium.

Microbiological analyses

Bacterial identification

Nasal swabs were cultured onto mannitol salt agar (Merck, Darmstadt, Germany) with and without antibiotics using cefoxitin (6 mg/L) and incubated for 18-24h at 37°C to screen for methicillin resistance. Mannitol-fermenting colonies were subjected to Gram staining, catalyse, coagulase, oxidase and DNase tests. API Staph (BioMerieux, Marcy-L'etoile, France) was used to confirm species identification. Pure *S. aureus* and *S. haemolyticus* colonies were stored at −20°C in tryptone soya broth supplemented with 20% glycerol.

Phenotypic screening

The package of MRSA, VISA, GISA, ROSCO Diagnostica Kits (Taastrup, Denmark) was used to screen MRSA, VISA, GISA according to the manufacturer instructions.

Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) were determined by the broth micro-dilution method for ampicillin, cefoxitin, ciprofloxacin, erythromycin, fusidic acid, gentamycin, linezolid, moxifloxacin, oxacillin, rifampicin, streptomycin, tetracycline, teicoplanin, trimethoprim-sulfamethoxazole and vancomycin. The European Committee on Antimicrobial Susceptibility testing (EUCAST) breakpoints (EUCAST, 2016) were used for interpretation of the results and *S. aureus* ATCC 29213 was used as the control.

Genomic analyses

DNA Isolation

The GenElute® bacterial genomic DNA kit (Sigma-Aldrich, St. Louis, MO, USA) was used for genomic DNA extraction according to the manufacturer's instructions. The extracted genomic DNA was verified by agarose gel electrophoresis and quantified via NanoDrop spectrophotometer and fluorimetric analysis (Qubit®).

Genome Sequencing

Genomic DNA was used for library preparation with multiplexed paired-end libraries (2×300 bp) prepared using the Nextera XT DNA sample preparation kit (Illumina, San Diego, CA, USA). An Illumina MiSeq machine was used for the sequencing with 100× coverage, at the National Institute of Communicable Diseases Sequencing Core Facility, South Africa.

Genome Assembly

The raw reads were checked for quality, trimmed and *de novo* assembled using the CLC Genomics Workbench version 10 (CLC, Bio-QIAGEN, Aarhus, Denmark) and SPAdes version 3.5 (Bankevich et al., 2012) to overrule any gaps from both software.

Genome Analysis

The *de novo* assembled reads were uploaded to the NCBI PGAP (https://www.ncbi.nlm.nih.gov/genome/annotation_prok/), RAST (http://rast.nmpdr.org/; Aziz et al., 2008) and ARG-ANNOT (http://en.mediterranee-infection.com/) for annotation. ResFinder (Zankari et al., 2012), VirulenceFinder (Joensen KG et al., 2014), PlasmidFinder (Carattoli et al., 2014) were used for identification of resistance genes, virulence factors and plasmids. The multi-locus sequence type (MLST) was determined from the WGS data. The *S. aureus* G703N1B1 contigs were mapped against the MSSA AUS0325 (Accession number: NZ_LT615218) for visualization of the genome.

Nucleotide sequence accession number

This whole-genome shotgun project PRJNA417366 of *S. aureus* and *S. haemolyticus* strains G703N2B1, G703N1B1, G811N2B1 and A109N1B1 has been deposited at DDBJ/EMBL/GenBank under accession numbers PGWZ00000000, PGXA00000000, PGWX00000000 and PGWY00000000, respectively. The version described in this paper are the versions PGWZ01000000, PGXA01000000, PGWX01000000 and PGWY01000000, respectively.

Results

Phenotypic and genotypic analyses

A total of 45 nasal swabs were obtained from 75 patients at three-time points. Multiple mannitol-fermenting colonies growing on selective agar were isolated from 9 (20%) patients at different time-points. Intriguingly, although all isolates were mannitol-fermenting, *S. aureus*

was isolated from only one patient (11%) at admission (G703N1B1) and after 48 hours (G703N2B1) in the tertiary hospital, with the other mannitol-fermenting colonies being *S. haemolyticus*, *S. epidermidis*, and *S. hominis*. The *S. haemolyticus* selected for the molecular characterization were the most resistant and were isolated from two patients in the tertiary (G811N2B1) and district (A109N1B1) hospitals, at admission and after 48h.

The WGS analyses identified several resistance genes in both *S. aureus* isolates, G703N1B1 and G703N2B1. More specifically, the beta-lactamase (*blaZ)*, tetracycline (tetK), fluoroquinolone including Topo-isomerase (ParC, ParE, norA) and DNA gyrase (gyrA, gyrB), fosfomycin (fosD, fosB) Teicoplanine (TcaA, TcaB, TcaR), trimethoprim (dfrG) and multi-drug resistance efflux pumps (MATE, AcrB) genes were detected (Table 1). In addition, both isolates of *S. aureu*s harboured eighteen virulence genes including aureolysin (*aur*), beta-hemolysin (*hlb*), gamma-hemolysin chain II precursor (*hlg*A), gamma-hemolysin component B precursor (*hlg*B), gamma-hemolysin component B precursor (*hlg*C), leucocidin D component (lukD), leucocidin E component (lukE), staphylokinase (sak), staphylococcal complement inhibitor (scn), enterotoxin G (seg), enterotoxin I (sei), enterotoxin M (sem), enterotoxin N (sen), enterotoxin O (seo), enterotoxin U (seu), serine protease splA, serine protease splB and exfoliative toxin A (*eta*) (Table 1).

In contrast, *S. haemolyticus* isolates carried more resistance genes than *S. aureus* especially against aminoglycosides [ant(6)-Ia, aac(6')-aph(2''), aph(3')-III, aac(6')-aph(2'')], fusidic acid (fusB), MLS [(msr(A), mph(C), vga(A)], fluoroquinolones (parC, parE, gyrA and gyrB), teicoplanin (TcaS, TcaB). The multidrug and toxin extrusion (MATE) transport protein, mepA encoding for resistance to tigecycline, the multidrug exporter AcrB and mexE from the resistance-nodulation-cell division (RND) family, the multi-drug resistant (MDR) efflux pump (qacA) and small MDR (SMR) family (qac) were additionally detected (Table 1). It is important to mention that none of the *S. haemolyticus* carried virulence factors.

Three plasmid replication proteins including repL(pDLK1), rep(SAP060B), rep(SAP015B) were detected in the *S. aureus* isolate G703N2B1. PlasmidFinder detected repA(VRSAp) in one *S. haemolyticus* isolate (A811N2B1). MLST-analyses revealed that both MSSA strains belonged to ST121 with 100% identity among all seven housekeeping genes (6-5-6-2-7-14-5) and all *S. haemolyticus* were assigned to ST25.

Discussion

This study undertook the molecular characterization of *S. aureus* and *S. haemolyticus* isolated from hospitalized patients in uMgungundlovu district, South Africa. Our results show that the highly-virulent community-acquired methicillin susceptible *S. aureus* (CA-MSSA) ST121 is present in South Africa. This concurs with the study from Rao et al., (2015) which revealed that the genetic lineage ST121 is an emerging and hypervirulent clone. Our *S. aureus* ST121 isolates are, in contrast to the rest of the world, implicated in nasal carriage from where they could subsequently cause infections ranging from minor superficial to severe invasive infectious diseases. To the best of our knowledge, this is the first report of CA-MSSA ST121 isolated from a nasal carriage sample in the country.

The detection of 18 virulence genes in the CA-MSSA ST121 in carriage samples of hospitalized patients revealed that this genetic lineage could probably contribute to severe outbreak situations not only in communities but also in hospitals if this clone spreads to immune-compromised people and infection control measures are not sufficiently implemented. Although not methicillin resistant, the CA-MSSA ST121 isolates were resistant to several antibiotics including erythromycin, gentamicin, ciprofloxacin, tigecycline, chloramphenicol, vancomycin, doxycycline and tetracycline, and were consequently MDR. We speculated that the presence of the numerous MDR efflux pumps belonging to MATE such as mepA, as well as the multidrug exporter AcrB fitting into the RND family, could have likely contributed to the observed multi-drug resistance.

Despite the fact that both CA-MSSA isolates were detected in the same patient at admission and after 48h in the district hospital, and harbored similar resistance genes, the *S. aureus* A703N2B1C1 isolated after 48h additionally carried the tet(K) gene and three plasmid incompatibility groups [rep(pDLK1, rep(SAP060B) and rep(SAP015B)] while the *S. aureus* A703N1B1C1 detected at admission did not harbor plasmid. This intimated the acquisition of the tet(K) gene and plasmids by horizontal transmission between bacterial species. In fact, since numerous staphylococcal species are natural colonizers of the human flora, they are close enough to allow for genetic exchange to occur through conjugation, transduction and transposition. Several studies have already revealed the possible horizontal transmission of resistance genes between *S. haemolyticu*s and *S. aureus* (Berglund and Söderquist, 2008; Rossi et al., 2016). A Swedish study showed that MRSH had horizontally transferred the *SCCmec* to an MSSA ST45 strain, resulting in MRSA responsible of an outbreak (Berglund and

Söderquist, 2008). Similarly, Rossi et al., (2016) described horizontal transmission of mupirocin resistance from *S. haemolyticus* to *S. aureus* through conjugative and mobilizable plasmids (Rossi et al., 2016). Even though direct evidence for *S. haemolyticus* mediated horizontal gene transfer to *S. aureus* is missing, this is indeed possible because of the genomic plasticity of the identified *S. haemolyticus*, especially the isolates A109N1B1 and G811N2B1 which harbored a total of 20 and 25 resistance genes, respectively (Table 1).

Our study showed that all MRSH isolates belonged to ST25 were not only methicillin resistant but also MDR (Table 1). They possessed many antibiotic resistance genes responsible for the high resistance to antibiotics observed (Table 2). The identification of the *S. haemolyticus* ST25 in two different levels of care and at two different time-points (admission and after 48h), demonstrates that they probably disseminate in South African public hospitals. *S. aureus* virulence factors were highlighted and contextualized against what is considered a "benign" specie, *S. haemolyticus*. Although there was no clear evidence of genetic exchange of resistance or virulence genes from one species to another, there appears to be the potential for horizontal gene transfer that could create a highly resistant and virulent *S. aureus* and/or *S. haemolyticus* strain. Horizontal transfer of antibiotic resistance genes from *S. haemolyticus* to *S. aureus* or virulence factors from *S. aureus* to *S. haemolyticus* should thus be further investigated. Genomic data of these bacteria could provide better understanding of their evolution to guide interventions for their containment.

Conclusion

The study evidenced the presence of highly-virulent *S. aureus* and MDR *S. haemolyticus* in nasal carriage samples in South African public hospitals. The possibility of horizontal transfer of antibiotic resistance genes from *S. haemolyticus* to *S. aureus* or virulence factors from *S. aureus* to *S. haemolyticus* should be investigated in order to palliate to potential outbreaks. The observed resistance to last resort antibiotics is of concern, necessitating antibiotic stewardship, routine screening of patients and stringent infection control measures.

Author contributions

R.C.F co-conceptualized the study, undertook sample collection, microbiological laboratory analyses, prepared tables and figures, contributed to bioinformatics analysis, interpreted results and drafted the manuscript. **L.L.F** undertook sample collection, microbiological laboratory analyses, contributed to bioinformatics analysis and vetting of the results. **M.S.** undertook bioinformatics analyses. **A.I.** performed whole genome sequencing analysis. **S.Y.E** co-

conceptualized the study, undertook vetting of the results and critically reviewed the manuscript. All authors read and approve the manuscript.

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Conflict of Interest

Professor Essack is a member of the Global Respiratory Infection Partnership sponsored by an unrestricted educational grant from Reckitt and Benckiser. All other authors declare that there is no competing financial interest.

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Table 1. Demographic, phenotypic and genotypic data of the MSSA and MRSH isolates

Isolate name	Patient's details		Hospital	Time-points	Resistance genes	Virulence factors	Plasmids	MLST
	Gender	Age						
S. aureus								
G703N1B1C1	F	37	Tertiary	Admission	blaZ, nor(A), fosD, dfrG, parC, parE, gyrA, gyrB, fosB, TcaA, TCaB, MATE, AcrB	Aur, hlb, hlgA, hlgB, hlgC, lukD, lukE, sak, scn, seg, sei, sem, sen, seo, seu, splA, splB, eta	/	ST121
G703N2B1C1				After 48h	blaZ, nor(A), fosD, dfrG, tet(K), parC, parE, gyrA, gyrB, fosB, TcaA, TCaB, MATE, AcrB	Aur, hlb, hlgA, hlgB, hlgC, lukD, lukE, sak, scn, seg, sei, sem, sen, seo, seu, splA, splB, eta	repL(pDLK1), rep(SAP060B), rep(SAP015B)	ST121
S. haemolyticus								
A109N1B1	F	78 District		Admission	mecA, blaZ, aac(6')-aph(2"), aac(6')-Ia, ant(6)-Ia, norA, msr(A), mph(C), dfrG, mepA, qac, bacA, MATE, AcrB, TcaA, TcaB, parC, parE, gyrA, gyrB	/	/	ST25
G811N2B1	F	30	Tertiary	After 48h	mecA, blaZ, aac(6')-aph(2"), aac(6')-Ia, aph(3')-IIIa, ant(6)-Ia, norA, fusB, msr(A), mph(C), vga(A), dfrG, mepA, qac, qacA, mexE, fusB, MATE, AcrB, TcaA, TcaB, parC, parE, gyrA, gyrB	/	RepA(VRSAp)	ST25

Table 2. Antimicrobial susceptibility to selected antibiotics in individual staphylococcal isolates

Isolate name	Cefoxitin	Amikacin	Gentamicin	Tobramycin	Ciprofloxacin	Moxifloxacin	Erythromycin	Clindamycin	Linezolid	Teicoplanin	Vancomycin	Tetracycline	Doxycycline	Tigecycline	Fusidic acid	Trimethoprim	Nitrofurantoin	Antibiotic resistance genes
S. aureus																		
G703N1B1C1	≥64	4	0.5	0.5	≥512	32	≥512	2	32	64	16	≥512	128	128	128	≥512	64	blaZ, nor(A), fosD, dfrG, parC, parE, gyrA, gyrB, fosB, TcaA, TCaB, MATE, AcrB
G703N2B1C1	≥64	2	0.25	0.5	≥512	32	≥512	2	32	64	16	≥512	128	64	128	≥512	32	blaZ, nor(A), fosD, dfrG, tet(K), parC, parE, gyrA, gyrB, fosB, TcaA, TCaB, MATE, AcrB
S. haemolyticus																		
A109N1B1	≥512	32	256	128	≥512	32	256	2	16	64	16	8	4	16	32	≥512	64	mecA, blaZ, aac(6')-aph(2"), aac(6')-Ia, ant(6)-Ia, norA, msr(A), mph(C), dfrG, mepA, qac, bacA, MATE, AcrB, TcaA, TcaB, parC, parE, gyrA, gyrB
G811N2B1	32	2	1	2	8	0.5	1	1	32	64	8	16	1	2	64	≥512	32	mecA, blaZ, aac(6')-aph(2"), aac(6')-Ia, aph(3')-IIIa, ant(6)-Ia, norA, fusB, msr(A), mph(C), vga(A), dfrG, mepA, qac, qacA, mexE, fusB, MATE, AcrB, TcaA, TcaB, parC, parE, gyrA, gyrB

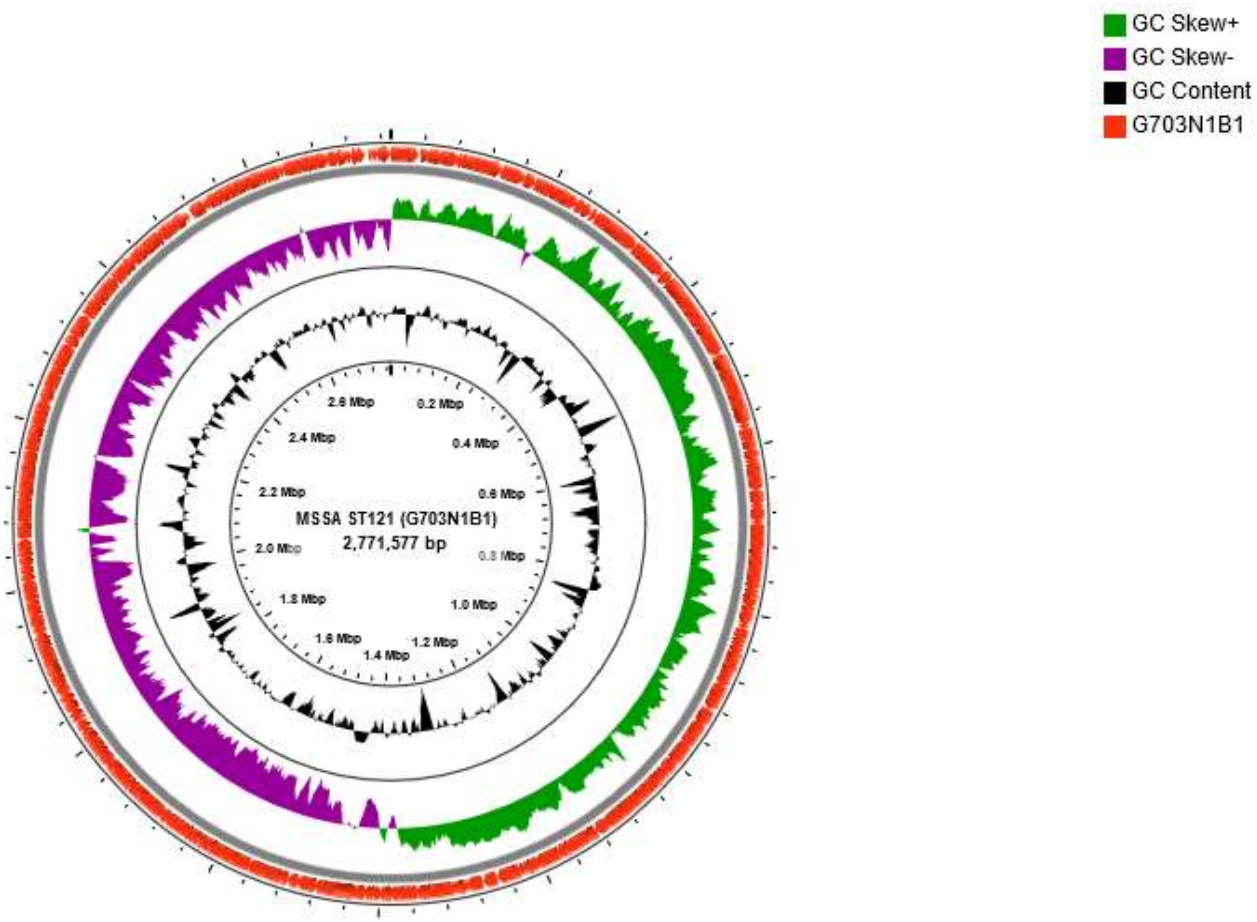

Figure 1. MSSA G703N1B1 ring representation using CGView Server V 1.0 (Grant et al., 2012). The inner ring shows the percent of identity comparing MSSA G703N1B1 and the complete genome of MSSA AUS0325 (NZ_LT615218). The first inner rings show the GC content. The next inner ring, alternating green and purple represents the GC skew. The last outer ring presents the genome of *S. aureus* G703N1B1.

CHAPITRE V.

ARTICLE 4. Clinical and Economic Impact of Antibiotic Resistance in Developing Countries: A Systematic Review and Meta-Analysis[4]

Author contributions:

- Raspail Carrel Founou, as the principal investigator, co-conceptualized the study, searched the literature, screened titles and abstracts, extracted and collated data, performed the quality assessment and statistical analyses, and drafted the manuscript.
- Luria Leslie Founou extracted and collated data, performed the quality assessment and statistical analyses, and reviewed the manuscript.
- Sabiha Yusuf Essack, as principal supervisor, co-conceptualized the study, screened titles, abstracts and full-texts, and undertook critical revision of the manuscript.

Objective met: This paper addresses the objective six i.e. to comprehensively analyse published literature on clinical and economic implications of AMR in developing countries, to ascertain the impact of resistant bacteria and to underscore the necessity to implement and improve antimicrobial stewardship programs and infection prevention and control measures in these nations using a review paper.

[4] This paper has been accepted for publication, pending corrections by PloS One

Clinical and economic impact of antibiotic resistance in developing countries: a systematic review and meta-analysis

Raspail Carrel Founou[1,2,*], Luria Leslie Founou[1,3] , Sabiha Yusuf Essack[1]

[1]Antimicrobial Research Unit, School of Health Sciences, University of KwaZulu-Natal, Durban, South Africa

[2]Department of Clinical Microbiology, Centre of Expertise and Biological Diagnostic of Cameroon, Yaoundé, Cameroon

[3]Department of Food Safety and Environmental Microbiology, Centre of Expertise and Biological Diagnostic of Cameroon, Yaoundé, Cameroon

***Corresponding author**

Email: czangue@yahoo.fr (RCF)

Abstract

Introduction: Despite evidence of the high prevalence of antibiotic resistant infections in developing countries, studies on the clinical and economic impact of antibiotic resistance (ABR) to inform interventions to contain its emergence and spread are limited. The aim of this study was to analyze the published literature on the clinical and economic implications of ABR in developing countries.

Methods: A systematic search was carried out in Medline via PubMed and Web of Sciences and included studies published from January 01, 2000 to December 09, 2016. All papers were considered, and a quality assessment was performed using the Newcastle-Ottawa quality assessment scale (NOS).

Results: Of 27 033 papers identified, 40 studies met the strict inclusion and exclusion criteria and were finally included in the qualitative and quantitative analysis. Mortality was associated with resistant bacteria, and statistical significance was evident with an odds ratio (OR) 2.828 (95%CI, 2.231-3.584; p= 0.000). ESKAPE pathogens was associated with the highest risk of mortality and with high statistical significance (OR 3.217; 95%CIs; 2.395-4.321; p=0.001). Eight studies showed that ABR, and especially antibiotic-resistant ESKAPE bacteria significantly increased health care costs.

Conclusion: ABR is associated with a high mortality risk and increased economic costs with ESKAPE pathogens implicated as the main cause of increased mortality. Patients with non-communicable disease co-morbidities were identified as high-risk populations.

Introduction

Antimicrobial resistance (AMR) is the ability of bacteria, parasites, viruses and fungi to grow and spread in the presence of antimicrobial medicines that are normally active against them. AMR occurs via a range of resistance mechanisms, such as a modified antimicrobial target, enzymatic hydrolysis/degradation, efflux and impermeability. This resistance is mediated by diverse resistance genes that evolve as a result of antimicrobial selection pressure exerted by the appropriate and/or inappropriate use of antimicrobial medicines, and is aggravated by the void of new antimicrobial agents in the current therapeutic pipeline (1, 2). AMR increases health-care costs, length of stay in hospitals, morbidity and mortality in both developed and developing countries (3). A recent report estimated that 10 million deaths will be attributed to AMR by 2050, and 100 trillion USD of the world's economic outputs will be lost if substantive efforts are not made to contain this threat (1, 4, 5).

The World Health Organization (WHO) published the first global surveillance report on antibiotic resistance (ABR) in 2014 to show the clinical impact of resistant bacteria in WHO regions across the world. This report showed that five out of the six WHO regions had more than 50% resistance to third generation cephalosporins and fluoroquinolones in *Escherichia coli* and methicillin resistance in *Staphylococcus aureus* in hospital settings. Similarly, more than 50% resistance to third generation cephalosporins and carbapenems was reported in *Klebsiella pneumoniae*. The report attributed 45% of deaths in both Africa and South-East Asia to multi-drug resistant (MDR) bacteria. It further revealed that *K. pneumonia*e resistant to third generation cephalosporins was associated with elevated deaths in Africa (77%), the Eastern Mediterranean region (50%), South East Asia (81%) and Western Pacific region (72%) (2).

Several resistant bacteria have been increasingly involved in infectious diseases in humans, specifically, *Enterococcus spp.*, *S. aureus*, *K. pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter spp*. They are collectively termed ESKAPE and recently gained further global attention by being listed by the WHO as priority antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics (5). The particularity of these bacteria is their ability to develop high level resistance to multiple drugs, thereby limiting therapeutic options and increasing morbidity and mortality. Numerous studies have confirmed that ESKAPE bacteria and their resistant clones, are actively transmitted in hospitals and communities

in both developed and developing countries. The threat posed by these resistant bacteria is however exacerbated in developing countries due to sub-optimal hygiene conditions, poor infection, prevention and control measures, lack of surveillance and the dearth antimicrobial stewardship programs (6, 7). Reports have shown high isolation rates of methicillin resistant *S. aureus* (MRSA) in healthcare settings in Cameroon (72%), South Africa (52%), Ethiopia (42.8%), Nigeria (29.6%), Kenya (27.7%), Ivory Cost (16.8%) and Morocco (14.4%) (2, 8-10). In 2008, the prevalence of nosocomial acquired and MDR infections due to Enterobacteriaceae isolated from blood cultures were 57.1% and 15.4% respectively, in South Africa (11). Likewise, rapid increases in the rates of infections due to carbapenemase-producing *K. pneumonia*, metallo-beta-lactamase-producing *A. baumannii* (MBL-AB), metallo-beta-lactamase-producing *P. aeruginosa* (MBL-PA), and extended-spectrum beta-lactamase (ESBL) producing *Enterobacter spp.* have been reported across the world (12-14). In Saudi Arabia, the rate of *P. aeruginosa* producing carbapenemase was 33%, of which 27% were MBL-producers (15), while in India, a 22.4% prevalence of *P. aeruginosa* producing MBLs was reported in tertiary care hospitals (16).

MDR-ESKAPE bacteria have been reported in hospital acquired infections (HAI), particularly in intensive care units (ICUs) where immune-compromised patients suffering from some non-communicable diseases (NCDs) including diabetes, cancers, chronic lung, cardiovascular and kidney diseases were highly affected (6, 17-22). The emergence and spread of these highly resistant bacteria in hospital care settings could thus have negative health repercussions and be an obstacle for the treatment of infections of patients with these NCDs (18, 23).

Despite the evidenced threat posed by ABR, information on its clinical and economic impact is limited in developing countries, and thus impede appropriate interventions for its containment (24, 25). Heightened awareness of policy-makers, health care workers, and the general population about the risks associated with ABR is essential to preserve antibiotics for future generations (26, 27). Hence, the aim of this study was to analyze the published literature on the clinical and economic impact of ABR in developing countries, in order to inform containment strategies such as antimicrobial stewardship programs and infection prevention and control measures in these nations.

Methods

The Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) and Meta-analysis of Observational Studies in Epidemiology (MOOSE) guidelines were followed (28, 29).

Ethical consideration

This systematic review and meta-analysis was based on published reports, and was therefore exempt from ethical approval.

Systematic review of the literature

A systematic search was carried out independently by RF and LF, in Medline via PubMed and Web of Sciences from January 2000 to December 09, 2016, using a combination of boolean operators (AND/OR), Medical Subject Heading (MeSH) and pre-defined keywords. Only papers published after 2000 were considered to ensure that the analysis focuses on contextual literature that depict current resistance patterns, infection rates, prevention measures, and clinical practice guidelines. Peer-reviewed papers in English and French on the clinical and/or economic impacts of ABR in developing countries were retrieved and independently evaluated for eligibility by RF and LF based on titles and abstracts (Table 1). Thereafter, the full-texts of eligible papers were assessed according to pre-defined inclusion and exclusion criteria (Table 1), with inconsistencies and disagreements being resolved by consensus. Efforts were made to contact the authors when data was missing and full-texts could not be retrieved, and a hand search was conducted in the reference list of all selected papers.

Table 1. Eligibility criteria

Inclusion criteria
- Original research - Minimum of 20 patients - Studies conducted in developing countries as defined by World Bank criteria - Report on association between resistant bacteria and clinical outcome and/or financial impact - Antimicrobial susceptibility testing done by either disk diffusion, broth micro-dilution, agar dilution, E-test or VITEK using - CLSI/EUCAST/SFM guidelines - Papers published in French and English - Studies published from January 1, 2000
Exclusion criteria

- Reports of antibiotic resistance unrelated to clinical outcome nor economic impact
- Reports on parasites, viruses and fungi
- Reports on treatment comparisons
- Studies conducted in developed countries as defined by World Bank criteria
- Reports published in languages other than French and English
- Antibiotic resistance in wildlife, companion and aquatic animals
- Grey literature, conference abstracts, reviews, meta-analysis, letters to editor, correspondence, editorials, comments and case reports.
- Studies published before January 1, 2000

Screening and data extraction process

Papers were managed using EndNote (version X7.7.1, Thomson Reuters) and the data from eligible papers was abstracted independently by two authors (RF and LF) using a standardized data extraction spreadsheet in Excel® (Microsoft® Office Excel 2016). Relevant data from papers included countries, WHO regions, World Bank classification, publication year, type of study, participant characteristics (number of participant, diseases, age), hospital' ward, bacteria, follow-up period, length of stay in hospital, mortality related to resistant bacteria, and, costs as described in Table 2.

Table 2. Description of eligible papers included in the systematic review

Country	Year	Type of study	Study population	Infection type	Hospital' ward	Bacteria	Sample size cases/ controls	Length of stay[2] (%)		Mortality[3] n/N (%)		References
								Case group	Control group	Case group	Control group	
STUDIES REPORTING IMPACT OF ABR ON THE MORBIDITY ONLY												
Turkey	2015	Retrospective cohort	NR	Nosocomial BSI	ICU	*A. baumannii*	41/45	25.49 days (%NR)	22.80 days (%NR)	NR	NR	Gulen et al., 2015 (3)
Turkey	2008	Prospective case—control	Adults>16 years old	Nosocomial Infections	ICU and others	*A. baumannii*	66/57	20.8 days (65.2%)	15.4 days (40.4%)	NR	NR	Baran et al., 2008 (30)
STUDIES REPORTING IMPACT OF ABR ON THE MORTALITY ONLY												
Brazil	2009	Retrospective case-control	Adults >14 years old	Nosocomial infections	Medical-surgical ICU	*P. aeruginosa*	63/182	NR	NR	31/63 (49%)	61/182 (33%)	Furtado et al., 2009 (31)
Brazil	2009	Case-control	Adults > 18 years old	BSI	NR	*E. coli* and *K. pneumoniae*	30/64	NR	NR	7/30 (23.3%)	12/64 (18.8%)	Serefhanoglu et al., 2009 (32)
China	2004	Case-control and Retrospective cohort	All ages	MDR-HAI	Various wards[1]	*P. aeruginosa*	44/68	NR	NR	24/44 (54.5%)	11/68 (16.2%)	Cao et al., 2004 (33)
China	2012	Retrospective	Children < 15 years old	Pneumonia	Pediatric ICU	*A. baumannii*	115/45	NR	NR	21/115 (18.26%)	2/45 (4.44%)	Cai et al., 2012 (34)
China	2015	Retrospective Case-Control	NR	MRSA infections	Various	*S. aureus*	57/116	NR	NR	12/57 (21%)	9/116 (8%)	Yao et al., 2015 (35)
Colombia	2014	Case-Control	All ages	CR-KP Infection	ICU	*K. pneumoniae*	61/122	NR	NR	31/61 (50.8%)	25/122 (20.4%)	Gomez et al., 2014 (36)
India	2014	NR	Neonates	BSI	Neonatal ICU	*A. baumannii*	33/32	NR	NR	9/33 (27.3%)	3/32 (9.4%)	Kumar et al., 2014 (37)
Malaysia	2009	Case-control	NR	Nosocomial AB BSI	NR	*A. baumannii*	53/56	NR	NR	25/53 (47.2%)	14/56 (25%)	Deris et al., 2009 (38)

Malaysia	2011	Cross-sectional descriptive and case-control	NR	IR-*A. baumannii* BSI	NR	*A. baumannii*	15/41	NR	NR	9/15 (64.3%)	15/41 (40.5%)	Deris et al., 2011 (39)
Mexico	2000	Case-control	Children	Pneumoniae	NR	*S. pneumoniae*	25/24	NR	NR	11/25 (44%)	7/24 (29%)	Gomes et al., 2000 (40)
Thailand	2011	Case-control	Adults >15 years old	MDR-*A. baumannii* bacteremia	In and out-patient departments	*A. baumannii*	24/25	NR	NR	22/24 (91.7%)	12/25 (48%)	Anunnatsiri et al., 2011 (41)
Thailand	2012	Case-control	Adults >15 years old	ESBL-producing bacteria in septicemia	In and out-patient departments	*E. coli*	32/113	NR	NR	9/32 (29%)	13/113 (11.5%)	Anunnatsiri et al., 2012 (42)
Thailand	2015	Case-control	Adults>18 years old	HAI	ICU and general wards	*A. baumannii*	139/132	NR	NR	79/139 (57%)	3/132 (2%)	Chusri et al., 2015 (43)
Thailand	2015	Retrospective cohort	Adults	Ventilator Associated Pneumoniae	ICU	*A. baumannii*	220/33	NR	NR	125/220 (56.8%)	7/33 (21.2%)	Inchai et al., 2015 (44)
STUDIES REPORTING IMPACT OF ABR ON THE MORBIDITY AND MORTALITY												
Brazil	2015	Case-control	Cancer children <18 years old	MDR-GNB Infection	Oncology pediatric ICU	Gram Negative Bacteria	47/54	8 days (63.8%)	2 days (37%)	12/47 (25.5%)	9/54 (16.7%)	Costa et al., 2015 (17)
Brazil	2006	Retrospective cohort	>1-year-old	BSI	Various wards[1]	*S. aureus*	61/50	>10 days (65.9%)	>10 days (34.1%)	33/61 (54.9%)	12/50 (24.7%)	Guilande et al., 2006 (45)
Brazil	2006	Retrospective cohort	All ages	BSI	Various wards[1]	*K. pneumoniae*	56/52	>10 days (56.2%)	>10 days (43.8%)	18/56 (69.2%)	8/52 (30.8%)	Marra et al., 2006 (46)
Brazil	2008	Case-control	Adults	VAP	ICU	*S. aureus*	29/32	>8 days (89.7%)	>8 days (90.6%)	11/29 (37.9%)	8/32 (25%)	Moreira et al., 2008 (47)
Brazil	2012	Case-control	Adults > 18 years old	Bacteremia	ICU	*P. aeruginosa*	29/48	43 days (NR)	43.1 days (NR)	13/29 (44.8%)	26/48 (54.2%)	Tuon et al., 2012 (22)
China	2012	Retrospective cohort	> 1 year old	BSI	Various wards[1]	*S. aureus*	75/43	55.3 days (NR)	38.7 days (NR)	25/75 (33.3%)	8/43 (18.6%)	Chen et al., 2012 (48)

China	2015	Retrospective	Geriatric inpatients	Bacteremia	Various wards[1]	*A. baumannii*	39/86	36.7 days (NR)	36.1 days (NR)	31/39 (79.5 %)	38/86 (44.2%)	Fu et al., 2015 (49)
China	2015	Retrospective case-control	NR	Enterococci infections	Various wards[1]	*Enterococci*	44/176	37 days (NR)	17 days (NR)	3/44 (6.8%)	3/176 (1.7%)	Jia et al., 2015 (50)
Colombia	2014	Prospective cohort	Adult	CR-*A. baumannii* Infections	ICU	*A. baumannii*	104/61	19 days (NR)	16.2 days (NR)	42/104 (40%)	13/61 (21%)	Lemos et al., 2014 (51)
India	2014	Observational	Adults	Septicemia	Various wards	GNB and GPB	133/87	14 days (NR)	11 days (NR)	16/133 (12%)	2/87 (2%)	Chandy et al., 2014 (52)
Jordan	2015	Matched case-control	Cancer patients	Nosocomial *A. baumannii* infections	Medical-surgical ICU	*A. baumannii*	161/262	12 days (NR)	3 days (NR)	118/161 (73.3%)	142/232 (61.2%)	Nazer et al., 2015 (53)
Palestine	2009	Prospective case—control	Neonates	Nosocomial septicemia	Neonatal ICU	*A. baumannii*	40/100	20 days (62.5%)	20 days (35%)	15/40 (37.5%)	12/100 (13.2%)	Aljarousha et al., 2009 (54)
Senegal	2016	Classic retrospective cohort and retrospective parallel cohort	All ages	ESBL-producing Enterobacteriaceae	Various wards[1]	*K. pneumoniae Enterobacter E. coli*	110/76	22.6 days (NR)	14 days (NR)	52/110 (47.3 %)	17/76 (22.4 %)	Ndir et al., 2009 (55)
Thailand	2007	Prospective case—control	Adults	HAI	Various wards[1]	*E. coli* and *K. pneumoniae*	74/74	22.5 days (NR)	17.5 days (NR)	26/74 (35.1%)	12/74 (16.2%)	Apisarnthanarak et al., 2007 (56)
Thailand	2008	Cohort	Adults	Community-onset BSI	Various wards[1]	*E. coli* and *K. pneumoniae*	36/108	8 days (NR)	6 days (NR)	13/36 (36%)	16/108 (15%)	Apisarnthanarak et al., 2008 (57)
Thailand	2014	Retrospective cohort	Adults>18 years old	HAI	Various wards[1]	*A. nosocomialis* and *A. pittii*	25/58	9 days (NR)	4 days (NR)	3/25 (12%)	20/58 (35%)	Chusri et al., 2014 (58)
Thailand	2009	Retrospective cohort	Adult> 15 years old	Nosocomial BSI	Various wards[1]	*A. baumannii*	67/131	37 days (NR)	27 days (NR)	35/67 (52.2%)	26/131 (19.9%)	Jamulitrats et al., 2009 (59)
Thailand	2006	Cross-sectional	All ages	Community-acquired pneumoniae	NR	*S. pneumoniae*	22/42	12.2 days (NR)	15.5 days (NR)	2/22 (9.1%)	4/42 (9.5%)	Reechaipichikul et al., 2006

												(60)
Thailand	2009	Case-control	Adult>18 years old	Nosocomial BSI	Various wards[1]	*E. coli* and *K. pneumoniae*	51/94	26 days (NR)	16 days (NR)	26/51 (51.0%)	28/94 (29.8%)	Superti et al., 2009 (61)
Thailand	2013	Retrospective Case-control	Neonates	CR- *A. baumannii* Bacteremia	Neonatal ICU	*A. baumannii*	14/44	34 days (NR)	24.5 days (NR)	6/14 (42.9%)	3/44 (5.9%)	Thatrimontrichai et al., 2013 (62)
Thailand	2016	Retrospective Case-control	Neonates	VAP	Neonatal ICU	*A. baumannii*	63/25	51 days (NR)	41 days (NR)	10/63 (15.9%)	0/25 (0%)	Thatrimontrichai et al., 2016 (19)
Turkey	2015	Observational retrospective cohort	All ages	HAI	ICU	*K. pneumoniae*	47/51	19 days (37.3%)	11 days (29.94%)	21/47 (44.7%)	26/51 (51%)	Candevir et al 2015 (63)
Turkey	2000	Retrospective	Adults	Bacteremia	ICU	*S. aureus*	46/55	50.3 days (NR)	32.7 days (NR)	15/46 (32.6%)	7/55 (12.7%)	Topeli et al., 2000 (21)
Turkey	2015	NR	NR	Nosocomial infections	Emergency ICU and Pediatric ICU	*P. aeruginosa*	32/8	20.58 days (NR)	6.33 days (NR)	14/32 (43.8%)	2/8 (25%)	Amer et al., 2015 (64)

LOS: Length of stay; NR: Not reported; BSI: Bloodstream infection, HAI: Hospital-acquired infection, VAP: Ventilator-Associated Pneumoniae; CR: Carbapenem-resistant; GNB: Gram negative bacteria; GPB: Gram positive bacteria, 1 various wards; 2 LOS attributed to the specific bacteria responsible of the infections, 3: Overall mortality attributed to the specific bacteria responsible of the infections, ICU: Intensive Care Unit.

Statistical analysis

Meta-analyses were undertaken using Comprehensive Meta-analysis software (Biostat, Inc., New Jersey, USA) version 3 for Windows, to determine overall mortality risk associated with resistance. Sub-group analyses for mortality were conducted for the data by WHO region, World Bank classification, countries, group of bacteria, and bacterial species where there were three or more studies that could be combined. Forest plots were performed to assess the significance of the results and generated using 95% confidence intervals (CIs). Analyses were undertaken across sub-groups for the selected outcome and the results presented as odds ratios. Studies were weighted in favor of those with narrower confidence intervals (more precise results), and the random-effects method was used to provide more confident data considering heterogeneity within and between reports. The *I-square* (I^2) statistic with cut-off values of 25, 50 and 75% was used to assess low, moderate and high heterogeneity respectively, and a *p*-value of <0.05 was considered statistically significant. Publication bias was evaluated using the funnel plot and statistical egger's test.

Quality assessment

Quality assessment was performed independently by RF and LF using the Newcastle-Ottawa quality assessment scale (NOS) for each study included in the systematic review and meta-analysis (65). NOS assesses methodological quality, based on three-dimensional criteria and included (i) selected population, (ii) comparability of groups, and (iii) outcome/exposure of interest. Studies were scored using a scale with a possible maximum of eight points where a score ≥ 6 indicated high-quality studies, a score between 3-6 as moderate and a score ≤ 3 as low quality.

Results

Literature search and study selection

The systematic search conducted in the two electronic databases generated 27 033 papers. A total of 24 057 papers were screened for probable inclusion according to titles and abstracts after de-duplication. Of these, the full texts of 92 eligible papers were fully evaluated based on predefined inclusion and exclusion criteria. One article was added following a hand-search in the reference lists of included papers. Forty studies were finally eligible for the qualitative and quantitative analysis (Fig 1), of which 18 were of high quality, while 15 and seven were moderate and low quality respectively.

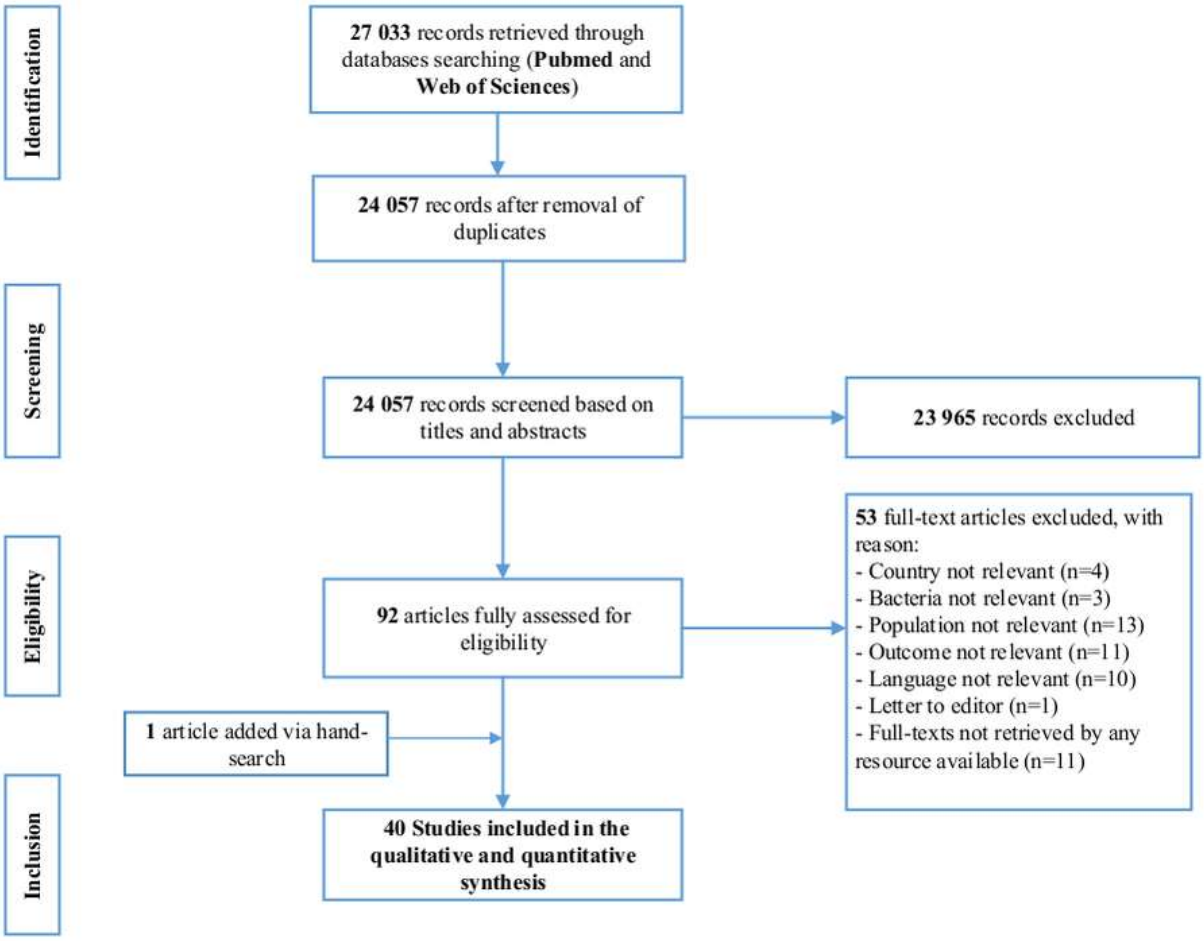

Fig 1. Prisma Flow-chart illustrating the study selection process.

Description and characteristics of studies included in systematic review

The majority of data analyzed were obtained from single center studies conducted in 11 countries. Thirty percent (n=12) of the observational studies on ABR were conducted in hospitals and communities in Thailand, the rest were performed in 10 different-countries namely Brazil (n=7; 17.5%), China (n=6; 15%), Turkey (n=5; 12.5%), Colombia (n=2; 5%), Malaysia (n=2; 5%), India (n=2; 5%), Mexico (n=1; 2.5%), Jordan (n=1; 2.5%), Palestine (n=1; 2.5%), and Senegal (n=1; 2.5%) (Table 2 and Fig 2).

Fourteen studies investigated the impact of ABR on mortality, two reported its impact on morbidity only (Table 2) while 24 considered both morbidity and mortality concomitantly. Eight studies reported on the economic consequences of ABR (Table 3). *A. baumannii* (n=14; 35%), *K. pneumoniae* (n= 6; 15%), *S. aureus* (n=5; 12.5%), *P. aeruginosa* (n=4; 10%) represented the main pathogens reported with ICUs being the principal hospital ward concerned (Tables 2 and 3).

Fig 2. Graphical representation of AMR in developing countries included in the study.

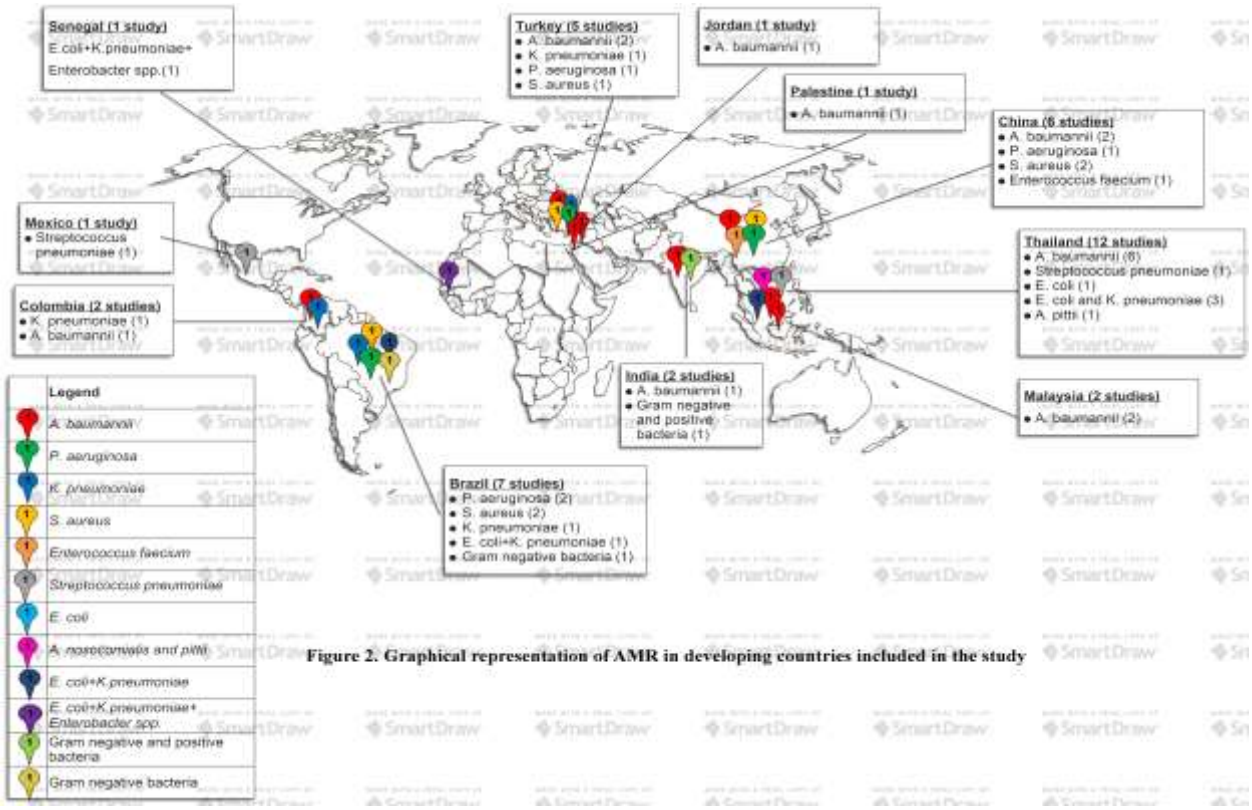

Figure 2. Graphical representation of AMR in developing countries included in the study

Table 3. Studies describing mortality rate associated with resistant and MDR ESKAPE bacteria

Authors	Hospital Wards	Bacteria	Mortality rate	P-value	References
Al Jarousha et al. (2009)	Neonatal ICU	MDR-*A. baumannii* (15/40)	37.5%	0.001	(54)
		Susceptible *A. baumannii* (12/100)	12%		
Anunnatsiri et al. (2011)	ICU	MDR-*A. baumannii* (22/24)	91.7%	0.001	(41)
		Susceptible *A. baumannii* (12/25)	48%		
Amer et al. (2015)	Emergency ICU /Pediatric ICU	CR-MBLP-*P. aeruginosa* (14/32)	43,8%	0.2	(64)
		CR-MBLN-*P. aeruginosa* (2/8)	25%		
Furtado et al. (2009)	ICU	Imipenem-resistant *P. aeruginosa* (31/63)	49%	0.02	(31)
		Imipenem-susceptible *P. aeruginosa* (61/182)	33%		
Marra et al. (2006)	ICU	ESBL-producing *K. pneumoniae* (18/56)	32.14%	0.042	(46)
		Non-ESBL *K. pneumoniae* (8/52)	15.38%		
Moreira et al. (2008)	ICU	ORSA (11/29)	37.9%	0.41	(47)
		OSSA (8/32)	25%		
Serefhanoglu et al. (2009)	ICU	MDR-ESBL-producing-*E. coli* and *K. pneumoniae* (7/30)	23.3%	0.606	(32)
		Non-MDR-ESBL- producing-*E. coli* and *K. pneumoniae* (12/64)	18.8%		
Tuon et al. (2012)	ICU	Carbapenem-resistant *P. aeruginosa* (13/29)	54.2%	0.043	(22)
		Carbapenem-susceptible *P. aeruginosa* (26/48)	44.8%		

Table 3. End

Authors	Hospital Wards	Bacteria	Mortality rate	P-value	References
Chen et al. (2012)	ICU	MRSA (25/75)	33%	0.01	(48)
		MSSA (8/43)	18.6%		
Fu et al. (2015)	ICU	XDR *A. baumannii* (31/39)	79.5%	0.1	(49)
		Non-XDR *A. baumannii* (38/86)	44.2%		
Jia et al. (2015)	ICU	Linezolid non-susceptible Enterococci (3/44)	6.8%	0.521	(50)
		Linezolid-susceptible Enterococci (2 /44)	4.5%		
		Un-infected Control patients (3/176)	1.7%		
Yao et al. (2015)	ICU	MRSA (12/57))	21%	0.002	(35)
		MSSA (9/116)	8%		
Gomez Rueda et al. (2014)	ICU	Carbapenem resistant *K. pneumoniea* (31/61)	50.8%	0.042	(36)
		Carbapenem-susceptible *K. pneumoniae* (20/61)	32.7%		
		Un-infected control patients (25/122)	20.4%		
Kumar et al. (2014)	ICU	Carbapenem-resistant *A. baumannii* (9/33)	27.3%	0.074	(37)
		Carbapenem-susceptible *A. baumannii* (3/32)	9.4%		
Nazer et al. (2015)	ICU	MDR-*A. baumannii* (118/161)	73.3%	0.015	(53)
		Non-MDR-*A. baumannii*(142/232)	61.2%		
Deris et al. (2011)	ICU	Imipenem-resistant -*A. baumannii* (6/15)	42.9%	0.201	(39)

		Imipenem-susceptible *A. baumannii* (9/41)	24.3%		
Inchai et al. (2015)	ICU	MDR-*A. baumannii* (10/72)	13.9%	0.001	(44)
		XDR- *A. baumannii* (88/220)	40%		
		PDR-*A. baumannii* (7/12)	58.3%		
Jamulitrat et al. (2009)	ICU	Imipenem-resistant-*A. baumannii* (35/67)	52.2%	0.001	(59)
		Imipenem-susceptible *A. baumannii* (26/131)	19.9%%		
Thatrimontrichai et al. (2016)	ICU	Carbapenem-resistant *A. baumannii* (10/63)	15.9%	0.01	(19)
		Carbapenem-susceptible *A. baumannii* (1/13)	7.7%		
		Un-infected control patients (0/25)	0%		
Topeli et al. (2000)	ICU	MRSA (15/46)	32.6%	0.02	(21)
		MSSA (7/55)	12.7%		

CR: Carbapenem-resistant; CS: Carbapenem susceptible; MBL: Metallo-beta-lactamase; IS: imipenem sensitive; IR: imipenem resistant; ICU: Intensive Care Unit; OSSA: Oxacillin-sensitive-*S. aureus;* ORSA: Oxacillin-resistant-*S. aureus*; PDR: Pan drug resistant; XDR: Extensive drug resistant

Statistical analysis

Primary analyses

Pooled estimates revealed 90% prevalence (95%CI, 2.852-3.557; p=0.000) of mortality attributable to infections in developing countries with greater mortality associated with ABR at an odds ratio (OR) 2.828 (95%CI, 2.231-3.584; p= 0.000) (Fig 3A).

Subgroup analyses

The subgroup analyses were performed by World Bank classification, WHO region, country, group of bacteria and bacterial species. Figure 3B presents a forest plot of mortality due to AMR categorized per World Bank classification. The risk of mortality due to resistant bacteria was high in upper middle-income countries (OR 2.769, 95% CIs, 2.142-3.579; p= 0.000), with studies from lower-middle and low-income nations not being evaluated due to insufficient data.

Four out of the six WHO regions were included in the analysis, with three showing a high risk of mortality (Fig 3C). High statistical significance was observed in the Americas (OR 2.126, 95% CIs; 1.546-2.925; p=0.000), South East Asia (OR 3.754, 95% CIs; 2.333-6.041; p=0.000) and the Western Pacific (OR 3.746, 95% CIs; 2.463-5.697; p=0.000) (Fig 3C). Results from Europe were not statistically significant and insufficient reports precluded analysis in Africa.

Subgroup analyses per country showed high statistical significance (OR 2.665, 95%CIs; 2.074-3.425, p=0.000) (Fig 3D) in favor of mortality. Brazil, China and Thailand, had statistically significant risk of mortality with OR being 1.825 (95%CIs; 1.239-2.689; p=0.002), 3.746 (95%CIs; 2.463-5.697; p=0.000), 3.928 (95%CIs; 2.116-7.293; p=0.000) respectively, in contrast to Turkey, which was not statistically significant (Fig 3D). In other countries, the number of reports was insufficient (less than three) to perform the meta-analysis.

Studies were categorized into three groups of bacteria namely ESKAPE, non-ESKAPE, and mixed (both ESKAPE and non-ESKAPE). The ESKAPE group was associated with the highest risk of mortality with a high statistical significance (OR 3.217; 95%CIs; 2.395-4.321; p=0.001) (Fig 3E). Although, the non-ESKAPE group was not associated with the risk of mortality (OR 1.167; 95%CIs; 0.385-3.534; p=0.000), when combined with ESKAPE within a study, it became statistically significant (OR 2.634; 95%CIs; 1.858-3.734; p=0.000) (Fig 3E).

High risk of mortality due to antibiotic-resistant *A. baumannii* was observed with high statistical significance (OR 4.636; 95%CIs; 2.954-7.277; p=0.000), followed by *S. aureus* (OR 2.842; 95%CIs; 1.868-4.324; p=0.000). *P. aeruginosa* (OR 2.076; 95%CIs; 0.833-5.177; p=0.117) and

K. pneumoniae (OR 2.026; 95%CIs; 0.733-5.598; p=0.173) were not significantly associated with mortality (Fig 3F).

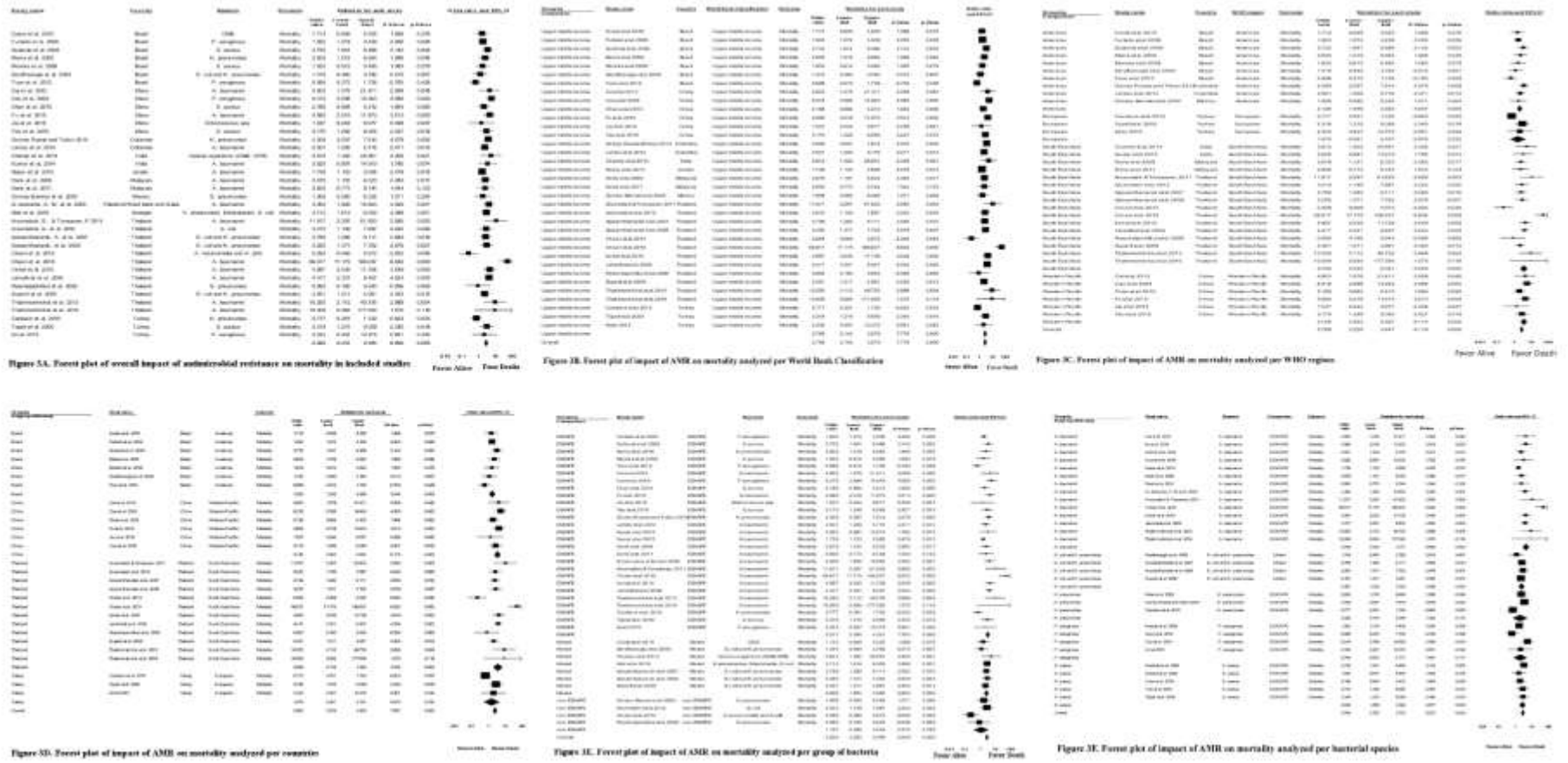

Fig 3. Forest plot of Impact of ABR on Mortality and sub-group analyses per World Bank Classification, WHO regions, Countries, group of bacteria and bacteria species. 3A. Forest plot of overall impact of antibiotic-resistance on mortality in included studies. 3B. Forest plot of impact of ABR on mortality analyzed per World Bank Classification. 3C. Forest plot of impact of ABR on mortality analyzed per WHO regions. 3D. Forest plot of impact of ABR on mortality analyzed per countries. 3E. Forest plot of impact of AMR on mortality analyzed per group of bacteria. 3F. Forest plot of impact of ABR on mortality analyzed per bacteria species.

Discussion

AMR is a global public health threat that affects human health, particularly hospitalized patients, and has substantive health and financial consequences. This study analyzed the published literature on the clinical and economic implications of ABR in developing countries from 40 eligible studies. Antibiotic-resistant bacteria were associated with increased mortality (OR 2.8341, 95%CIs; 2.2180-3.6213; P=0.000), consistent with several reports in both developed and developing countries (66-69). The main ward involved was the ICU, possibly due to the heavy use of antibiotics and hence the selection pressure for ABR development and prevalence in these units (4, 23, 70, 71). This concurred with studies from Mexico, Brazil, China, Thailand, France and Serbia, that reported high mortality due to antibiotic-resistant bacteria in ICUs (17, 49, 67, 71-73). The study further showed that ABR research is neglected in developing countries with only one report from low-income (Senegal), two from lower-income (Palestine and Jordan), and 37 from upper-middle income nations (Table 1 and Figure 2). Developing countries are thus far behind high resource settings in the fight against AMR and that requiring considerable efforts to reduce its consequences (74). Three WHO regions, i.e., the Americas, South East Asia and the Western Pacific region showed the highest risk of mortality associated with MRSA and *K. pneumoniae* resistant to third generation cephalosporins. Our results concurred with the 2014's WHO report, which showed a significant increase of mortality due to antibiotic-resistant *K. pneumoniae* and *S. aureus* in hospitals particularly in ICU across WHO regions (2). Resistance levels could be explained by the practices of self-medication and the purchase of antibiotics over-the-counter common in these settings. Policies and regulations promoting rational antibiotic use are also minimal or non-existent. Additionally, limitations in managing nosocomial infections, sub-optimal infection control measures, unsafe water, poor hygienic conditions, lack of knowledge and inadequately trained personnel might also be associated with the prevailing resistance in these regions. Comprehensive studies are needed to provide accurate and reliable data to inform decision-makers about the danger of ABR in developing countries and suggest a way forward for the alleviation of the resulting implications.

Resistant ESKAPE bacteria including carbapenem-resistant *A. baumannii*, MBL- producing *P. aeruginosa*, ESBL-producing *K. pneumoniae*, and MRSA represented the most common resistant bacteria associated with increased mortality. These bacteria were the main cause of morbidity and mortality in bloodstream infections in hospital settings, with a high statistical significance (OR

2.978, 95%CIs; 2.362-3.753; p=0.000) (Fig 3F). This concurred with the WHO Global Antimicrobial Surveillance System (GLASS), which recognized *A. baumannii*, *K. pneumoniae*, and *S. aureus*, as priority pathogens in blood specimens and list them together with *P. aeruginosa* as priority antibiotic resistant-bacteria for research and development in 2017 (4, 5).

According to the meta-analysis, MDR-ESKAPE were associated with a greater risk of mortality than mono-drug (including imipenem, methicillin, and linezolid) resistant bacteria, with a high statistical significance (OR 2.846, 95% CIs; 1.744-4.643; p= 0.000; versus OR 2.301; 95%CIs; 1.718-3.082; p=0.000; Table 3). Moreover, when comparing the mortality risk between resistant- and susceptible-ESKAPE pathogens (Table 3), results showed that carbapenem-resistant *A. baumannii* (CRAB) were associated with higher mortality risk than susceptible strains with a high statistical significance (2, 5). The pooled estimate of mortality rate ranged from 15.9 to 91.7% (p=0.001), consistent with a report from Taiwan, where a significant increase of mortality from 14% to 46% (p=0.0001) was associated with carbapenem-resistant-*A. baumannii* implicated in HAIs during 2003-2008 (75).

Although the mortality attributable to ESKAPE pathogens is indisputable compared to non-ESKAPE pathogens, we observed that when these two groups infected patients concomitantly, they were associated with a long length of hospital stay (LOS) and a higher mortality. This concurred with studies from Senegal (55), Turkey (3) and China (35, 50) which have reported high LOS and death due to MDR-*A. baumannii*, ESBL-producing *Enterobacteriaceae* and MRSA, respectively.

Eight studies reported that ABR increased health care costs with resistant ESKAPE bacteria being the main causative agents associated with high hospital costs (Table 4). Four out of the eight revealed that length of stay had an impact on hospital costs. LOS was also a risk factor for acquisition of nosocomial infections, and thereby increased mortality. Overall, health-care costs in all studies for case and control groups were 8,107.375 USD versus 5,469.487 USD respectively. Two studies indicated health care costs >10 000 USD in Thailand and Colombia (19, 51) while one report showed cost ≥ 35 000 USD in Turkey (3). In contrast, three studies reported overall hospital costs ≤ 1000 USD (55-57), with one below 250 USD in Senegal (55). These differences are attributed to the diverse socio-economic characteristics of the countries concerned.

Table 4. Summary of data on health care costs associated with resistant infections

Country	WHO Region	World Bank classification	Settings	Follow-up period	Overall Health care costs			References
					Case group	Control group	p-value	
Colombia	Americas (PAHO)	Upper Middle Income	Tertiary hospital	30 days	11 822 USD	7 178 USD	< 0.001	(51)
India	South East Asia (SEARO)	Upper middle income	Tertiary hospital	NR	1 478 USD	790 USD	< 0.001	(52)
Senegal	Africa (AFRO)	Low income	Hospital	NR	228 USD	122 USD	< 0.0001	(55)
Thailand	South East Asia (SEARO)	Upper middle income	University Hospital	34 days	935 USD	122 USD	< 0.05	(56)
Thailand	South East Asia (SEARO)	Upper middle income	University Hospital	43 days	615 USD	214 USD	< 0.05	(57)
Thailand	South East Asia (SEARO)	Upper middle income	University Hospital	NR	2731 USD	1 199 USD	< 0.001	(58)
Thailand	South East Asia (SEARO)	Upper middle income	University Hospital	NR	11 773 USD	7 797.9 USD	< 0.05	(19)
Turkey	Europe (EURO)	Upper middle income	University Hospital	28 days	35 277 USD	26 333 USD	<0.282	(3)

In terms of the limitations of the study, several papers were not included in the meta-analysis because they did not provide sufficient information regarding clinical and/or economic impact of ABR in developing countries. We were unable to present the genomic characteristics of antibiotic-resistant bacteria due to the scarcity of data. In addition, we did not focus on antibiotic classes and resistance patterns due to the lack of standard methods for identification and interpretation in developing countries. Moderate heterogeneity (I^2=58.88%, p=0.000) was reported, which could be due to various external factors, such as different type of studies (retrospective, retrospective cohort, retrospective case-control, prospective cohort, prospective case-control, etc.), diverse populations (adult, children, neonates), infection prevention and control measures and antimicrobial stewardship practices. Moreover, minor publication bias was observed in the funnel plot (fig 4) which could possibly be attributed to the lack of reports from lower-middle and low-income countries. We tried to limit the influence of heterogeneity and publication bias in our statistical analysis by using the random effects model that considers differences within and between studies, as well as by including articles in different languages (English and French).

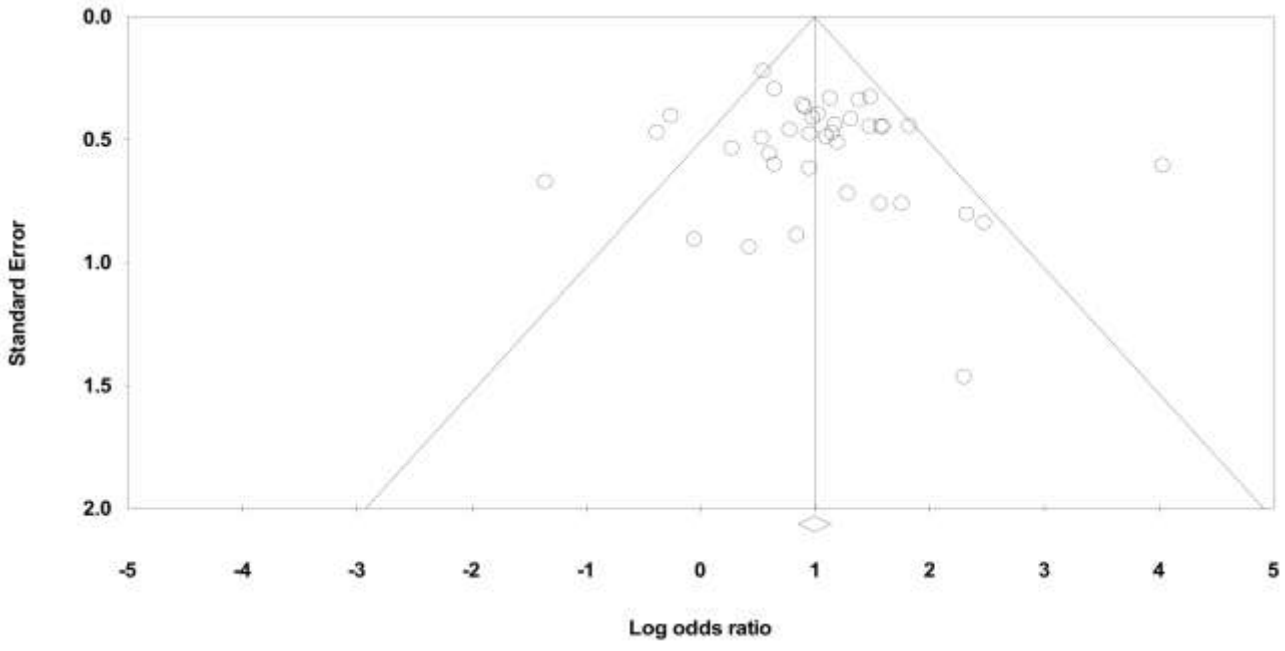

Fig 4. Funnel plot of Standard Error by log odds ratio

Conclusion and recommendations

The key findings of this study confirm that ABR, particularly antibiotic-resistant ESKAPE pathogens are associated with a high risk of mortality and greater economic costs. Developing countries need to optimize their management of communicable and non-communicable diseases, implement infection, prevention and control (IPC) measures, as well as antimicrobial stewardship programs (ASP) in both hospital and community settings to reduce morbidity, mortality and the costs associated with ABR. Furthermore, optimization of rational antibiotic use at regional and national levels, is essential to ensure a high quality and effective of therapeutic options (76). Substantial and sustainable efforts to develop rapid diagnostics, new antibiotics and vaccines are required. An international platform for global real-time surveillance and monitoring of antimicrobial resistance could advance containment of this threat.

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Supporting Information

S1 Table 1. PRISMA Checklist

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	3-4
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	4
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	NA
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	5, Suppl. Table 2
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	4
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	4, Suppl Table 3
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	4, Figure 1.
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	4-5
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	4-5, Suppl Table 2
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the	5-6

		study or outcome level), and how this information is to be used in any data synthesis.	
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	6
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	6-7
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	Figure. 4
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	6-7
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	6, Figure 1.
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	6, Table 1
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	NA
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	6, Figure 3
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	6-7; Figures 3A-3F
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	Figure 4
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	6-7; Figures 3A-3F
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	7-8
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	8-9
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	9
FUNDING			

Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	9

S2 Table 2. Search strategy performed in Pubmed and Web of Science.

Initial search terms	**Refining terms (AND/OR)**	**Restriction (NOT)**
"Antimicrobial resistan*"* *"Antibiotic resistan*"* *"Drug* resistan*"* *"Multi-drug resistan*"* *"Multidrug resistan*"* *"Multiple-drug resistan*"* *"Multiple drug* resistan*"* *Bacterial* resistan** *Cross infection* *Nosocomial* *Hospital-acquired* *Healthcare acquired* *Hospital associated* *Healthcare associated* *Community acquired* *Community-associated* *Community* *Hospital* *Hospitals* *Hospitalized* *Intensive care* *Critical care* *Inpatients* *Outpatients* *Community patients*	Afghanistan, Albania, Algeria, American Samoa, Angola, Argentina, Armenia, Azerbaijan, Bangladesh, Belarus, Belize, Benin, Bhutan, Bolivia, Bosnia and Herzegovina, Botswana, Brazil, Bulgaria, Burkina Faso, Burundi, Cabo Verde, Cambodia, Cameroon, Central African Republic, Chad, China, Colombia, Comoros, Congo, Dem. Rep. Congo, Rep. Costa Rica, Cote d'Ivoire, Cuba, Djibouti, Dominica, Dominican Republic, Ecuador, Egypt, Arab Rep, El Salvador, Equatorial Guinea, Eritrea, Ethiopia, Fiji, Gabon, Gambia, The Georgia, Ghana, Grenada, Guatemala, Guinea, Guinea-Bissau, Guyana, Haïti Honduras, India, Indonesia, Iran, Islamic Rep Iraq, Jamaica, Jordan, Kazakhstan, Kenya, Kiribati, Korea Dem. People's Rep, Kosovo, Kyrgyz, Republic Lao PDR, Lebanon, Lesotho, Liberia, Libya, Macedonia FYR, Malawi, Malaysia, Maldives, Mali, Marshall Islands, Mauritania, Mauritius, Mexico, Micronesia. Fed Sts, Moldova, Mongolia, Montenegro, Morocco, Mozambique, Myanmar, Namibia, Nepal, Nicaragua, Niger, Nigeria, Pakistan, Palau, Panama, Papua New Guinea, Paraguay, Peru, Philippines, Romania, Russian Federation, Rwanda, Samoa, Sao Tome and Pincipe, Senegal, Serbia, Sierra Leone, Solomon Islands, Somalia, South Africa, South Sudan, Sri Lanka, St Lucia, St Vincent and the Grenadines, Sudan, Suriname, Swaziland, Syrian Arab Republic, Tajikistan, Tanzania, Thailand, Timor-Leste, Togo, Tonga, Tunisia, Turkey, Turkmenistan, Tuvalu, Uganda, Ukraine, Uzbekistan, Vanuatu, Venezuela, RB Vietnam, West Bank and Gaza, Yemen, Rep. Zambia, Zimbabwe	*Review* *Letter* *Case reports* *Meta-analysis* *Editorial* *Conference abstract* *Meta-analysis* *Correspondence* *Comment* *Year Restriction (2000)*

CHAPTER VI. CONCLUSION

I. Introduction and key findings

A total of 71 non-duplicate ESKAPE bacteria collected from carriage samples (nasal and rectal swabs) at three time-points (admission, after 48h and discharge) as well as from clinical specimens in a rural district and an urban, tertiary hospital in uMgungundlovu, South Africa, between May and July 2017, were investigated via conventional, multiplex and real-time PCR, as well as WGS with a view to delineate antibiotic resistance genes, virulence factors, plasmids and MLST profiles.

The following are the main conclusions from the study:

- The overall prevalence of carriage of MDR Gram-negative ESKAPE bacteria was 37.21% (16/43), 42.31% (11/26) and 57.14% (4/7) at admission, after 48 h and at discharge respectively.
- The prevalence of MDR Gram-negative ESKAPE bacteria in faecal carriage (46%) was higher than clinical samples (28%).
- Colonization was mainly associated with referral from district to tertiary hospital with high statistical significance (OR: 14.40, 95% CI 0.98-210.84).
- *bla*$_{\text{CTX-M-group-9}}$, *bla*$_{\text{CTX-M-group-1}}$ and *bla*$_{\text{SHV}}$ were the main resistance genes identified through conventional, multiplex and real-time PCR.
- Several patients carried more than two different isolates in both district and tertiary hospitals and the clonality reveals that *K. pneumoniae* (K1) was circulating within wards and between hospitals.
- ESBL-producing MDR Gram-negative ESKAPE bacteria are widespread in public hospitals in uMgungundlovu district.
- The overall prevalence of faecal VRE carriage was 53% with patients of the district hospital being more likely to be colonized by VRE at admission (44%), after 48h (64%) and discharge (100%) than those of the tertiary level.
- High genetic diversity and clonal dissemination of various *E. faecalis* and *E. faecium* lineages were observed across wards and within hospitals in uMgungundlovu district.

- Gender, antibiotics use, co-morbidity, previous hospitalization, referral from district to tertiary hospital were the main risk factors identified at admission in both hospitals while hospitalization in the surgical wards increased the odds to being colonized by VRE after 48h.
- High level of antibiotic resistance in *S. aureus* were attributed to the multi-drug resistant efflux pumps mepA, mexE, AcrB, MATE, qac and qacA.
- Genome analysis revealed that the circulating *S. aureus* isolates belonged to the extremely virulent ST121 clone with a total of 18 virulence genes identified. Chromosomal integration of several plasmid replicons was also evident.
- ABR is associated with a high mortality risk and increased economic costs in developing countries with ESKAPE pathogens implicated as the main cause of increased mortality with high statistical significance (OR 3.217; 95%CIs; 2.395-4.321; p=0.001).
- ABR, and especially antibiotic-resistant ESKAPE bacteria significantly increased health care costs.
- Patients with non-communicable disease co-morbidities were identified as high-risk populations.

These findings are comparable to those reported elsewhere in Africa and South Africa, particularly in terms of the predominance of CTX-M among Gram-negative ESKAPE bacteria. However, the detection of VRE harbouring VanC-1 gene and genetic lineage *S. aureus* ST121 in carriage samples are unprecedented in South Africa.

II. Study significance

This study presents an overview of the current status of antibiotic-resistant ESKAPE bacteria in a rural, district and an urban, tertiary public hospitals in uMgungundlovu district, KwaZulu-Natal, South Africa. The study adds new knowledge on the different resistance mechanisms associated with ESKAPE pathogens and confirms these bacteria as serious health treat. The results provide preliminary evidence for policy-makers to consider ABR containment strategies on the basis of screening for and surveillance of EKAPE pathogens.

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III. Limitations

The main limitation of this study is that the sample size was relatively small and limited to a two-month period for two public hospitals. Although giving an interesting overview of the epidemiology of antibiotic resistance at two levels of care in uMgungundlovu District, the results generated preclude extrapolation to the province and the country as a whole.

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IV. Recommendations

- The high genetic diversity and pathogenicity of circulating ESKAPE bacteria, especially *E. faecalis*, *E. faecium* and *S. aureus*, underlines the urgent necessity to develop and implement policies, guidelines, activities and interventions to contain their spread in South African hospitals.
- Similarly, the high prevalence of ESBL-producing MDR Gram-negative ESKAPE bacteria in carriage and clinical samples among hospitalized patients urges the implementation of regular screening and surveillance of MDR Gram-negative ESKAPE bacteria in communities and hospitals, to monitor epidemiological changes, ascertain socio-economic impact and inform antibiotic treatment.
- Screening and surveillance measures should be coupled with strict infection prevention and control programmes and antimicrobial stewardship programmes (ASP) essential to address ABR in South African healthcare settings.
- Heightening awareness and education of policy-makers, prescribers, pharmacists, laboratory scientists, both in- and out-patients and the general population about rational antibiotic use and ABR as well as associated clinical and societal implications is essential to curb emergence and spread of ABR and preserve potent antibiotics and secure the health of future generations on the country.
- Forthcoming whole genome sequencing-based epidemiological studies should be initiated together in public and private hospitals to delineate the true burden of antibiotic-resistant ESKAPE bacteria in the country.

V. Conclusion

The sub-optimal infection prevention and control measures as well as extensive antibiotic use not only in hospitals but in communities lead to the wide dissemination of antibiotic-resistant ESKAPE bacteria in carriage and clinical samples of hospitalized patients in South Africa. Consequently, patients, healthcare workers and the general population are at high risk of being infected with these resistant bacteria which are associated with high mortality rates. Similarly, the asymptomatic carriage of resistant ESKAPE bacteria among hospitalized patients could lead to hospital acquired infections especially in immune-compromised patients. These bacteria could ultimately cause outbreaks in both communities and healthcare settings where they will increase healthcare costs, morbidity and mortality.

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APPENDICES

Appendix 1. Ethical approval (BREC) letter

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

10 January 2017

Mr RCF Zangue (216076503)
Discipline of Medical Microbiology
School of Laboratory Medicine and Medical Sciences
Czangue@yahoo.fr

Dear Mr Zangue

Title: Molecular epidemiology of antibiotic resistant ESKAPE pathogens isolated from public sector in KwaZulu-Natal, South Africa.
Degree: PhD
BREC REF NO: BF512/16 (*sub-study of BCA444/16*)

The Biomedical Research Ethics Committee (BREC) has considered the abovementioned application at a meeting held on 11 October 2016.

The study was provisionally approved by BREC pending appropriate responses to queries raised. Your responses dated 14 November 2016 to queries raised on 31 October 2016 have been noted and approved by the Biomedical Research Committee at a meeting held on 13 December 2016. The conditions have now been met and the study is given **full ethics approval** and may begin as from 10 January 2017.

This approval is valid for one year from **10 January 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).

Pg. 2/...

Biomedical Research Ethics Committee
Professor J Tsoka-Gwegweni (Chair)
Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Telephone: +27 (0) 31 260 2486 Facsimile: +27 (0) 31 260 4609 Email: brec@ukzn.ac.za

Appendix 2. Provincial (KZN) Health Department approval

health
Department:
Health
PROVINCE OF KWAZULU-NATAL

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

DIRECTORATE:
Health Research & Knowledge Management (HKRM)

Reference: HRKM108/17
KZ_2017RP3_437

29 March 2017

Dear Prof S Y Essack
(University of KwaZulu-Natal)

Subject: Approval of a Research Proposal

1. The research proposal titled **'The "One Health" Approach to Containing Antibiotic Resistance'** was reviewed by the KwaZulu-Natal Department of Health (KZN-DoH).

   The proposal is hereby **approved** for research to be undertaken at Edendale, Greys & Appelsbosch Hospitals and also Bruntville, Appelsbosch Gateway, East Boom & Imbalenhle Clinics.

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

Dr E Lutge
Chairperson, Health Research Committee
Date: 29/03/17

Fighting Disease, Fighting Poverty, Giving Hope

Appendix 3. uMgungundlovu Health District manager approval

health
Department:
Health
PROVINCE OF KWAZULU-NATAL

DISTRICT MANAGERS OFFICE

Enquiries: Mrs. N. M. Zuma-Mkhonza
27 OCTOBER 2016

TO: Professor Sabiha Essack and Mlisana
B. Pharm., M. Pharm., PhD
South African Research Chair in Antibiotic Resistance & One Health
Professor: Pharmaceutical Sciences
Director: Antimicrobial Research Unit
College of Health Sciences
University of KwaZulu-Natal
Private Bag X54001
Durban,4000
South Africa

Dear, Professor Sabiha Essack

RE: THE" ONE HEALTH" APPROACH TO CONTAINING ANTIBIOTIC RESISTANCE

I have pleasure in informing you that support and permission have been granted to you by the District Office to conduct a research in ***THE" ONE HEALTH" APPROACH TO CONTAINING ANTIBIOTIC RESISTANCE.***

PLEASE NOTE THE FOLLOWING

1. Please ensure that you adhere to all policies, procedures, protocols and guidelines of the Department of Health with regards to this research.
2. This research will only commence once this office has received the full ethics approval has been received and the confirmation from the Provincial Health Research Committee in the KZN Department.
3. Please ensure that this office is informed before you commence your research.
4. The District Office will not provide any resources for this research.
5. You will be expected to provide feedback on your findings to the District Office.

Thank you

P.P

MRS N.M. ZUMA - MKHONZA
DISTRICT DIRECTOR
UMGUNGUNDLOVU HEALTH DISTRICT

UMnyango Wezempilo. Departement van Gesondheid

Fighting Disease, Fighting Poverty, Giving Hope

Appendix 4. Certificate of NIDA Clinical Trials Network

NIDA Clinical Trials Network

Certificate of Completion

is hereby granted to

Raspail Carrel Founou Zangue

to certify your completion of the six-hour required course on:

GOOD CLINICAL PRACTICES

MODULE:	STATUS:
Introduction	N/A
Institutional Review Boards	Passed
Informed Consent	Passed
Confidentiality & Privacy	Passed
Participant Safety & Adverse Events	Passed
Quality Assurance	Passed
The Research Protocol	Passed
Documentation & Record-Keeping	Passed
Research Misconduct	Passed
Roles & Responsibilities	Passed
Recruitment & Retention	Passed
Investigational New Drugs	Passed

Course Completion Date: 9 November 2016

CTN Expiration Date: 9 November 2019

Tracee Williams, Training Coordinator
NIDA Clinical Coordinating Center

This training has been funded in whole or in part with Federal funds from the National Institute on Drug Abuse, National Institutes of Health, Department of Health and Human Services, under Contract No. HHSN272012010000024C.

Appendix 5. Information sheet

Date: _____/_____/_____

Dear volunteers

My name is Raspail Carrel Founou Zangue; I am a Doctoral student of the University of KwaZulu-Natal (UKZN) School of Health Sciences, College of Health Sciences in South Africa. I am the principal investigator of a Doctoral Research Project entitled "**MOLECULAR EPIDEMIOLOGY OF ANTIBIOTIC RESISTANT ESKAPE PATHOGENS ISOLATED FROM PUBLIC SECTOR IN UMGUNGUNDLOVU DISTRICT IN KWAZULU-NATAL**". This project is under the supervision of Professor Sabiha Yusuf Essack from University of KwaZulu-Natal, the College of Health Sciences and with the support the University. All the information about the different ethical approvals obtained in South Africa is detailed above.

This study is carried out in three hospitals namely Greys, Edendale, Appelbosch in uMgungundlovu district. During two-months, we will look at ESKAPE group of bacteria and their resistant clones in hospital particularly on in-patients. The latter have been recently identified like main agents of hospital acquired infection. These bacteria are able to perpetuate infection in spite of antibiotic therapy because they have become resistant to numerous antibiotics and involved in higher length of stay, hospitalization costs, in hospital mortality. Find out the clinical and economic impacts of these bacteria in hospital particularly in-patient, will serve to implement effective prevention measures, strategies to counteract nosocomial infection, reduce antibiotics use in the hospital, reduce consequently the mortality due to antimicrobial resistance in hospitals.

The objective of this study is to identify these bacteria in hospitals to reduce the incidence of the latter, optimize the success of treatment. Before taking part to this study, it is important to read and understand very well all the information concerning the project. This information sheet presents and explains the objectives, procedures, potential risks, benefits and inconvenience of the study. If you feel that something is unclear while reading this document, please do not hesitate to ask for more clarification from the study personnel. If you would like to participate to the study, please sign as appropriate below.

OVERARCHING AIM AND OBJECTIVES OF THE STUDY

The principal objective of this study is to find out if you have any infection and whether or not it has resistant to antibiotics caused by the ESKAPE group pathogens involved diseases for the hospitalized patients. I am soliciting your voluntary participation in this research because you comply with the inclusion criteria of the study. Please, be sure that your participation is completely voluntary. If you decline your participation in this study, there will not have any negative consequences for you. However, if you agree, please, you also need to be conscious of the fact that you may stop participating at any time and you may decide not to answer any question.

If you voluntary to participate in this study, you should know the following acts will occur:

- You will have automatically a unique participant identification number; this number will be used to ensure the traceability of your results and provide confidentiality. Your name will not be asked or recorded on any study materials.
- You will receive a questionnaire— including sex, age, residence location, principal profession, date of recent hospitalization, recent surgery, etc.
- Your samples including rectal swab or stool sample and nasal swab will be collected according to the decision of the doctor.

TIME REQUIRED FOR PARTICIPATION

The time of your participation will take about 30 minutes above to respond to the questionnaire.

POTENTIAL RISKS OF THE STUDY

The procedure to record your information and collect your sample has been done by the researcher to minimize the risk of discomforts for yourself. If any act and question that you should answered, make uncomfortable yourself, you could able to skip this question and stop the collect.

BENEFITS

For yourself, no payment will be asked and no reimbursement will be made for your participation in the study. Your participation is necessary to improve data on the different pathogens and resistance mechanisms existing in public hospitals in South Africa in order to implement strategies and policies to contain antimicrobial drug resistance. Your results will allow the doctor to choose the correct antibiotics and provide information on the most appropriate therapy.

CONFIDENTIALITY

After the study, if you decide to withdraw at any time, all the material required concerning your identification —questionnaire, informed consent, data recorded and sample—will be destroyed except if you give authorization to the researcher to use it after your withdrawal. If you choose to be enrolled, all your responses and information will be confidential and kept properly with limited access to allowed person only. Neither your name nor your address will be recorded, and no other person will know your answers. Your data will be conserved as described by the following measures which are applied for all applicants.

During the research:

- Your name will be codified, and nobody will know your answers.
- All the research document and material will be stored in a workbook with key.
- The database containing numerical data will have an access granted by a password detained by the researcher only.
- Numerical data will be protected in files with access granted by a password detained by the researcher.
- Only the researcher and authorized persons will have access to the data containing names and codes which will be stored separately from the material of research, from the data and informed consent.

During the dissemination of the results:

- The participant names will never appear in a report, publication or scientific presentation
- The results will be presented globally to avoid the identification of participant
- The results of the study will be published in scientific journals and nobody will be recognized.
- The participants desirous to have their resume of the results should provide their Email or Postal address in the blank space provided below.

THE ELECTRONIC OR POSTAL ADDRESS IF YOU WOULD LIKE TO RECEIVE A RESUME OF THE RESULTS:

At the end of the study:

- The collected data and materials used during the research may be used later for other research
- All the material and data will be destroyed at later in 10 years.
- Measures have been done for persons desirous to know their results.
  i. The results will be sent to participants who will have done the request by indicating their address at the space provided.
  ii. The results will not be available before the end of the year 2017.
  iii. If this address changes by this date, I encourage you to inform us of your new address.

VOLUNTARY CONSENT AND PARTICIPATION IN THE STUDY

You have read the information sheet above concerning the study; you have received an opportunity to ask for any questions and explanation about the procedure, benefits and risks of the study; you understand the purpose, process and risks of the study. If you would like to be freely enrolled in the study, please sign the informed consent form below. A copy of this consent will be given to you for your record.

For further interrogation concerning the study, your rights as participant or the researcher, please be free to contact the researcher or the Biomedical Research Ethics Committee as detailed below:

Principal Investigator: Raspail Carrel Founou Zangue
BSc., MSc., PhD Student
Discipline of Pharmaceutical Sciences
School of Health Sciences
College of Health Sciences
University of KwaZulu-Natal
Durban 4000
South Africa
Tel: +237 675 94 35 67
Email: czangue@yahoo.fr

Supervisor: Prof. Sabiha Yusuf Essack
B. Pharm., M. Pharm., PhD (Pharmaceutical Microbiology)
Professor: Pharmaceutical Sciences
South African Research Chair in Antibiotic Resistance & One Health

University of KwaZulu-Natal
South Africa
Tel: +27(0) 31 2602486
Fax: +27 (0) 31 2604609
Email: essack@ukzn.ac.za

Biomedical Research Ethics Administration
Research Office, Westville Campus
Govan Mbeki Building
University of KwaZulu-Natal
Private Bag X 54001, Durban, 4000
KwaZulu-Natal, South Africa
Email: BREC@ukzn.ac.za

Appendix 6. Informed consent

I _____certify that I have been invited to participate in a study entitled "**MOLECULAR EPIDEMIOLOGY OF ANTIBIOTIC RESISTANT ESKAPE PATHOGENS ISOLATED FROM PUBLIC SECTOR IN UMGUNGUNDLOVU DISTRICT IN KWAZULU-NATAL**" with Mr Raspail Carrel Founou Zangue from the School of Health Sciences, College of Health Sciences of the University of KwaZulu-Natal in Durban-South Africa as Principal Investigator.

- I certify that I have read and understood the purpose, procedures, benefits and risks of the study
- I have been given an opportunity to ask for more clarification and I have received answers to my satisfaction
- I understand that my participation is completely voluntary, that my information will be strictly confidential and that I may withdraw at any time without affecting my treatment and legal rights,
- I understand that if I have further interrogations concerning the study I may freely contact the researcher Raspail Carrel Founou Zangue at the address below:

Principal Investigator: Raspail Carrel Founou Zangue
BSc., MSc., PhD Student
Discipline of Pharmaceutical Sciences
School of Health Sciences
College of Health Sciences
University of KwaZulu-Natal
Durban 4000
South Africa
Tel: +237 675 94 35 67
Email: *czangue@yahoo.fr*

- I additionally understand that for any questions about my rights as participant or about the researcher, I may contact the UKZN Biomedical Research Ethics Committee at the address mentioned below:

Biomedical Research Ethics Administration
Research Office, Westville Campus
Govan Mbeki Building
University of KwaZulu-Natal
Private Bag X 54001, Durban, 4000
KwaZulu-Natal, South Africa
Tel: +27(0) 31 2602486
Fax: +27 (0) 31 2604609

By signing this document, I freely agree to be enrolled in this study and give the authorization under the specified conditions to the PhD student researcher Mr Raspail Carrel Founou Zangue from the University of KwaZulu-Natal to use my samples and isolates (where applicable) for the actual and where necessary, future studies concerning antimicrobial resistance.

_____ _____

Signature of Participant **Date**

_____ _____

Signature of guardian **Date**

(Where applicable)

Participant code: _____

Location: _____

Gender: ☐ Male ☐ Female

Appendix 7. Questionnaire

Date: _____/_____/_____

Patient ID _____

Please, understand that questions are personal to you and please be assured that your answers will be confidential. We would appreciate your honesty and complete response to each question. It should take approximately 30 minutes to complete.

Instructions to all participants: Check ☑ the appropriate box or write your answer in the space provided.

Part I: On admission

Section 1: Demographic Information

These questions are about socio-demographic information. Remember, your answers will remain strictly confidential.

1. What is your gender?

   ☐ Male

   ☐ Female

2. What is your age?

   _____

3. Your current residence location is UMgungundlovu?

   ☐ Yes

   ☐ No

4. What is your principal profession?

   _____

5. Do you have an occupation related to hospital?

   ☐ Yes

   ☐ No

Section 2: Clinical History

The next questions concern your medical history.

6. In the past 12 months have you been hospitalized? (If no, skip to the next question)

□No □yes

7. In which ward have you been hospitalized?
   - □ Intensive care unit
   - □ Paediatric unit
   - □ Endocrinology
   - □ Emergency
   - □General medicine
   - □ Surgical unit
   - □Other units_____

8. Are you currently under antibiotic treatment?

   Yes □ No □ (if no, skip to the next question)

9. Which antibiotic?

   _____

<u>Part II</u>: On discharge

10. Have you received antibiotics during your hospitalization? Yes □ No □

    If yes, which drug have you received?

    - □Name _____
    - □Route_____
    - □Duration _____

11. How many antibiotics have you received during your hospitalization?
    - □one
    - □two
    - □more than three

12. How many days have you been hospitalized?
    - □one week
    - □two weeks
    - □three weeks
    - □more than three weeks

Are you currently under antibiotic treatment? Yes □ No □