COLLEGE OF HEALTH SCIENCES

SCHOOL OF LABORATORY MEDICINE AND MEDICAL SCIENCES

DEPARTMENT OF MEDICAL MICROBIOLOGY

Prevalence, Phenotypic and Genotypic Characterization of Resistant Clinical ESKAPE and *Escherichia coli* Isolates, at Kamuzu Central Hospital, Lilongwe Malawi

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Prevalence, Phenotypic and Genotypic Characterization of Resistant Clinical Gram-Negative Isolates at Kamuzu Central Hospital, Lilongwe Malawi

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

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

This is to certify that the content of this thesis is the original research work of MS Faheema Ebrahim Choonara, carried out under our supervision at the Antimicrobial Research Unit (ARU), School of Health Sciences, University of KwaZulu-Natal (UKZN), Durban, South Africa.

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DECLARATION

I, Ms. Faheema Ebrahim Choonara, declare that:

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DEDICATION

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

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ACRONYMS

AME: Aminoglycoside-modifying enzymes

AMP: Ampicillin

AmpC: AmpC β-lactamase

AMR: Antimicrobial resistance

API: Analytical profile index

AST: Antimicrobial susceptibility testing

ATCC: American type culture collection

ATP: Adenosine triphosphate

AZT: Aztreonam

BORSA: Borderline oxacillin resistant *Staphylococcus aureus*

BSI: Blood stream infection

CA-MRSA: Community acquired methicillin resistant *Staphylococcus aureus*

CAZ: Ceftazidime

CDC: Centres for Disease Control and Prevention

CFU: Colony forming units

CGE: Centre for Genomic Epidemiology

CHINET: China antimicrobial surveillance unit

CIP: Ciprofloxacin

CLI: Clindamycin

CMS: Clinical microbiology services

CSF: Cerebrospinal fluid

CTX: Cefotaxime

CTX-M: Cefotaximase-München

CXM: Cefuroxime

EARS: European Antimicrobial Resistance Surveillance Network

ERY: Erythromycin

ESBL: Extended spectrum β-Lactamase

ESKAPE: *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter* spp.

EUCAST: European Committee on Antimicrobial Susceptibility Testing

FOX: Cefoxitin

FQ: Fluoroquinolone

FUS: Fusidic acid

GEN: Gentamicin

GLASS: Global Antimicrobial Resistance Surveillance System

HAI: Hospital- acquired infection

HA-MRSA: Hospital acquired methicillin resistant *Staphylococcus aureus*

HLAR: High-level aminoglycoside resistance

I: Intermediate

ICU: Intensive care unit

IMP: Imipenemase

INFORM: International Network for Optimal Resistance Monitoring

KCH: Kamuzu Central Hospital

LMIC: Low- and Middle-Income Country

MATE: Multi-drug and toxic compound extrusion

MBL: Metallo- β-lactamases (MBL)

MDR: Multidrug resistant

MEM: Meropenem

MEML: Malawi essential medicine list

MFP: Membrane fusion protein

MFS: Major facilitator superfamily

MRSA: Methicillin resistant *Staphylococcus aureus*

MSSA: Methicillin sensitive *Staphylococcus aureus*

MSTG: Malawi standard treatment guidelines

NCTC: National collection of type cultures

NDM: New Delhi metallo- β-lactamases

OXA-48: Oxacillinase-48

PACE: Proteobacterial antimicrobial compound efflux

pAmpC: Plasmid mediated AmpC

PBP: Penicillin –binding protein

PEN: Penicillin

R: Resistant

RND: Resistance-nodulation-cell division

S: Sensitive

SEM: Sau Paulo MBL

SHV: Sulfhydryl reagent variable

SMR: Small multi-drug family

SPA: *Staphylococcus aureus* protein A

SSI: Surgical site infection

ST: Sequence types

SUL: Sulphonamide

SXT: Trimethoprim-sulfamethoxazole

TB: Tuberculosis

TEM: Temoneira

TMP: Trimethoprim

TOB*:* Tobramycin

TZP: Pipercillin-tazobactam

UTI: Urinary tract infection

VAN: Vancomycin

VIM: Verona integrin encoded metallo β-lactamase

VISA: Vancomycin intermediate resistant *Staphylococcus aureus*

VRE: Vancomycin resistant Enterococci

WGS: Whole genome sequencing

WHO: World Health Organisation

ABSTRACT

Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter spp. (ESKAPE) pathogens are the leading cause of nosocomial infections worldwide. They are highly virulent, multi-drug resistant (MDR) and therefore difficult to treat posing major public health and clinical challenges globally, particularly in developing countries where resources and microbiology diagnostic services are limited or not available. The aim was to investigate prevalence, phenotypic and genotypic resistant profiles of selected ESKAPE and other important bacterial pathogens isolated from adult patients admitted at Kamuzu Central Hospital (KCH)

Escherichia coli and *Staphylococcus aureus* were the dominant species isolated. Multi-drug resistance and extended spectrum β-Lactamase -production was evident in *K. pneumoniae* (n=20/29; 69%) and *E. coli* (49/92; 53%). *Pseudomonas aeruginosa* was resistant to meropenem but none were carbapenemase producers. MRSA was detected in 10.5% (n=9/86) of *S. aureus*. These MDR isolates were mostly isolated from pus specimens from the surgical department

Genotypically, the CTX-M type (55/60; 92%) and CMY type (16/21) were most prevalent among phenotypically-positive ESBL and pAmpC β-lactamases respectively. Both CTX-M and CMY were most prevalent in *E.coli* with 71% (15/21) carrying both CTX-M and CMY

The most common sequence type in the CTX-M group 1 and CTX-M group 9 positive *E.coli* was ST410 (n=14/29; 48%) and ST131 (n=5/7; 71%) respectively; all of which contained the $bla_{\text{CTX-M-15}}$ resistance gene. In CMY positive *E. coli*, ST410 was the most prevalent and all contained $bla_{\text{CMY-2}}$ resistance gene. All the *E.coli* isolates carrying both CTX-M and CMY were ST410 and contained both $bla_{\text{CMY-2}}$ and $bla_{\text{CTX-M-15}}$ resistance genes. All phenotypically confirmed methicillin resistant *Staphylococcus aureus* (MRSA) contained *mec*A gene and t064 was most common *spa* type. *Spa* type t355 was most common in *S. aureus* that were negative for *mec*A gene

Findings demonstrate the need for continuous antibiotic resistance surveillance at the hospital to inform antibiotic treatment options. There is also a need for the establishment of antibiotic stewardship programs to sustain the efficacy of antibiotics in Malawi

CHAPTER I.

INTRODUCTION AND LITERATURE REVIEW

1. History of Antibiotics,

Antibiotics are medicines that are used to treat or prevent infections and are compounds that inhibit or destroy bacterial growth (1). Prior to the antibiotics era (19th century) infectious diseases such as pneumoniae, tuberculosis (TB) and diarrhea were the leading cause of deaths and accounted for one-third of all deaths. Globally, the average life expectancy at birth was relatively low; an average of 47 years (46 years for men and 48 years for women) (2).

Pyocyanase was one of the first antibiotics to be used to treat human infections. It was discovered by Rudolf Emmerich (1856–1914) and Oscar Lowe (1844–1941) from *Pseudomonas aeruginosa*; when they observed that the green bacteria (*P. aeruginosa*) found in bandages of injured patients was inhibiting the growth of microbes. However, it was only in 1909 that Paul Ehrlich (1854–1915) discovered salvarsan- an arsenic based chemical used to treat syphilis that the antibiotic era began (3).

In 1928, the discovery of the antibiotic –penicillin by Sir Alexander Fleming (1881-1955) marked the beginning of the antibiotic revolution. Ernest Chain and Howard Florey purified the first penicillin, penicillin G. It became widely available for use in medicine in 1945. This marked the antibiotic era and a major breakthrough for medicine (2). Penicillin provided a solution for quick and complete treatment of infections that were previously untreatable with few side effects (4).

Post- antibiotic discovery, the number of deaths significantly declined and only 4.5% of deaths were attributed to infectious diseases such as pneumonia. Life expectancy rose to 78.8 years and the elderly population increased from 4 to 13% (2). This significant decline in death was multifactorial and a result of introduction of antibiotics, improved public health, improved housing and sanitation as well as animal and pest control antibiotics. Infectious disease now became a disease of the elderly and those with underlying illnesses, surgical patients and immuno-compromised patients (2).

The era between the 1950's and 1970's was named the "Golden Era". In this era, the search for antibiotics began and many novel antibiotics were developed and introduced on the market (3). Initially, the best source of new agents was from other naturally occurring microorganisms but this changed after the discovery of streptomycin from *Streptomycin griseus*- a microorganism found in soil in 1944 (3). Thereafter, vancomycin was discovered from *Streptomyces orientalis* found in soil from Borneo and was made available for use in 1952. By this time, resistance was becoming apparent and scientists now looked at ways to improve existing agents to combat the obstacle of resistance. To combat resistance, in 1952, Beecham developed methicillin; the first penicillinase resistant β-lactam antibiotic. Thereafter,

in 1961, ampicillin was developed followed by cephalosporins and in the late 1970's 3rd generation cephalosporins (3). Since the golden era, there have been no new classes of antibiotics discovered, rather modification of the existing antibiotics.

Antibiotics played a key role in achieving major advances in medicine and surgery. Infections were treated successfully in ICU patients and patients with chronic illnesses. Also, antibiotics have significantly contributed to control of infectious diseases that were leading causes of mortality and morbidity (2). Success in reducing mortality and morbidity associated with infectious diseases during the first three quarters of the 20th century, led to complacency rather than continued research and development for new, innovative antimicrobial treatment options. However, re-emergence of multi-drug resistant (MDR) TB strains and the general increase in the mortality due to infectious diseases provided evidence that as long as microbes evolve, new diseases will continue to arise (4).

Resistance dates back to the early days of antibiotic use, but at the time, there was a steady flow of antibiotics. Therefore, it was possible to switch treatment once resistance to a specific antibiotic was observed. Eventually, the pool of novel antibiotics decreased and we entered the era of antibiotic discovery void. Since 1987, there has been a lack of innovation in this field and only few novel antibiotics such as cefiderocol have reached the market (5). Some of the challenges that have resulted in slow discovery of antibiotics include: (i) difficulty to develop antibiotics that are clinically effective without being toxic at therapeutic concentrations, (ii) financial constraints and hurdles from regulatory bodies since each new formulation takes almost 10 years to develop with each formulation having to undergo rigorous testing for quality, efficacy and patient safety which is also time consuming and not always guaranteed to succeed, (iii) lack of specialized personnel and loss of skill in this field due to technical difficulty in developing antibiotics, and, (iv) poor financial incentives which has resulted in pharmaceutical companies scaling down or abandoning antibiotic development programs (6). While antibiotic discovery has slowed or stalled, bacteria are continuously evolving and the emergence of resistance is faster than antibiotic development compromising the use of existing antibiotics and therefore resulting in low profits and financial loss on the part of pharmaceutical companies (6).

Resistance poses great challenges and the consequences of this have been seen worldwide. Antibiotic resistance is on the rise and more bacterial infections are becoming difficult to treat, particularly infections caused by Gram-negative bacteria. There are limited alternatives available for treatment particularly for infections caused my multi-resistant *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter spp.* (ESKAPE) pathogens. These ESKAPE pathogens are of importance because they are responsible for the majority of nosocomial infections, associated with highest risk of mortality, have the ability to escape the actions of antibiotics and are listed on the WHO list of pathogens (7) that need new antibiotics urgently (8).

2. Antibiotic Resistance Mechanisms

Antibiotic resistance is the ability of the bacteria to survive in the presence of the antibiotic and is a result of the bacteria mutating or acquiring genetic information from other bacteria to develop resistance (9). It is a natural phenomenon; whereby bacteria are forced to evolve mechanisms against attack from chemicals/antibiotics that are produced by other microbial flora or introduced into their environments in order to protect their ecological niches (10). Bacteria over the course of time and exposure to antibiotics have been able to develop mechanisms by which they can expel, destroy or withstand compounds such as antibiotics (10). Antibiotics misuse, overuse and inappropriate prescribing are major drivers of antibiotic resistance (11)

Resistant infections decrease the available treatment options and increase mortality and morbidity as opposed to susceptible infections; hence the need to understand antibiotic resistance mechanisms which is critical to minimizing the threat to human health and limit the spread of drug-resistant infections (12).

Antibiotic resistance is genetically encoded and occurs as a result of point mutations or acquisition of new genes via horizontal gene transfer or overexpression or duplication of existing resistance genes (12). Resistance can occur in one or more of the following ways: 1) Reduced permeability into the cell, 2) efflux 3) modification or degradation by enzymes, 4) target site modification that prevents the antibiotic from binding or reduces its affinity (12)(13). Resistance mechanisms vary in Gram-positive and Gram-negative bacteria (14). Mechanisms of resistance can be specific (for e.g. aminoglycoside modifying enzymes will only modify certain aminoglycoside substrates) or non-specific (cell membrane of bacteria is impermeable to many small molecules) (15)(16).

- ***Reduced permeability into the cell***

Bacteria have cell membranes which act as natural barriers against certain type of molecules. Gram-negative bacteria contain an additional outer membrane which is made up largely of lipopolysaccharide that acts as a barrier against certain molecules providing intrinsic resistance against lypophillic agents. In bacteria with outer membranes, substances enter through porin channels. Changes in these porin channels affect permeability or uptake of antibiotics. This occurs in two ways; 1) number of porins are reduced and 2) mutations that result in changes in the selectivity of the porin channel. Members of the *Enterobacterales* are known to become resistant to carbapenems by reducing the number of porins or stopping the production of certain porins completely. In *Enterobacter aerogenes*, mutations in porins result in resistance to imipenem and cephalosporins (14). The outer membrane acts as a barrier but it does not do so individually rather in conjunction with porin channels and/or efflux pumps. Due to the

absence of an outer cell membrane in Gram-positive bacteria, resistance associated with this mechanism is less common, but not uncommon. In Enterococci, polar molecules have difficulty entering the cell wall, hence making them intrinsically resistant to aminoglycoside. Vancomycin intermediate resistant *S. aureus* (VISA), thickens its cell wall making it difficult for vancomycin to enter the cell resulting in its intermediate resistance to vancomycin (17).

- ***Efflux***

Genes encoding for efflux pumps are found on plasmids and/or the chromosome (17). The genes for chromosomally encoded efflux pumps are expressed constitutively, some are induced and some are expressed only in the presence of a stimuli or when a suitable substrate is available (14). The primary function of these efflux pumps is to get rid of toxic substances including antibiotics from inside the bacterial cell. Pumping antibiotics out of the bacterial cell results in decreased concentration of the antibiotic inside the cell. This enables the bacteria to survive despite high external concentrations of the antibiotic, leading to survival and resistance (14). Some efflux pumps have the ability to transport a wide variety of substances, molecules, or more than one type of antibiotic class, thereby conferring multi-drug resistance. These type of efflux pumps are known as MDR-efflux pumps (17). There are six main families of efflux pumps and are classified based on their structure and energy source, viz.: 1) adenosine triphosphate (ATP)-binding cassette (ABC) family, 2) multi-drug and toxic compound extrusion (MATE), 3) major facilitator superfamily (MFS), 4) resistance-nodulation-cell division (RND) family, 5) small multi-drug family(SMR), and 6) proteobacterial antimicrobial compound efflux (PACE) (17)(14). All of these except for the RND family efflux pumps function as single component pumps which transport substrates across the cytoplasmic membrane. The RND family efflux pumps are multi component pumps that span both the inner and outer membrane of the bacterial cell. They function in association with a periplasmic membrane fusion protein (MFP) and an outer membrane protein (OMP-porin) to transport substrates across the entire cell membrane (14). The RND and PACE family efflux pumps are unique to Gram-negative bacteria whereas the ABC, MATE, MFS and SMR efflux pumps are found in both Gram- positive and Gram-negative bacteria (14).

In Gram-negative bacteria, the most clinically significant efflux pump are members of the RND family. These RND pumps are highly conserved among different species, however high levels of homology have been observed among the different RND pumps (*Escherichia coli* (AcrB), *P. aeruginosa* (MexB), *A. baumannii* (AdeB)) found in different species of Gram-negative bacteria (14). The most well characterised RND efflux system is *E. coli* AcrAB-TolC system. This *E. coli* AcrAB-TolC system, like other members of the RND family, is made up of three components: 1) an inner membrane transporter (AcrB), 2) an outer membrane protein channel (TolC) and, 3) a periplasmic adaptor protein (AcrA).

This efflux pump confers resistance to penicillins, chloramphenicol, macrolides, fluoroquinolones, and tetracycline (14). Some of the efflux pumps of the RND family are antibiotic or antibiotic class specific whereas some have the ability to recognize a wide variety of substrates, and are therefore associated with MDR; for example the MexAB-OprM pump in *Pseudomonas aeruginosa* confers resistance to more than one class of antibiotics such as β-lactams, tetracycline, tigecycline, chloramphenicol, trimethoprim, sulfamethoxazole and norfloxacin (14).

In addition to the above, MDR is also observed in the RND family efflux systems in Gram-negative bacteria due to overexpression of chromosomally encoded efflux pumps which arise as a result of regulatory mutations (14). These efflux pumps are commonly observed in isolates of *A. baumannii* and *P. aeruginosa*; both of which are nosocomial pathogens that are difficult to treat and have limited treatment options. In *A. baumannii*, overexpression of the RND efflux pump AdeABC is most commonly associated with MDR and is found to confer resistance to aminoglycosides and decreased susceptibility to fluoroquinolones, tetracycline, tigecycline, chloramphenicol, erythromycin, trimethoprim, netilmicin and meropenem (14). AdeABC is also known to be associated with tigecycline resistance; a last line antibiotic used to treat MDR infections in Gram-negative bacteria. An evaluation of AdeB (the gene coding for AdeABC pump) in bloodstream isolates of *A. baumannii* found a 54-fold increase in expression of AdeB in tigecycline resistant isolates versus tigecycline susceptible isolates thus indicating that overexpression of AdeABC plays a role in resistance of tigecycline and other antibiotics as previously mentioned (18) .

In Gram-positive bacteria; efflux pumps also play a significant role in emergence of resistance. In *S. aureus*, there are more than twenty MDR efflux pumps that are chromosomally encoded (19); of which the NorA efflux pump is one of the most studied efflux systems in this species. It is coded on the chromosome by the gene *norA* and was first described in 1986 in a Japanese hospital isolated from a fluoroquinolone-resistant *S. aureus* isolate (19). It belongs to the MFS family of efflux pumps and uses proton motive force to energise the transport of antimicrobial compounds across the cell membrane using an H+: drug antiport mechanism (19). When expressed at basal level, *nor*A has been associated with reduced susceptibility to fluoroquinolones. However, overexpression of the *nor*A gene results in increased resistance to fluoroquinolones. The overexpression of the *norA* gene can be a result of mutations in the *norA* promoter region or inducible through the action of regulatory proteins (20).

- ***Modification or Degradation by Enzymes***

This is the most common antibiotic resistance mechanism exhibited by ESKAPE pathogens. It involves irreversible destruction or neutralization of the antibiotics (13). Bacteria inactivate antibiotics in two

main ways: 1) actual degradation of the antibiotics by hydrolyzing enzymes (e.g., hydrolytic cleavage of the β-lactam ring by β-lactamases) and 2) transfer of a chemical group (acetyl, phosphoryl, and adenyl) to the antibiotic using transferase enzymes whereby a chemical group is added to the antibiotic molecule, thus modifying the antibiotic and impairing its ability to bind to the target (14) (e.g., aminoglycoside-modifying enzymes [AMEs]) (13). Antibiotics have chemical bonds made of amides and esthers which are hydrolytically susceptible and can be cleaved by enzymes (14). There are many different enzymes that can cleave chemical bonds but the predominant enzymes and that are of major interest due to the threat they pose in treatment of infections are the extended spectrum β-lactamases (ESBLs) (16).

- *β-lactamases*

The β-lactam antibiotics are the most commonly used antibiotics and they all share a specific core structure; the four sided β-lactam ring. One of the ways in which bacteria confer resistance to these β-lactam antibiotics is through production of enzymes known as β-lactamases. β-lactamases can be divided into: 1) enzymes with serine residues at their active site similar to bacterial penicillin binding proteins and 2) metalloenzymes with zinc ion as a co-factor (21). β-lactamases have been around since the time when β-lactams first came into clinical use and have since co-evolved with the β-lactam antibiotics. The enzymes were first observed in *S. aureus*. Later they spread to bacteria that previously lacked the enzyme such as *Haemophilus influenzae* and *Neisseria gonorrhoeae*, indicating that resistance genes can be acquired and transferred by different mechanism of conjugation, transformation and transduction via horizontal gene transfer from one bacterial species to another (22). Production of β-lactamases is the most common method of resistance mechanism utilized by Gram-negative bacteria as a defense against β-lactam antibiotics (21). β-lactamases can be found innately on the bacterial chromosomes or can be acquired via plasmids and other mobile genetic elements (MGEs) (14). They inactivate the β-lactam antibiotics by hydrolyzing a specific site in the β-lactam ring causing the ring to open. The opening of the ring alters the structure of the antibiotic thus preventing it from binding to its target penicillin binding protein (14).

As more β-lactam antibiotics (such as cephamycin, cephalosporins with an oxyimino side chain, carbapenems, and the monobactam aztreonam) were being introduced and utilized; the bacteria responded as defense mechanism with production of a wide range of new β-lactamases such as the extended spectrum β-lactamases (ESBL), plasmid mediated AmpC β-lactamases (pAmpC) and carbapenem-hydrolyzing β-lactamases (carbapenemases) (21).

β-lactamases are classified into two systems based on their primary structure and on the basis of their substrate spectrum and response to inhibitors (19, 20, and 22). The Ambler system classifies the β-

lactamases based on their structure-amino acid sequence and consist of four classes; A, B, C and D. For classes A, C and D, the active site contains serine and Class B contains zinc dependent metallo enzymes. The Bush-Medeiros-Jacoby system is based on degraded β-lactam substrates and the effects of inhibitors. It classifies β-lactamases into three groups: Group 1, Group 2 and Group 3. Group 1 includes class C cephalosporinases, group 2 includes β-lactamases except those already incorporated into Group 1 which have serine active sites and Group 3 include metallo β-lactamases corresponding to Amblers Class B classification. Class C β-lactamases are encoded by the *AmpC* gene and are found in Enterobacterales. They confer resistance to cephamycin, cephalosporins and penicillins. These are classified as Group 1 by Bush-Medeiros-Jacoby classification. Class D also known as oxacillinases have ability to hydrolyse oxacillin, and contain serine as their active site. They are classified as Group 2 by Bush-Medeiros-Jacoby classification. Metallo- β-lactamases (MBL) are Group 3 of Bush and Jacoby's classification and Class B of Ambler classification. MBL's have the ability to hydrolyse all β-lactam antibiotics except monobactam. MBL have zinc as their active site and therefore they can be inhibited by chelating agents such as EDTA (23). Some of MBLs reported include imipenemase (IMP), Verona integrin encoded MBL (VIM), Sau Paulo MBL (SEM) and MBL from New Delhi (NDM) (23).

- *Extended spectrum β-lactamases (ESBLs)*

ESBLs are defined as transmissible β-lactamases, have the capability to hydrolyse penicillins and broad-spectrum cephalosporins and monobactams and can be inhibited by clavulanic acid, tazobactam and sulbactam (20, 22). However ESBLs do not confer resistance to cephamycin and carbapenems (24). ESBLs are serine β-lactamases and belong to class A of the Ambler classification (24). ESBLs are epidemiologically important β-lactamases which have become epidemic worldwide, causing life threatening infections as there are limited available treatment options to treat infections caused by ESBL-producing bacteria. ESBLs are found in many species of Enterobacterales as well as *P. aeruginosa* and *A. baumannii* (24).

ESBL genes are diverse with unique characteristics and can be grouped in different families; SHV, TEM, CTX-M to mention a few. Some of these ESBL families are closely related like SHV and TEM which only differ by a few amino acid substitutions whilst other ESBLs like CTX-M type are more genetically diverse (24). Currently, the CTX-M type is the most common type of ESBL (22)

- SHV type

The SHV type ESBLs are assumed to have originated from *Klebsiella* spp. (22)(24) as the progenitor SHV-1 is found universally in all *K. pneumoniae* (22) within the bacterial chromosome. It is assumed that the gene for SHV-1 evolved as a chromosomal gene and it was later incorporated into the plasmid

of *K. pneumoniae* and spread to other Enterobacterales (22). SHV-1 confers resistance to broad-spectrum penicillins such as ampicillin, tigecycline and piperacillin but not to the oxyimino substituted cephalosporin (22). SHV-1 accounts for 20% of plasmid mediated ampicillin resistance in *K. pneumoniae* (22). There are 228 sequence variants of SHV; of which SHV-5 and SHV-12 are the most prevalent SHV types in Enterobacterales worldwide (24)(21). In Europe, a survey to determine the spread of ESBL and carbapenemases conducted on 45,335 Gram-negative bacilli collected in 18 European countries as part of the International Network for Optimal Resistance Monitoring (INFORM) global surveillance programme from 2013 to 2017 found that 3.1-17.0% of the ESBLs were of SHV type and most prevalent in Greece (25). However, this prevalence was much lower in comparison to CTX-M-type ESBL. In another study, investigating β-lactamase characteristics in ceftazidime resistant isolates from hospitalized patients with complicated urinary tract infections (UTI) and complicated intra-abdominal infections found SHV-type ESBLs were rarely encountered (14/284 were SHV type) and were only found in isolates that also produced plasmid mediated AmpCs or carbapenemases (24). Likewise, in Malawi SHV type ESBLs are also rarely encountered notwithstanding that data is limited to *E.coli* only. Whole genome sequencing conducted in Blantyre on 94 *E.coli* samples isolated from blood and CSF from hospitalized patients found that SHV type ESBLs were only found in 1/94 of the *E.coli* (26). In another study in Lilongwe, Malawi, WGS was conducted on 58 *E.coli* collected from a variety of samples from hospitalized patients from 2012-2018 found no SHV-type ESBL (27). SHV-type ESBLs are thus infrequently encountered in clinical isolates of *E. coli*. For Malawi, limited data exists for Enterobacterales, *P. aeruginosa and A. baumannii*, hence there is need to explore SHV prevalence in these bacteria.

- TEM type

These are ESBLs that are variants of the original plasmid mediated β-lactamases TEM-1 that was described in the early 1960's (22). TEM is closely related to SHV type ESBL. They have similar substrate and inhibition profiles with only differences of a few amino acids. TEM-1 has the ability to hydrolyse penicillins and first generation cephalosporins, however it is unable to hydrolyse oxyimino cephalosporins. This enzyme was first identified in *E.coli* in 1965 (22), isolated from a blood culture of a Greek patient named Temoneira; hence the enzyme was named after this patient. TEM-2 type was the first derivative of TEM-1 and had a single amino acid substitution compared with TEM-1 (22). TEM-2 acts as a progenitor for the other TEM variants (24). They are 243 different TEM variants however not all of them have the ESBL phenotype (24). TEM-3 reported in 1989, was the first TEM type ESBL to show ESBL phenotype (24)(22). The prevalence of TEM type ESBLs vary according to regions. TEM-3 was more prevalent in France but was rarely isolated in the USA (24) whereas TEM-10 and TEM 26 were more prevalent in the USA (21). Just like SHV-type ESBL, the TEM-type ESBLs are

also becoming more infrequent. Less than 1% of ESBL producing *E.coli* and *K. pneumoniae* in Europe were of TEM-type according to INFORM survey (25). In contrast, prevalence of TEM-type in particular TEM-1 type ESBLs were much higher in Malawi in comparison to the European INFORM surveillance program (25).

In comparison to the European INFORM surveillance programme, which comprises 18 countries, a WGS of 58 *E. coli* isolates from a variety of specimens from hospitalised patients in Lilongwe, Malawi (central region of Malawi), revealed a higher prevalence of TEM type ESBL (24). In Lilongwe, TEM-1 was detected in 43/58 (74%) of isolates. Similarly in Blantyre Malawi (southern region of Malawi), WGS of 94 *E.coli* isolated from blood culture and CSF also detected a high prevalence (74/94; 79%) of TEM-1 type ESBL (27). Other TEM-type ESBLs detected from Lilongwe were similar to those found in the INFORM surveillance program, e.g., TEM-113. TEM-150, TEM-214 and TEM-235 were detected in 1/94 (1%) isolate and TEM-209 in 2/94 (3%) isolates of *E.coli* (26). In other regions of Africa, TEM-type ESBLs have also been observed. For instance, TEM-1-type ESBLs were identified by WGS in a two-month observational prevalence study characterizing ESBL-producing K. pneumoniae from rectal swabs of hospitalised patients in Kwazulu Natal in two hospitals in the uMgungundlovu district, South Africa in 2017 (26). They found 9 ESBL producing *K.pneumoniae*, all of which contained the TEM-1-B ESBL gene.

In Tanzania, a prevalence study conducted on 1,260 clinical specimens (blood, wound swabs, urine and pus) isolated 92 ESBL-producing *K. pneumoniae* which were evaluated for ESBL resistance genes. Seventy percent (49/92) of the *K. pneumoniae isolates* contained TEM-1 type ESBLs (28). A systematic review of prevalence of ESBL-producing Enterobacterales and associated genes at community and hospital settings in East, Central, and Southern African countries involving 27 studies found that 7% of the studies detected equal proportions of TEM-type and CTX-M type ESBLs (29). This shows that TEM-type ESBLs may not be as infrequent in Africa as they are in more developed countries. This also highlights the need to further investigate molecular characterization of ESBLs to establish their burden in African regions as currently limited data exists.

- *CTX-M type*

CTX-M type ESBL are β-lactamases that have the ability to hydrolyse cefotaxime and are better inhibited by β-lactamase inhibitor tazobactam rather than sulbactam and clavulanate. They originated from chromosomal ESBL genes found in *Kluyvera* spp., an opportunistic pathogen found in the environment (22). They were first reported in the late 1980's and were emerging sporadically in different countries and were given different names. CTX-M came from cefotaxime in Munich Germany, FEC-1 and Toho-1 in Japan, and MEN-1 in France. They then spread across the globe causing outbreaks

in many countries and was referred to as the CTX-M pandemic (24). Since 2000s, CTX-M type ESBLs are the most common genetic variant and largest group of ESBL to date (21)(22), replacing SHV and TEM as the dominant ESBLs (24). CTX-M β-lactamases are class A β-lactamases according to Ambler classification and exclusively found in Group 2 according to Bush and Jacoby classification. They are found mainly in *E.coli* particularly in UTI isolates (14) but is also found in other Enterobacterales, *P. aeruginosa* and *Acinetobacter* spp.(22)(24). CTX-M ESBLs are transmitted via horizontal gene transfer from other bacteria using conjugative plasmid or transposons (22); thus CTX-M ESBLs are more transmissible and widespread.

There are 238 variants that have been sequenced and can be divided into 5 groups based on their amino acid sequence (22): CTX-M-Groups 1, 2, 8, 9 and 25. In CTX-M group 1, the most common is CTX-M-15 followed by CTX-M-3 and CTX-M-1. In CTX-M group 9, CTX-M-9 and CTX-M-14 are the most common and more recently CTX-M-27 (24). In the other groups (CTX-M groups 2, 8 and 25), CTX- 2, CTX-M- 8 and CTX-M-25 were the most common in their own groups respectively (24). The earlier variants of CTX-M type ESBL had limited activity against ceftazidime, however the newer variants such as CTX-M-15 from CTX-M Group 1 and CTX-M-27 from CTX-M Group 9 have shown capability to hydrolyse ceftazidime (24). CTX-M-15 are capable of hydrolyzing ceftazidime as opposed to their ancestors CTX-M-3 as a result of a single amino acid change in position 240 (Asp to Gly) (24). The Asp240 residue which is located in the terminal part of the B3 β-strand is responsible for the flexibility, accommodating ceftazidime which is a bulkier molecule than cefotaxime (24).

CTX-M-15 prevalence has increased globally over time and is now the predominant genotype in most regions except China, South East Asia, Japan, South Korea and Spain (30). In these regions, the predominant CTX-M ESBL type are from CTX-M- group 9 such as CTX-M-14 and CTX-M-2. (30). As previously reported CTX-M type ESBLs are more prevalent in *E.coli,* e.g. CTX-M-15 is observed more frequently in *E.coli* and specifically in *E.coli* ST131(31). This association between ST131 and CTX-M-15 is said to be responsible for the increase in the prevalence and global spread of the CTX-M- 15 as a result of clonal expansion of *E.coli* ST131 which has also been observed in commensal *E.coli* in the gut. A study investigating phenotypic and genotypic prevalence of ESBLs in *E.coli*, conducted from 2020-2021 on 205 *E.coli*, isolated from out-patients with UTI and acute diarrhea at Mina University Hospital, Egypt, found that 84.8% (89/105) of the *E.coli* from UTI and 47% (47/100) of the intestinal *E.coli* were of ST131 type (31). Of these, 78.9% (45/57) of urinary *E.coli* and 53% (35/66) of intestinal *E.coli* contained CTX-M-15 gene. CTX-M-15 was also found in the majority of the *E.coli* regardless of sequence type and was more prevalent in intestinal isolates where 90% (64/71) of ESBL producing intestinal isolates and 68.4% (54/79) ESBL producing urinary isolates contained the CTX-M-15 resistance gene (31). This study shows the association between ST131 and CTX-M-15 and higher prevalence in intestinal isolates; both of which could be a factor responsible for the increased spread of the CTX-M-15 gene worldwide.

According to the INFORM global surveillance programme, CTX-M-15 was the most common variant and was detected in 77-90% of ESBL positive isolates in Northern/Western, Eastern and Southern Europe. In individual countries of these regions except for Greece, CTX-M-15 was found in more than 70% of ESBL positive isolates (25). For ESBL positive *E.coli*: 55-71% of the isolates from European sub regions contained CTX-M-15 gene and more than 43% of ESBL positive *E.coli* from the individual European countries except for Netherlands contained the CTX-M-15 gene (25). Similar to Europe, CTX-M-15 is also predominant in the African regions. The systematic review of prevalence of ESBL-producing Enterobacterales and associated genes at community and hospital settings in East, Central, and Southern African countries involving 27 studies found that CTX-M-15 was the predominant ESBL gene detected in the majority of the countries (22/27; 82%). Similarly in Malawi, a study on WGS of *E.coli* in Lilongwe found that CTX-M-15 was predominant (25). WGS of 58 *E.coli* isolates from a variety of specimens from hospitalized patients found that 26/58 (44.8%) contained the CTX-M-15 gene and CTX-M-15 was also most prevalent in ST131 type *E.coli*. These findings concur with the European INFORM survey (25), the systemic review of ESBLs in Africa (29) and the ESBL prevalence study conducted from 2020-2021 in Egypt (31). In Blantyre, WGS conducted on 94 *E.coli* isolated from blood culture and CSF (26) found that CTX-M-15 (20/94; 21.3%) was common, however it was superseded by narrow-spectrum TEM-type β-lactamases (74/94; 78.7%). The study in Blantyre was conducted in 2017 versus the Lilongwe study which was conducted from 2012 – 2018, therefore it could be that there is a shift from narrow-spectrum TEM β-lactamases to CTX-M type ESBLs. This shows that Malawi is also following the same trend as other countries across the globe; however data is still limited. As of now, only *E. coli* data are available. There is therefore a need for more surveillance studies to allow for a clearer understanding of ESBL gene distribution in other bacterial species.

- ***Target Site Modification***

Changes in the target site of the antibiotic affect and limit the interaction between the antibiotic and the bacteria. This prevents the bactericidal/bacteriostatic effect of the antibiotic thus promoting resistance. Limitations of the interactions arise as a result of low binding affinity due to change in the structure of the binding site or reduction in the number of binding sites (14). This mechanism of resistance is most commonly associated with resistance to β-lactams in Gram-positive bacteria. Resistance to β-lactams arise due to alterations in the structure of the penicillin –binding proteins (PBPs) and/or changes in the number of PBPs. PBPs are involved in synthesis of the cell wall (14). β-lactams target these PBPs to inhibit the cell wall synthesis by inhibiting the transpeptidation step of the cell wall bio-synthesis by acting as a substrate analog of the D-Ala-D-Ala peptidoglycan side chain. The β-lactams then forms a covalent acyl-enzyme complex with the nucleophilic serine of the PBPs active site inhibiting cell wall

transpeptidation resulting in defective cross linking of the cell wall and ultimately resulting in destruction of the cell wall. In *S. aureus*, target site modification confers resistance to methicillin. More specifically in *S. aureus*, resistance to methicillin is associated with the replacement of PBPs with a new PBPs called PBP2a. PBP2a is encoded by *mec*A gene on the chromosome and has a low affinity for β-lactams. Resistance conferred by PBP2a is manifested in two ways; 1) reduced rate of β-lactams enzyme acylation and 2) the absence of high affinity for β-lactams (32).

This mechanism of resistance is also observed at other antibiotic target sites; such as the ribosome subunit, DNA gyrase or topoisomerase IV and RNA during mRNA transcription. Resistance is conferred to antibiotics that target ribosome subunits via ribosomal mutations, ribosomal subunit methylation or ribosomal protection. Mutations cause structural changes to DNA gyrase (e.g. *gyrA*) and topoisomerase IV (e.g. *grlA)* and prevent or decrease the ability of the antibiotics (fluoroquinolones) to bind conferring resistance. Single step point mutation of the rpoB gene which codes for the RIF binding pocket on the β sub-unit of the RNA polymerase results in amino acid substitutions which confers resistance to rifampicin (33).

3. ESKAPE Pathogens

ESKAPE pathogens are major causes of nosocomial infections particularly in critically ill and immuno-compromised patients. These ESKAPE pathogens are MDR organisms, and are capable of escaping the biocidal action of antibiotics (34). ESKAPE pathogens comprise of both Gram-positive and Gram-negative bacteria and include *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species. Each of the ESKAPE pathogens are described in more detail in the following sections:

Gram-negative ESKAPE pathogens

- *A. baumannii*

A. baumannii is an aerobic, pleomorphic, non-motile Gram-negative bacillus primarily associated with hospital acquired infections. It is an opportunist pathogen and commonly causes bloodstream infections (BSI), wound infections, soft tissue infections, UTI, and ventilator associated pneumonia. It is only in the last decade that *A. baumannii* has been associated with community acquired infections. *A .baumannii* has been labeled as an emerging pathogen because of its antibiotic resistance properties and not because of its virulence as would be the case for other emerging pathogens (35). *A. baumannii* is intrinsically resistant to a wide variety of antibiotics (35). It has the ability to intrinsically express

diverse resistance mechanisms that can alter membrane permeability by reducing outer membrane porins or efflux pumps hence resulting in insufficient concentrations of antibiotics to inhibit the bacterial cell targets (36). It possesses two key features that make it a successful pathogen; (i) it has sufficient number of resistant elements (35), and, (ii) the capacity to colonise any environment and survive in extreme environmental conditions (35)(23). *A. baumannii* has been referred to as pan-drug resistance pathogen since many countries have found it to be resistant to all currently available antibiotics. *A.baumannii* has been reported as resistant to β-lactams, broad-spectrum cephalosporins, aminoglycosides and fluoroquinolones, however since 1991, *A. baumannii* has also been found to be resistant to carbapenems (35).

According to the 2013 CDC report, multi drug-resistant *A. baumannii* was classified under the CDC antibiotic resistance threats level as serious. By 2019, the threat level of the pathogen has changed to urgent (37), and now the focus is on carbapenem resistant *A. baumannii*. Carbapenem resistant *A. baumannii* are categorized as critical according to WHO priority pathogen list for development and research of new antibiotics. With limited treatment options and no new antibiotics in development to treat these infections, there was need to escalate the threat levels from serious to urgent (7). This further highlights the need for surveillance of antibiotic resistance for such pathogens in countries like Malawi that are already under resourced, so that prevalence of resistance types can be understood and strategies can be put in place to hinder resistance from spreading and escalating to levels where no antibiotic treatment is available.

A. baumannii has both acquired and intrinsic mechanisms of resistance and are difficult to treat regardless of colonization site. It is one of the most successful MDR bacterial colonisers in hospitals. Its infection is commonly treated with tetracycline, aminoglycosides and β-lactams and for MDR *A. baumannii* infections, carbapenems, colistin, and other alternative therapies are being used. However, *A. baumannii* is also becoming resistant to colistin; the last and only treatment option. Hence, WHO has declared it as a pathogen that must be closely monitored for hetero- and colistin-resistance in order to develop and explore new antibiotics (35)(38).

Categorizing of this pathogen as urgent by CDC and critical by WHO as well as continuous monitoring has shown positive impact on hindering the spread of resistant *A. baumannii*. According to CDC report on antibiotic threats (37), it was found that there was decrease in the number of carbapenem resistant *A. baumannii* infections from 2012-2017 in the USA.

Currently, the prevalence of antibiotic resistance (multi-drug and pan-drug resistance) *A.baumannii* is increasing globally (39). A 5-year retrospective study from 2010-2015 analysing antimicrobial susceptibility data of 577 ESKAPE pathogens isolated from urine, rectal swabs, nasal swabs and blood from a primary hospital in Brazil found 8% prevalence of *A. baumannii* (40). Higher resistance rates were observed in *A. baumannii* in comparison to other ESKAPE pathogens, ranging from 100%

resistance to imipenem to 91.9% resistant to ciprofloxacin, cefotaxime and ceftazidime, 88.8% to cefepime, 86% to meropenem and 82.2% to levofloxacin (38). A cross-sectional study conducted at Felegehiwot referral hospital in Ethiopia from April 1 to July 31, 2018 on 238 patients with blood stream, urinary tract and surgical site infections (SSI) found a 3.8% (9/238) prevalence of *A. baumannii* infection from wound, urine and blood specimens, of which 100% were resistant to ampicillin and piperacillin, 88.9% were resistant to amoxicillin-clavulanic acid, ceftriaxone and cefotaxime, and 77.8% were resistant to tetracycline and ceftazidime, 44.5% were resistant to ciprofloxacin and 33.3% resistant to meropenem (41). All of the *A. baumannii* were MDR with resistance to three to six antibiotics of different antibiotic classes. Three of the 9 (33.3%) *A. baumannii* were resistant to all six antibiotics. In South Africa, a five year antibiotic surveillance study from August 2011- December 2015 on antibiotic resistance trends of ESKAPE pathogens isolated from a variety of clinical specimens (blood, urine, catheters, wounds, respiratory specimens) from 9 hospitals in two districts of KwaZulu Natal also observed similar patterns of high antibiotic resistance (42). A total of 64,502 ESKAPE clinical isolates formed the sample, of which 8010 (12.4%) were *A. baumannii* that were resistant to piperacillin tazobactam (81%), ceftazidime (77%), meropenem (73%), imipenem (72%), ciprofloxacin (71%), gentamicin (68%), amikacin (18%) and colistin (4.8%). Seventy percent of *A. baumannii* were MDR and resistant to all antibiotics except colistin, amikacin and gentamicin (42).

In Malawi, a surveillance study on antibiotic susceptibility patterns of 194,539 blood cultures from adults (79 095; 40·7%) and children (115 444; 59·3%) at a tertiary hospital over a 19 year period (1998-2016) with resistance found an *A. baumannii* prevalence of 1-9% (n=545) (43). Of these 31.2% (n=119) were resistant to amoxicillin, co-trimoxazole, chloramphenicol, gentamicin, ciprofloxacin, and ceftriaxone. These findings are similar to findings in other countries. Even if prevalence for Malawi is relatively low, the isolates were resistant to more than one antibiotic and exhibited MDR phenotypes. The low prevalence may be attributed to the limited number of studies carried out because of possible lack of resources to have surveillance systems in place (43).

In a literature review (44) of 29 low income countries (LIC) determining *A. baumannii* susceptibility patterns for the past 20 years (2000-2020), most studies reported 3-100% MDR *A. baumannii* cases. An interesting observation from this review was that, despite poor hygiene, lack of treatment options, poor understanding of transmission mechanisms, widespread infectious diseases, and practices such as over-the-counter prescription of antibiotics, empirical treatment of infections, and community prescribing pressure on clinicians without proper investigation, the prevalence of antibiotic-resistant *A. baumannii* was much lower in LMICs than in developed countries. The low number of *A. baumannii* reports in the literature could be attributed to lack of surveillance systems due to restricted financial resources. In order to address this gap, WHO's Global Action Plan to tackle Antimicrobial Resistance (AMR) focused on improving surveillance capacity in LICs in both the governmental and private sectors (44). Like other LICs, Malawi lacks comprehensive AMR data on *A. baumannii*, thus making it pertinent to

understand the status of its resistance patterns in Malawi. This in turn will contribute to global data, bridge information gaps, align the findings with existing data and track the AMR trends of this bacterium to limit its spread.

- *P. aeruginosa*

P. aeruginosa is a Gram-negative, non-fermenting bacteria. It has the ability to survive and colonise in natural, clinical and artificial settings. It is one of the five most common pathogens that are responsible for nosocomial infections (45) particularly in intensive care units (ICU) (46). *P. aeruginosa* causes infections such as pneumoniae, BSI, UTI and SSI (37). The infections are usually life threatening and difficult to treat due to their ability to alter the host immune responses and cause severe tissue damage. *P. aeruginosa* is increasingly becoming resistant to all antibiotics including carbapenems and it is MDR (37). *P. aeruginosa* acquired MDR after multiple mutations which resulted in decreased permeability of its outer membrane and changing regulation of efflux pumps (35). Carbapenems are used to treat MDR *P. aeruginosa*, however recently carbapenem resistant *P. aeruginosa* have emerged and caused nosocomial outbreaks. Surveillance studies in UK and Europe have noted an increase in prevalence of carbapenem resistant *P. aeruginosa*; from 4% in the 1990's to 30% in 2000s (45).

Carbapenem resistance is a result of multiple resistance mechanisms such as production of carbapenemases including MBLs [New Delhi MBL (NDM), VIM and IMP], class A carbapenemases (KPC and GES) and OXA-type β-lactamases, over expression of efflux pumps, and, loss of porins in the outer membrane together with production of ESBLs and AmpC β lactamases (45). Of all the resistant mechanisms, carbapenemase production is of utmost importance since it is associated with high mortality in comparison to non-carbapenemase producing carbapenem resistant *P. aeruginosa* (45). Two major features of *P. aeruginosa* are; (i) they are highly virulent since they are able to form biofilms in order to protect themselves from the harsh environments such as those containing antibiotics and heavy metals, and, (ii) they possess intrinsic and acquired resistance phenotypes. β-lactams, monobactams, cephalosporins, carbapenems, polymixin and aminoglycosides are used for treatment of infections caused by *P.aeruginosa* (35).

Based on WHO's priority pathogen lists for antibiotic development and research, carbapenem resistant *P. aeruginosa* is classified as critical (37) and the CDC antibiotic resistance threats level classified MDR *P. aeruginosa* as serious (7). About 2-3% of carbapenem- resistant *P. aeruginosa* carry mobile genetic elements which makes a carbapenemase enzyme. These mobile genetic elements can be easily shared between bacteria, promoting the rapid spread and increase of this resistance around the world. Resistance also limits treatment options and poses a financial burden on health systems. Hence the need

to continuously monitor and track the resistance profiles of *P. aeruginosa*. According to CDC there has been a decline in cases of MDR *P. aeruginosa* in USA from 40,000 in 2012 to 32,600 in 2017. However, in other countries MDR *P. aeruginosa* continues to rise (37).

A retrospective study conducted in a university teaching hospital in Sao Paulo Brazil, determined the prevalence of MDR and resistance profiles of *P. aeruginosa* isolated from ICU patients for about four years (46). The study found that 48.7% of the *P. aeruginosa* isolates were MDR and reported an increase in resistance to carbapenems, i.e., imipenem from 50% in 2010 to 71.4% in 2013 and meropenem from 44.4% in 2010 to 71.4%. Similarly, in a retrospective study of 740 adult patients diagnosed with *P. aeruginosa* nosocomial pneumonia from 12 hospitals across 5 countries (United States; n = 3; France; n = 2; Germany; n = 2; Italy; n = 2; and Spain; n = 3) (47) determined the risk factors associated with MDR strains of *P. aeruginosa* acquisition and found that 30.5% (n=226/740) of the *P. aeruginosa* were MDR. Of these, 26.5% were resistant to cephalosporins and 15% were resistant to carbapenems. MDR was highest in Germany (44.2%) and Spain (43.4%) followed by France (33.3%), Italy (22.2%) and the United States (20.5%) (47).

A South African study on ESKAPE pathogens found a 17.4% prevalence of *P. aeruginosa*. There was a decreasing trend in antibiotic resistance of *P. aeruginosa* to ceftazidime (17-13%), piperacillin (27-21%), ciprofloxacin (22-18%) and meropenem (18-10%) (42). The prevalence and resistance rates were lower compared to other research mentioned here. In Malawi, there is limited data on *P. aeruginosa* however the Blantyre, Malawi study mentioned above found a prevalence of 1.5%. This study did not have comprehensive data on antibiotic susceptibility profiles for *P. aeruginosa* since antibiotics such as ceftazidime were not always available and therefore only 9 were tested for antibiotic susceptibility. Seven of the 9 were resistant to ceftazidime (43). It is seen from the surveillance study in Malawi that prevalence of *P. aeruginosa* is low but this is not representative of the country and all specimen types. This study was conducted in the southern region of Malawi only and only covered blood cultures. In a two-month prospective study of swabs performed at the Queen Elizabeth Central Hospital (QECH) in Blantyre, Malawi, from June to August 2000, where *P. aeruginosa* prevalence was 4.5%, it was found that pus specimens collected at surgical sites and burns units were more likely to contain *P. aeruginosa* infections (48). This study at QECH in the burns unit (48) was carried out more than 10 years ago and the prevalence may have increased now that utilization of microbiology services are being advocated, promoted and resources are available. Therefore, there is a need to conduct surveillance studies to bridge the gap that exists in the data for *P. aeruginosa* in Malawi. MDR *P. aeruginosa* may already exist and be spreading its resistance genes in silence to other Gram-negative bacteria. Therefore, there is a need for urgent surveillance of this particular microorganism.

- *Enterobacter* spp.

Enterobacter spp. are Gram-negative bacteria that belongs to the Enterobacterales family. They are saprophytic and can be found in the environment as well as are part of the commensal flora of the gastro-intestinal tract. It is an opportunistic pathogen that commonly causes infections in the immune-compromised patients and those that are on mechanical ventilation. Incidence of *Enterobacter* spp. is higher in ICU patients. It is commonly associated with urinary tract, gastrointestinal tract and respiratory tract infections (49).

Enterobacter spp. comprises of 22 species; six of which are grouped together and called the *E. cloacae* complex. *E.cloacae* complex consist of *E. asburiae*, *E. hormaechei*, *E. kobei*, *E. ludwigii* and *E. nimipressuralis* (49). *E. cloacae and E. aerogenes* are the most commonly isolated species from clinical specimens (50). *E. cloacae* is clinically significant and ranks third amongst all Enterobacterales in healthcare associated infections. It is listed in the WHO priority pathogen list for development and research of new antibiotics (7). It commonly causes nosocomial infections associated with the urinary tract, lower respiratory tract, skin and soft tissue, biliary tract, the central nervous system, catheters and intravenous devices. *E.cloacae* contains chromosomally encoded AmpC β-lactamases which make it intrinsically resistant to ampicillin, amoxicillin/clavulanic, cephamycin, and the 1st and 2nd generation cephalosporins (51). Due to increase in invasive procedures and a rise in severe co-morbid conditions there has been an emergence in carbapenem resistant *E .cloacae* since carbapenems have increasingly been used to treat these nosocomial infections. Carbapenem resistant *E. cloacae* are of importance because they are associated with higher mortality, longer hospitalisation, resistance to last resort antibiotics and higher costs posing a threat to public health (51). Hence, these carbapenem resistant *E. cloacae* (as part of carbapenem resistant Enterobacterales) are classified under the CDC antibiotic resistance threats level as urgent (37). According to the China antimicrobial surveillance unit (CHINET), carbapenem resistance in *E. cloacae* has increased from < 1% in 2007 to 6.9%, 7% and 8.2% in 2017 to imipenem, meropenem and ertapenam respectively (51).

Carbapenem resistance in *E. cloacae* occurs by various mechanism such as production of carbapenemases, decreased porin permeability, alteration or loss of non-specific porins together with overexpression of ESBL and/or AmpC (51). However production of carbapenemase is the most common mechanism which is utilized by *E. cloacae* to resist carbapenems. The genes for carbapenemases such as MBL are found on mobile genetic elements such as plasmids and transposons of *E. cloacae* (51) and this makes it easy for these resistance genes to be transferred to other bacteria by horizontal gene transfer posing an even greater public health threat that requires urgent attention to hinder it's spread (51)

An increase in resistance to 3rd generation cephalosporins has been observed in *E. cloac*ae. A study conducted in Malaysia examined 117 *Enterobacter* spp. isolates from a variety of clinical specimens

from 2013-2014 from Medical Microbiology Diagnostic Laboratory, University Malaya Medical Centre found a 64.9% (n=76/117) prevalence of *E. cloacae* (50). These were found to be resistant to cefotaxime (50%; 38/76), ceftriaxone (48.7%; 37/76) and ceftazidime (30.3%; 23/76) although none were resistant to meropenem (50).

In Brazil, a cross sectional retrospective study on antibiotic susceptibility profiles data of ESKAPE pathogens obtained from a primary public hospital for the period January 2010 to December 2015 (40) found that *Enterobacter* spp. were more resistant than isolates from Malaysia; 73.9% were resistant to ceftriaxone, 79.2% to cefoxitin and 87.5% to amoxicillin/clavulanate although the study did not differentiate between the different species of *Enterobacter* (40). In Turkey, a cross-sectional study evaluating a variety of clinical specimens from 223 outpatients and inpatients treated in Karabuk University Training and Research Hospital for the period January 2016 – December 2020 found an *E. cloacae* prevalence of 59.2% (132/223) (52). Of these, 29% (39/132) were resistant to ceftazidime and 60% (63/104) were resistant to cefepime. In addition, 4% (4/89) resistance to meropenem and imipenem was observed. In this study, resistance to carbapenem has been observed though the percentages were not as high as those observed in CHINET from 2017 (51).

The South African study (42) found a 6.6% *Enterobacter* spp. prevalence with low resistance to meropenem (2%), while resistance to ceftazidime (27%) was similar to those observed in Turkey (52) and Malaysia (50). Although meropenem resistance was not high, this could mark the emergence of carbapenem resistance in the African continent and therefore attention and caution must be exercised towards this pathogen. Currently for Malawi there is no reported data for *Enterobacter* spp., because of limited resources and laboratory capacity to identify such pathogens. This calls for more surveillance on the part of the public health and clinical laboratories in the country which is currently underway through funding that is now available for surveillance in Malawi.

- *K.pneumoniae*

K. pneumoniae is a Gram-negative, facultative anaerobic, non-motile, non-flagellated bacillus. It belongs to the family of Enterobacterales. It is a commensal flora of the gastrointestinal tract and is a common opportunistic pathogen (53). *K. pneumoniae* are among the top ten bacteria that cause hospital acquired infections and is most commonly isolated in the ICU (54). Usually, *K. pneumoniae* causes UTIs, cystitis, pneumonia, surgical wound infections and life threatening infections such as endocarditis and septicemia. It is also associated with community acquired infections causing infections such as necrotizing pyogenic liver abscess and endogenous endophthalmitis (55). Key features that make *K. pneumoniae* a successful pathogen include (i) carriage of plasmids that encode an array of antibiotic

resistance genes due to acquisition of transposons and other MGEs, and (ii) co-carriage of more than one antibiotic resistant plasmid (55).

In addition to being a highly prevalent bacteria, it is a major source of antibiotic resistance. Increased use of antibiotics and continuous exposure of *K. pneumoniae* to antibiotics has resulted in emergence of MDR *K. pneumoniae* (54). These MDR- *K. pneumoniae* have the ability to spread rapidly and cause outbreaks in hospitals. Most of these outbreaks are caused by ESBL producing *K. pneumoniae* that are increasing and have limited treatment option (54). They are classified according to CDC antibiotic resistance threat level-as serious (37) and as critical according to the WHO pathogen priority list (7). With the rise in ESBL producing *K. pneumoniae*, use of carbapenems has increased which has led to emergence of carbapenem resistant *K. pneumoniae*; which is a public health threat and a global health concern since these Enterobacterales are classified as urgent under the CDC antibiotic resistance threat level (37). According to the WHO pathogen priority list, carbapenem resistant *K. pneumoniae* is classified as critical priority for the research and development of new antibiotics (7).

Resistance in *K. pneumoniae* occurs in two ways; due to chromosomal mutations or acquisition of resistance genes via horizontal gene transfer via mobile genetic elements such as large conjugative plasmids, the latter being the most common method of acquisition of antibiotic resistance in *K. pneumoniae* (56). Carbapenem resistance in *K. pneumoniae* is by two main mechanisms: permeability defects combined with overexpression of β-lactamases with weak carbapenemase activity and production of carbapenemases (57). There are three different Ambler classes of carbapenemases enzymes; Class A which include the KPC-type enzyme, Class B which includes the metallo-β-lactamases enzymes, (NDM-, VIM-, and IMP-type) and class D which includes OXA-48–like β-lactamases. Of all of these, the KPC-type enzyme has been extensively reported in *K. pneumoniae* (55)(57).

Initially, the most prevalent ESBL in *K. pneumoniae* was TEM and SHV β-lactamases but now the CTX-M type are more prevalent (55). A review of prevalence and/or the molecular epidemiology of drug resistant strains of *K. pneumoniae* in some selected countries in Asia from 2005-2019 found CTX-M-1 (41.9%) to be most prevalent followed by SHV-11 (41.8%), TEM (39.5%), CTX-M-15 (35.3%), KPC-2 (14.6%) and NDM-1 (6.7%) (53).

According to European antimicrobial resistance surveillance network (EARS) surveillance from 2002-2015 (http://atlas.ecdc.europa.eu/public/index.aspx?Instance), resistance rates of *K. pneumoniae* have steadily increased over the years against the four major classes of antibiotics (3rd generation cephalosporin, aminoglycosides, carbapenems and fluoroquinolones) where 50-60% of *K. pneumoniae* are resistant to 3rd generation cephalosporins, fluoroquinolones and aminoglycosides (55). Occurrence of ESBL producing *K. pneumoniae* has increased worldwide reaching epidemic proportions in many countries (1). In 2005, all countries in Europe were free from carbapenem resistant *K. pneumoniae*,

however by 2015 this had changed and carbapenem producing *K. pneumoniae* were observed in 40-60% of isolates (55).

Increasing trends have been observed in *K. pneumoniae* both with regard to isolation rate and antibiotic resistance. The Brazilian study found high resistance of *K. pneumoniae* to cefepime (75.2%), cefotaxime (75.2%) and ertapenam (69.1%) (40). Similarly Karadiya *et al*. (2016) (58), aimed to detect the prevalence and antibiotic susceptibility of ESBL and AmpC- β-lactamase producing *E. coli* and *K. pneumoniae* from various clinical specimens in Jaipur India, investigated 108 *K. pneumoniae* and 180 *E. coli from* January to May 2014. They found high resistances to cephalosporins and carbapenems where 94% of *K. pneumoniae* isolates were resistant to cefepime and 14.3% to meropenem. They also found that more than half of *K. pneumoniae* were MDR (63.5%) and ESBL producers (63/108; 58.3%) (58). In Turkey, a study evaluating antibiotic susceptibility profiles of 2,452 *K. pneumoniae* isolates from various clinical samples submitted to Meram Medical Faculty Hospital between July 2015 - November 2019 found high resistance rates (>50%) to cefuroxime (91.8%; n=2252), ceftazidime (86.5%; n= 2122), ciprofloxacin (75.9%; n= 1862), meropenem (63.6%; n=1559), and gentamicin (52.6%; n=1290) (58).

In Saudi Arabia, a cross-sectional study to determine the prevalence of antibiotic resistance in 15,708 isolates of *K. pneumoniae* isolated from a variety of clinical samples from King Fahad Hospital, Medina from January 2014 – December 2018 found high resistance rates of greater than 50% for majority of antibiotics, i.e., 66.9% (n= 743) were resistant to ceftazidime, 77% resistant to cefotaxime, 61.1% (n=686) were resistant to ciprofloxacin, and 52.2% (n=543) were resistant to gentamicin (54). Lower resistance rates were observed for meropenem (46.1%; n= 371) and imipenem (38.4%; n=436). However, an increase in resistance rates was observed for imipenem between 2015 and 2018 from 34.6% (n=65) to 38.7% (n=135). This provides evidence that carbapenem resistance is on the rise in this country (53).

For the African region according to WHO global surveillance report (1), resistance data submitted by 13 countries showed that resistance to cephalosporins ranged from 8-77% (43), and resistance to carbapenems ranged from 0-4% (43). This data indicates that for the African continent, carbapenem resistance in *K. pneumoniae* are emerging and therefore whilst prevalence of these is still low when compared to countries like Turkey and Brazil, this is the right time to contain and prevent them from spreading.

In Southern Ethiopia, a cross sectional study of microbiology samples collected from inpatients and outpatients presenting with infection was conducted at the microbiology laboratory of Hawassa University Comprehensive Specialized Hospital (HUCSH), Hawassa, Ethiopia from January 2019 – December 2020 to determine the prevalence of the major pathogens and their antibiotic susceptibility profiles. The investigators observed that 32.6% (354/1085) of the specimens had bacterial growth (59).

Of all the Gram-negative isolated, *K. pneumoniae* was found with the highest resistance rates to ceftazidime (82%) and ciprofloxacin (80.9%). However, lower resistance rates were observed for meropenem (3.3%). *K. pneumoniae* was also found to be among organisms with the highest MDR at 87.6% (78/89).

The South African study (42) found that more than 50% of *K. pneumoniae* isolates were resistant to third generation cephalosporins (57.9% to ceftazidime and 58.3% to ceftriaxone). An increasing trend in resistance was noted for most antibiotics; an increase in ceftriaxone resistance from 54.6% to 65.5%, and meropenem resistance increased from 5% to 16 %.

In the Malawian study (43), 31.8% (n=121) of *Klebsiella* spp. were resistant to all antibiotics except carbapenem. In addition, according to the WHO GLASS -Early implementation report 2017-2018 (60), almost 80% of *K. pneumoniae* isolates were found to be resistant to ceftazidime and less than 5% were found to be resistant to meropenem. There are data gaps in terms of susceptibility profiles for Malawi that need to be addressed so that a clearer picture can be seen with regard to how antibiotic resistance is trending. Also meropenem resistance is still relatively low in Malawi from what has been found so far and therefore, continuous monitoring and surveillance of antibiotic susceptibility profiles will help to track and contain meropenem resistance.

- *E.coli*

Although *E.coli* is not recognized as an ESKAPE pathogen, it is of high importance and interest since antibiotic resistant *E.coli* present one of the largest clinical burdens both in human and animal health; hence there is need for this pathogen to be considered as a critical health concern (13). *E. coli* are Gram-negative rods belonging to *Enterobacterales*, and live in the intestinal tract of humans as commensals. They are major causes of UTI and BSI both in hospitals and in the community (61). In Africa, *E.coli* is the second most common Gram-negative bacilli that is associated with BSI and diarrhea particularly in under five children (26).

Resistance in *E.coli* is either by acquisition of mobile genetic elements via horizontal gene transfer or by mutations (13). Resistance to broad spectrum penicillins and 3rd generation cephalosporins is a result of acquiring MGEs and production of enzymes whereas fluoroquinolones resistance is a result of mutations. *E. coli* that are ESBL producers are also resistant to other antibiotics hence carbapenems are among the only treatment options (26). However, continuous use of carbapenems to treat ESBL producing *E.coli* has resulted in emergence of carbapenemase resistant *E.coli (*26).

In recent years there has been a significant increase in infections caused by *E.coli*; particularly those *E.coli* that are MDR and ESBL producing isolates, mainly CTX-M-producers (62). The spread of CTX-M enzyme-coding capacity occurs due to the mobilizing ability of their insertion sequences and

integrons. CTX-M isolates also have the ability to transfer ESBL plasmids between bacteria of the same and/or different species *via* horizontal gene transfer (63). CTX-M-15 are now the dominant and most prevalent ESBL type found in *E. coli* worldwide; CTX-M-15 is predominantly associated with ST131 clone (26).). In Malawi, a study on whole genome sequencing (WGS) of 94 *E. coli* isolates isolated from blood cultures and CSF from the period 1996-2014 also found that CTX-M-15 was the most prevalent ESBL gene type of *E. coli* (20/21; 94%) (26).

High resistance rates of resistance to aminopenicillins, fluoroquinolones, aminoglycosides, and third generation cephalosporins have been observed in *E. coli* worldwide (13). A study conducted in Tehran, Iran involving several major hospitals from May to November 2016 isolated 60 *E. coli* from urine specimens and found that all the 60 isolates were MDR with resistance rates of >50% to the following antibiotics ; cefepime (100%), cefalothin (74%), cefpodoxime (67%), nalidixic acid (63%), cotrimoxazole (54%), cefixime (50%), cephazolin (50%), and tetracycline (50%). No resistance to imipenem and meropenem were observed (61).

In Nigeria, a cross-sectional study aimed at determining prevalence of ESBL in *E. coli* and *K. pneumoniae* isolated from 200 urine samples from 2 hospitals (Grimmard Catholic hospital and Maria Goretti hospital) found an 82.1% (128/156) prevalence of *E. coli* (64). Of these 69% (54/78) *E. coli* were ESBL producers. All ESBL producers were highly resistant to both cefotaxime (100%) and ceftazidime (79.6%). Lower levels of resistance to imipenem (22.2%) was observed (64).

In Tanzania, a prospective study conducted on samples collected from inpatients and outpatients between October 2018 and September 2019 assessed the antibiotic susceptibility patterns of clinical bacterial isolates obtained from four referral hospitals. The study found a 14.8% (388/ 2620) positivity rate for bacterial growth (62). Of these 15.2% were *E. coli* that were highly resistant to ampicillin (100%), amoxicillin-clavulanic acid (75.0%), gentamicin (70.2%), tetracycline (70.2%) and ciprofloxacin (42.6%). Lower levels of resistance were observed against ceftriaxone (n=5/21; 23.8%) and meropenem (n=3/10; 30%). Only two isolates of *E. coli* were tested for ESBL both of which were negative. Unlike other studies, prevalence of ESBL was low but that could've been because not all isolates were tested for ESBL and key marker antibiotics for ESBL such as ceftazidime and cefotaxime were not tested. Although ESBL was not prevalent, resistances to other commonly used antibiotics was observed (62).

The Malawian study found all *E. coli* to be MDR and 17.8% were resistant to all antibiotics tested (43). Also according to the WHO GLASS -Early implementation report 2017-2018, almost 70% of the *E. coli* isolated from BSI in Malawi were resistant to ceftazidime and ceftriaxone and almost 80% isolated from urine are resistant to ceftazidime and ceftriaxone. According to GLASS less than 5% of *E. coli* isolated from urine and BSI were resistant to meropenem. Although data is fragmented for *E. coli* in Malawi, existing data shows that *E.coli* are highly resistant to some antibiotics and MDR and ESBL

profiles are evident. *E. coli* is highly resistant to majority of antibiotics except meropenem. Hence there is need to undertake an in-depth cross-sectional study to understand the status of *E. coli* antibiotic susceptibility profiles. It is a pathogen that quickly acquires resistance and can spread rapidly hence the need for this pathogen to be under surveillance (27).

- ***Gram positive ESKAPE pathogens***

    - *Vancomycin resistant Enterococci (VRE)*

Enterococci are Gram positive cocci bacteria and are part of the normal gut flora of both human and animals (65)(66). There are 50 different species of *Enterococcus*, However *E. faecalis* and *E. faecium* are the most commonly isolated from the human gut and are responsible for the majority of human enterococcal infections. These are opportunistic pathogens that can cause life threatening infections such as BSI and endocarditis. They are leading cause of hospital acquired infections and MDR infections. Of the two species; *E. faecium* is the prominent nosocomial pathogen and increasingly resistant to vancomycin. These vancomycin resistant Enterococci (VRE) are of concern since they are also resistant to other antibiotics raising concern that the antibiotics that are available to treat the VRE are rapidly dwindling. Hence, it is listed as high priority in the WHO pathogen priority list for research and development of new antibiotics (67) and as a serious threat under the CDC antibiotic resistance threat level (37). Enterococci has become a major nosocomial pathogen due to: (i) its intrinsic resistance to commonly used antibiotics such as penicillin, ampicillin and most cephalosporins, and, (ii) Its ability to acquire virulence and MDR determinants. Enterococci can rapidly develop resistance post exposure to antibiotics and during treatment including to the last resort antibiotics such glycopeptides, dalfopristin, linezolid, daptomycin and tigecycline (65).

Development of resistance has been through acquisition of resistant determinants by horizontal gene transfer of MGEs or via mutations that alter gene expression and binding sites in native genes (65) (66). These mechanisms are responsible for vancomycin resistance and is particularly prevalent in healthcare associated species of *E. faecalis* and *E. faecium* (66).

Resistance in Enterococci is said to have occurred in two waves. The first occurred in the 1980's in USA and was associated with introduction of third generation cephalosporins driving the emergence of vancomycin and ampicillin resistant *E. faecalis* (13). The second wave was dominated by vancomycin resistant *E. faecium*. It was first identified in the USA then it spread to other parts of the world (13). In 2016, a survey in Australia of BSI found 50% of *E. faecium* resistant to vancomycin (13). The most common sequence type in *E. faecium* is clonal complex 17 (CC17) and is responsible for the majority of nosocomial infections and is one that is vancomycin resistant (13). Unlike other ESKAPE pathogens,

outbreaks associated with VRE are much longer (approximately 11 months) and have been found to precede antibiotic exposure allowing for the resistant strains to become predominant species in the gut (13). Resistance to newer antibiotics such as linezolid, daptomycin, tigecycline is now emerging. There is therefore the need to use these antibiotics with caution and continuously monitor their susceptibility profiles (68).

In USA, there has been a decline in the number of infections caused by VRE, both in community acquired infections and hospital acquired infection, i.e., a decline from 84,800 cases in 2012 to 54,500 cases in 2017 was observed (37). However, VRE is still prevalent in the USA and other developed countries (68)

The Brazilian study found a 4% prevalence of *E. faecium*, of which 80% of *E. faecium* were found to be resistant to vancomycin (40). In addition, high resistance was observed to other antibiotics such as erythromycin (95.8%), ciprofloxacin (91.7%), ampicillin (91.7%) and penicillin G (91.7%). Low resistances were observed to linezolid (12.5%) and daptomycin (16.7%) (38).

In Germany, 10 year surveillance study (2007-2016) analyzing data submitted to the German national nosocomial infection surveillance system of VRE isolated from BSI, UTI and SSI found a total of 12, 659 infections caused by *Enterococcus* spp. across all specimen types. The prevalence of VRE increased from 1.4% in 2007/2008 to 10% in 2015/2016, whilst by individual specimen types VRE increased in BSI from 5.9-16.7%, in UTI from 2.9-9.9% and in SSI from 0.9-5% (69).

A systematic review including 291 studies that determined the frequency of antibiotic resistance to common antibiotics in enterococci isolated from blood isolates of hospitalized patients worldwide from January 2000 to May 2018 found a pooled total of 13238 isolates of *E. faecium* and 24,913 isolates of *E. faecalis*. Resistance profiles of *Enterococcus* isolated from BSI found pooled prevalence of *E. faecium* to be 8-13% (70). An increase over the years in resistance to vancomycin in *E. faecium* was observed, e.g., in 2000-2005 resistance rates were 8.2%, in 2005-2010 resistance rates were 9% and 2010-2016 resistance rates were 22%. By WHO regions, resistance to vancomycin was highest in the Americas (25.5% to 57%), followed by Western Pacific region (12-33%) , Eastern Mediterranean region (11-42.3%), Europe (5-8.6%), South East Asia (2-15.3%), and Africa (0.1-17%) (70).

A systematic review conducted from January 2000 to May 2018 on global prevalence and antibiotic resistance of enterococci strains isolated from BSI found that in Africa, resistance in *E. faecium* and *E. faecalis* are not as high as on other continents (69). In this review, the pooled prevalence of vancomycin resistance in *E. faecium* in Africa was 1.3% versus 23.2% in Eastern Mediterranean, 20.5% in the Western Pacific, 10.5% in America, 6.5% in Europe, 6% in South East Asia and the pooled prevalence of *E. faecalis* was 1% in Africa versus 4.5% in America, 4% in South East Asia and 5% in Eastern Mediterranean (70). The South African study found a 3.4% (2,217) prevalence of *E. faecium* (42), none

of which were resistant to vancomycin or linezolid (42). Similarly in an antibiotic susceptibility survey of wound infections in Gondar, Ethiopia, a cross-sectional study conducted from March to May, 2014 on 137 patients presenting with wound infections found 1.5% prevalence of *Enterococcus* spp of which only one of the two *Enterococcus* spp isolates were found to be resistant to vancomycin (71). These studies indicate that resistance to vancomycin is low or still emerging (42)(71).

The Malawian study (43) found a 0.8% prevalence of *E. faecalis* (n=220) and, 0.3% (n=76) prevalence of *E. faecium*. Unfortunately, vancomycin was not tested and therefore no data was available for resistance to vancomycin. An increase in VRE in developed countries and presence of VRE in low levels in African countries indicates that VRE is emerging even in Africa. Thus with limited data of VRE in Malawi, it is important to bridge the gap that exists for antibiotic resistance patterns data for *Enterococcus* sp. in Malawi. This is so that resistance profiles of this bacteria can be understood and the relevant steps to contain and prevent the spread of VRE can be put in place. Hence this calls for a cross-sectional surveillance study that covers the major regions of Malawi.

- *S. aureus*

S. aureus is a Gram-positive cocci which is part of the normal flora of the skin and nose (1). However, it is also an important pathogen that cause disease in humans. *S. aureus* commonly causes skin, soft tissue, bone, BSI, and post-operative wound infections (1). *S. aureus* produces toxins and can cause a variety of specific symptoms such as toxic shock syndrome and food poisoning (1).

Resistance in *S .aureus* dates back to the pre-antibiotic era. Even before penicillin was marketed some *S. aureus* strains possessed resistance mechanisms in the form of β-lactamases. These genes were found on plasmids and easily spread leading to resistance to penicillin. Penicillin was an effective first line treatment for *S. aureus* related infections (1). This led to development and marketing of more powerful antibiotics. Methicillin and cloxacillin plus combinations with inhibitors were introduced to treat these penicillin resistant infections (1). This only bought about more resistance. *S .aureus* acquired a novel gene (*mec*A) which is found on single genetic element called the staphylococcal cassette chromosome mec (SCC*mec*). The SCC*mec* is a genomic island that encodes methicillin resistance and contains two gene complexes; (i) the *mec* complex consisting of *mec*A and (ii) the *ccr* complex (33). *mec*A gene codes for penicillin binding protein 2a (PBP2a) which is a transpeptidase that has low affinity for most β-lactam antibiotics and takes over the transpeptidation reaction of the host PBP. Due to reduced affinity for β-lactam antibiotics, the PBP2a remains active in the presence of therapeutic levels of methicillin ensuring that the cross-linking of the glycan chains in the peptidoglycan continues (72). This results in the cell wall remaining intact, preventing the cell from lysing and thus gives rise to methicillin resistant *S. aureus* (MRSA) (72).

MRSA are classed as high priority under the WHO pathogen priority list for research and development of new antibiotics (7) and as serious under the CDC antibiotic resistance threat level (37). Methicillin resistance was first identified in 1961 and was only a problem in hospital acquired infections but this has now changed and MRSA is now being isolated from community acquired infections (13). Fortunately, community acquired MRSA (CA-MRSA) are still susceptible to non- β-lactam antibiotics unlike hospital acquired MRSA (HA-MRSA) which are MDR. In countries like the USA, cases of HA-MRSA are declining and CA-MRSA are on the rise (13). CA-MRSA emerged in the 1980s in Australia and in 1990s in USA and Canada. CA-MRSA are more often associated with skin and tissue infections whereas HA-MRSA causes more severe and serious infections such as pneumonia and BSI. However, the distinction between HA-MRSA and CA-MRSA is now becoming more and more difficult as CA-MRSA is now being isolated from BSI in hospitals (13). The burden of CA-MRSA and HA-MRSA varies across the different countries. For example, in China HA-MRSA clone ST239-t030 and ST239-t037 decreased from 2007-2018 from 20.3% to 1% and 18.4% to 0.1% respectively and was replaced by the ST 5-t2460 strain which emerged rapidly from 0% to 17.3% at the same time. CA-MRSA strains ST59 and ST398 also increased from 1.0%-5.8% and 1.8% to 10.5% respectively (13). MRSA infections are treated using glycopeptides such as vancomycin and teicoplanin which are only available in injection form and require close monitoring for adverse side effects (1).

A subtype of *S. aureus* that is given little attention is borderline oxacillin resistant *S.aureus* (BORSA) (12). It is found both in the community and in hospitals and characterized by intermediate resistance to penicillinase resistant penicillins with oxacillin MIC being between 1-8ug/ml (13). Since it lacks the *mec*A gene, it is not truly methicillin sensitive or resistant resulting in misidentification of these strains as MRSA which pose significant threat to patient treatment and outcome since BORSA may be nonresponsive to high doses of oxacillin (13)

Mechanisms of resistance of *S. aureus* include limited uptake of the antibiotic, modification of the antibiotic target site and inactivation of the antibiotic by production of an enzyme (13). Resistance in MRSA arise from expression of methicillin hydrolyzing β-lactamase and expression of an additional penicillin binding protein –PBP2a which was acquired from other species (73)(74). PBP2a has lower penicillin binding affinity and increased rates of release of the bound antibiotic compared to the normal penicillin binding protein (73)(74). PBP2a is encoded by the *mec*A gene and is found on the chromosomes of MRSA (13)

Different regions of the African continent have shown variations in resistance. *S. aureus* is more resistant to fusidic acid in North Africa (13-62%) than Sub-Saharan Africa (SSA) (0-2%) (75). *S. aureus* is also found to be more resistant in urban areas than rural areas; a result of increased use and prescription of these antibiotics in urban areas (75)

Methicillin resistance has exceeded 20% in all WHO regions and in some regions it has reached up to

80%, 21-90% in the Americas, 10-53% in East Mediterranean region, 0.3-60% in European region, 10-26% in South East Asia and 4-84% in Western Pacific region (76). In the Nordic countries (Denmark, Finland, Iceland, Norway and Sweden), a prevalence study aimed at comparing the emergence and dissemination of MRSA clones from 1997-2016 found a steady increase in MRSA cases throughout the study period. However, after the year 2000, the incidence rates of MRSA increased 6-35 fold except for Finland where the MRSA incidence rates remained steady after 2004 (incidence rates between 23.6-33.5 per 100,000 inhabitants (77).

In Africa, prevalence of MRSA is increasing but it is still below 50% and the prevalence is heterogeneous both inter- and intra-country. Data from nine countries shows that MRSA prevalence ranged from 12-80% (76). In East African countries such as Uganda, the prevalence ranged from 31.5-42%, 31-82% in Rwanda and 10-50% in Tanzania. For countries like South Africa where antibiotic stewardship and infection control practices are in place, a decline in prevalence rates were observed from 36% in 2006 to 24% for the period 2007-2011 (76)(78)

A systematic review conducted in Nigeria to examine the prevalence, trend and antimicrobial susceptibility of MRSA isolates from 2007-2017 assessed a total of 2,203 isolates of *S. aureus*, of which 582 were MRSA strains. The prevalence of MRSA increase from 18.3% in 2009 to 42.3% in 2013 (79).

In Kenya, a cross- sectional study conducted at two teaching and referral hospitals namely Thika and Kiambu hospitals aimed at characterization of MRSA phenotypes from swabs collected from various infected sites in both inpatients and outpatients between December 2017 and September 2018. The study found a 39.1% (n=54/138) prevalence of *S. aureus*, of which 40.74% (n=22/54) were found to be MRSA positive (80). The South African study (42), found a 38% (n=24495) prevalence of *S. aureus*. MRSA prevalence varied between 18% and 31% per year. Overall a decreasing trend in the proportion of MRSA was observed from 28% in 2011 to 18% in 2014 (42).

The Malawian study found a 6.6% (n=1925) prevalence of *S. aureus*. Of those that were tested for methicillin resistance; 9.6% (107/1118) were MRSA (43). Another study conducted at KCH involving 2056 patients and various clinical specimens from 2006-2007 determined susceptibility profiles of commonly isolated bacterial pathogens. The study found a 42% (n=154) prevalence of *S. aureus*, of which 46/147 (31.3 %) were MRSA (81). Although they were not genotypically confirmed as MRSA, this allows us to understand that MRSA is present in Malawi. There are many gaps in data on MRSA in Malawi with only a report of confirmed MRSA from one large study (43) that focused only on blood cultures. A cross-sectional study involving all specimen types may depict different results, since *S .aureus* may be more prevalent in pus and abscess than blood cultures (43).

For the majority of the African countries including Malawi, MRSA prevalence is below 50% as described in the studies above. This should not hinder countries like Malawi from conducting

surveillance on *S. aureus* but rather it should promote surveillance to prevent the spread of MRSA before it reaches levels that cannot be contained. Surveillance can aid in putting infection prevention strategies in place to prevent MRSA from spreading in hospitals and the community as it has been done in developing countries.

In summary, antibiotic resistance is a global public threat that needs to be addressed as a matter of urgency. All of this emphasizes the need to perform antibiotic surveillance in Malawi so that the gaps on current status of resistance can be filled. Surveillance can additionally allow the development of antibiograms to guide local prescription policies contribute to combating the global threat and contain antibiotic resistance.

Progress is being made in Malawi on antibiotic resistance surveillance. A national AMR coordinating center has been established and an AMR surveillance plan is in place with a budget. There are currently four surveillance sites enrolled for submitting data to GLASS. However, there are still gaps, for example, of the four labs only two are currently submitting data to GLASS. Continuous monitoring and tracking of antibiotic susceptibility profiles at all levels are needed.

Regrettably, there is no baseline data on the prevalence of pathogens in Malawi and their susceptibility profiles. Such baseline data will make it possible to understand and track the resistance in the country and prevent the spread of MDR pathogens. Also, such data is key for informing empirical treatment and forms a basis for control measures to prevent and decrease incidence of infections due to ESKAPE pathogens. Surveillance enables the assessment of incidence rates of resistance to various antibiotics globally, nationally, regionally, and at the hospital-level. Data can also be used to understand gaps and guide research efforts and develop new products to address these gaps.

4. Research aims and objectives of the study

- ***Overarching Aim***

To determine the prevalence, phenotypic and genotypic antimicrobial resistance/susceptibility profiles, and clonality of Gram negative ESKAPE pathogens and develop local antibiograms to support evidence based antibiotic treatment guidelines and infection prevention and control programmes.

- ***Specific objectives***

More specifically, the study objectives are:

I. To undertake passive sentinel surveillance of ESKAPE pathogens processed at Kamuzu Central Hospital (KCH) over a 6-month period.

II. To identify antibiotic resistant pathogens by phenotypically establishing the sensitivity/resistance profiles of isolates to a range of antibiotics by disk diffusion according to EUCAST guidelines.

III. To determine the prevalence of ESKAPE pathogens isolated from patients at KCH

IV. To undertake phenotypic and genotypic identification of extended spectrum β-lactamases, plasmid mediated AmpCs and carbapenemases in Gram negative ESKAPE pathogens and other clinically important bacterial pathogens.

V. To undertake phenotypic and genotypic identification of MRSA in *S. aureus* isolates and perform sequencing on these isolates using SPA typing.

VI. To undertake conventional, multiplex and real-time PCR as well as whole genome sequencing of selected isolates to delineate antibiotic resistance genes

VII. To correlate the phenotypic and genotypic results to inform empiric antibiotic treatment guidelines and infection prevention and control programs.

5. Study design and methodology

This was an observational study conducted over a 6-month period using passive, sentinel surveillance to ascertain the nature and extent of antibiotic resistance in ESKAPE pathogens and other clinically important bacteria. The study was conducted from June –December 2017 at KCH, a government referral hospital for the central region of Malawi serving a community of 6 million people with approximately 750 beds. KCH has four major hospital departments; medical, obstetrics and gynaecology, surgical and paediatric and is a referral centre for eight districts, six Christian Health Association of Malawi

(CHAM) hospitals and three level II hospitals. Patient information was collected using a standardised data form that also served as a specimen request form for submission to the laboratory. Unique identifiers were then allocated to each patient to ensure anonymity and confidentiality.

Antimicrobial susceptibility testing was performed using disk diffusion method as per the EUCAST guidelines and zone diameters were interpreted using the EUCAST clinical breakpoints version 4 (http://www.eucast.org/clinical_breakpoints/). Phenotypic identification of ESBLs, AmpC β-lactamases and carbapenemases was by ROSCO (Taastrup, Denmark) kits; ROSCO ESBL confirm kit (ROSCO) and ROSCO AmpC Confirm ID kit and ROSCO KPC/metallo-β-lactamase and OXA-48 Confirm kit respectively. Phenotypic identification of MRSA was performed using cefoxitin (30µg) disc and VRE was identified using vancomycin disc (30µg).

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, all), bla_{CMY}, bla_{CIT}, bla_{FOX}, bla_{Mox}, bla_{DHA}, bla_{ACC}, bla_{EBC}, and *mec*A genes associated with the relevant ESKAPE pathogens. Representative isolates underwent whole genome sequencing using MiSeq platform (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. Briefly, genomic DNA was purified using the EZ1 DNA Tissue kit (Qiagen, Hilden, Germany). DNA libraries were prepared using Nextera/Nextera XT kits (Illumina) followed by paired-end sequencing. Contigs were assembled using SPAdes v3.13.0. The presence of resistance genes/mutations were determined using Abricate 0.9.8 and NCBI - Bacterial Antimicrobial Resistance Reference Gene Database (PRJNA313047) as the reference database and ST were determined from WGS data using Mlst 2.16.2 database hosted by the Centre for Genomic Epidemiology (CGE) (http://cge.cbs.dtu.dk/services/MLST/). Acquired antibiotic resistance genes and chromosomal point mutations including the DNA gyrase *gyr*A, *parC* and *parE* genes (quinolone resistance) were annotated using ResFinder 4.1(https://cge.cbs.dtu.dk/services/ResFinder/). Plasmid replicon types were identified using PlasmidFinder 2.1 on the CGE website (https://cge.cbs.dtu.dk/services/PlasmidFinder/). SPA typing data was analysed using the databases from Centre for Genomic Epidemiology, DTU, Denmark; http://cge.cbs.dtu.dk/servicdes/spaTyper-1.0/

6. Thesis outline

The thesis is in the form of published and unpublished manuscripts together with an introduction and conclusion in five chapters as follows:

- **Chapter 1. Introduction and Literature Review**: this covers antibiotic history, resistant mechanisms and the various ESKAPE pathogens. It also includes aims, objectives, study design and methodology.
- **Chapter 2. Article I: Antimicrobial susceptibility profiles of clinically important bacterial pathogens at the KCH in Lilongwe, Malawi**: this original research paper has been submitted and published in Malawi Medical Journal. This paper reports the overall antimicrobial susceptibility profiles, phenotypic prevalence of resistance mechanisms associated with ESBL, pAmpC β-lactamases and carbapenemases in ESKAPE pathogens and other clinically important bacterial pathogens from clinical samples collected from adult hospitalised patients at KCH, in Lilongwe Malawi. It addresses objectives I, II, III and VII.
- **Chapter 3. Article II: Molecular epidemiological characterisation of ESBL- and plasmid mediated AmpC-producing *Escherichia coli and Klebsiella pneumoniae* at Kamuzu Central Hospital, Lilongwe, Malawi**: this research, original paper, has been submitted and published in Journal of Tropical Medicine and Infectious Diseases. This paper reports the genetic characterisation of selected Enterobacterales (*E.coli*. *K. pneumoniae*, *P. mirabilis* and *E. cloacae*) that were phenotypically positive for ESBL and -pAmpC β- lactamases isolated from clinical samples collected from adult hospitalised patients at KCH, in Lilongwe Malawi. It also includes whole genome sequencing of selected isolates of *E.coli* and *K. pneumoniae* which were positive for CTX-M PCR and pAmpC PCR. The paper further describes the clonal relationship and the distribution of sequence types. It addresses objectives IV, VI.
- **Chapter 4. Short Communication: Molecular Characterization of *S. aureus* isolates isolated from a tertiary referral Hospital in Lilongwe, Malawi**: this paper reports the genetic characterization of *S. aureus* including phenotypic positive MRSA isolates from clinical samples collected from adult hospitalised patients at KCH, in Lilongwe Malawi. It describes the resistance mechanisms of *S. aureus*, prevalence of *mec*A gene and the genetic diversities of the *S .aureus* as determined by SPA typing. It addresses objective V.
- **Chapter 5. Conclusion:** This chapter presents the extent to which the overarching aim was achieved by highlighting the findings of each objective. It describes the limitations, recommendations and the significance of the study.

CHAPTER 2

Article 1: Antimicrobial susceptibility profiles of clinically important bacterial pathogens at the Kamuzu Central Hospital in Lilongwe, Malawi[1]

Author contributions:

- Faheema E Choonara: Study design, sample processing, data collection, data analysis and interpretation, writing the manuscript
- Bjorg C Haldorsen: study design, data analysis, technical guidance, review of manuscript
- Isaac Ndhlovu: sample processing, data collection
- Osbourne Saulosi: sample processing, data collection
- Tarsizio Maida: sample processing, data collection
- Fanuel Lampiao: administrative guidance, review of manuscript
- Gunnar S Simonsen: study design and review of manuscript
- Sabiha Y Essack: Co-conceptualized the study, guided the literature review and ethical clearance application, enabled data collection and analysis and undertook the critical revision of the manuscript as co-supervisor
- Arnfinn Sundsfjord: study design, data analysis and interpretation, writing the manuscript

[1] This manuscript has been published in the Malawi Medical Journal

Original Research

Antimicrobial susceptibility profiles of clinically important bacterial pathogens at the Kamuzu Central Hospital in Lilongwe, Malawi

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Abstract

Background

The aim of this prospective study was to ascertain antimicrobial resistance (AMR) in clinical bacterial pathogens from in-hospital adult patients at a tertiary hospital in Lilongwe, Malawi.

Methods

Clinical specimens (blood culture, pus, urine and cerebrospinal fluid) collected during June to December 2017 were examined for bacterial growth in standard aerobic conditions. One specimen per patient was included. Antimicrobial susceptibility testing (AST) was performed using the disk diffusion method and interpreted according to EUCAST guidelines.

Results

A total of 694 specimens were collected during the study period, of which 336 (48%) specimen yielded visible bacterial growth. Of the 336 specimens, a total of 411 phenotypically different isolates were recovered. Of the 411 isolates, 84 isolates (20%) were excluded and the remaining 327 (80%) were further characterised. The characterised isolates were identified as ESKAPE pathogens (n=195/327; 60%), *Escherichia coli* (n=92/327; 28%), *Proteus mirabilis* (n=33/327; 10) or *Salmonella* spp. (n=7/327; 2%) and were included for further analysis. The excluded isolates (n=84) comprised of coagulase-negative staphylococci (n=25), streptococci (n=33), and low-prevalence Gram-negative bacilli (n=26). *E. coli* (n=92; 28%) and *S. aureus* (n=86; 26%) were the most dominant species. A multidrug resistant (MDR) extended spectrum β- lactamase (ESBL)-positive phenotype was detected in *Klebsiella pneumoniae* (n=20/29; 69%) and *E. coli* (n=49/92; 53%). One third of the *Pseudomonas aeruginosa* isolates were resistant to meropenem (MEM), but did not appear to be carbapenemase-producers. Methicillin resistant *Staphylococcus aureus* (MRSA) was molecularly confirmed in 10.5% of *S. aureus* (n=9/86).

Conclusion

The high proportion of the MDR ESBL-phenotype in clinical isolates of *Enterobacterales*, strongly limits antimicrobial treatment options and has consequences for empirical and targeted antimicrobial treatment as well as clinical microbiology services and hospital infection control. There is need for a continuous surveillance and an antimicrobial stewardship (AMS) program to contain and prevent the spread of AMR.

Keywords; ESKAPE, antimicrobial resistance, ESBL, AmpC

Background

Antimicrobial resistance (AMR) in bacterial pathogens is a global health threat[1-2]. However, the magnitude of the problem is still to be determined in many parts of the world. This is of particular concern in low- and middle-income countries (LMICs), often with a higher burden of infectious diseases, and where antimicrobial treatment guidelines are based on insufficient surveillance data due to limited diagnostic capacity[3-4]. In Malawi, a rapid expansion of extended spectrum β- lactamase (ESBL)-producing *Enterobacterales* and emerging methicillin resistant *Staphylococcus aureus* (MRSA) have been documented in blood cultures isolates on patient admission to Queen Elizabeth Central Hospital, Blantyre[5]. AMR was also reported in various clinical specimens obtained from patients at Kamuzu Central Hospital, Lilongwe (KCH), on admission during 2006-7[6]. One third of *S.aureus* was resistant to oxacillin, indicating MRSA, and 18.8% of Gram-negative bacteria were resistant to ceftriaxone[6]. In a study of burn patients at KCH in 2015, an increasing rate of wound colonization with multidrug resistant (MDR) *Enterobacterales* and/or MRSA up to 40% and 39%, respectively, were observed during hospitalisation[7]. However, the national AMR-data in Malawi is still limited and it is important to obtain a comprehensive understanding of the problem.

AMR is a particular problem in ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter* spp.) as they are major causes of hospital acquired infections (HAIs) in vulnerable patients[8]. Thus, it is important to

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investigate the antimicrobial susceptibility profiles of these pathogens to inform empiric treatment. The aim of the study was to ascertain AMR in clinical ESKAPE-isolates and selected prevalent bacterial pathogens from patients admitted at KCH, a tertiary hospital in Lilongwe, Malawi.

Methods

Study design

A prospective observational study was conducted over a 6 month period from June –December 2017 at KCH, a governmental 750-bed hospital that serves the Lilongwe district and offers referral services to the central region of Malawi, with 1.6 and 7.5 million people, respectively[9]. Microbiology specimens were collected from hospitalised adult patients (>18 years) suspected to have a clinical infection. Specimen types included urine, blood cultures, cerebrospinal fluid (CSF), other sterile fluids, and pus. The clinical staff collected the samples based on the clinical diagnosis of infection made by a physician. Specimens were submitted to the microbiology department in the hospital laboratory for analysis within 24 hours according to specific guidelines (Supplementary Figure 1). The clinical staff were trained in sample and data collection criteria prior to the study. A focal contact person was identified in each department to facilitate study implementation, specimen collection and data reporting.

Data collection and bacterial culture

Patient information was collected using a standardised data form that also served as a specimen request form for submission to the laboratory (Supplementary Figure 2). The collected data did not allow a distinction between community or hospital acquired infection. Data on HIV status of the patients was not collected. Unique identifiers were then allocated to each patient. Data on the overall admissions and the number of patients during the study period were obtained from the hospital's data collection system.

Pus and sterile site fluids (ascites, pleura, knee, and sinus), including positive blood culture samples were inoculated on chocolate agar, Columbia blood agar and MacConkey agar (Oxoid, Hampshire, UK) and incubated for 16- 24h at 35±1°C in ambient air except for chocolate agar which was incubated 16- 24h at 35±1°C in 5% CO^2. For urine specimens, a 1 µl calibrated disposable sterile loop inoculum was cultured on blood agar (Oxoid, Hampshire, UK) and MacConkey agar (Oxoid) at 35±1 0C for 16-20h. Mid-stream urines with $\geq 10^5$ CFU/ml and catheter-/suprapubic urines with $\geq 10^3$ CFU/ml were defined as significant bacteriuria and were selected for microbial culture and antimicrobial susceptibility testing (AST). For blood cultures, approximately 7-10 ml of blood was collected aseptically and inoculated into BD BACTECTM Plus Aerobic medium blood culture bottle (Becton and Dickinson, Franklin, US) and inverted 3-4 times. The bottles were then incubated in the BD BACTECTM 9050 instrument (Becton and Dickinson, Franklin, US) for 5 days. Anaerobic culture was not performed due to the lack of suitable equipment.

Bacteria were identified by Gram stain. Gram-negative bacteria were further identified by the standard oxidase test, and analytical profile index (API) 20E and 20NE systems (BioMerieux, Durham, US) for oxidase negative and positive bacteria, respectively according to manufacturer instructions. Gram-positive bacteria were further identified using the standard catalase test, coagulase test for *Staphylococcus* sp. and/or streptococcus latex agglutination tests and bile aesculin test for the identification of enterococci. *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 were used as quality control (QC) strains. The QC-strains were used to perform QC on reagents, media and antibiotic discs on a monthly basis and for each new batch of media or reagents. In addition, the laboratory was also enrolled onto the National Heath Laboratory Service external quality assurance scheme for bacteriology. Bacterial isolates identified as ESKAPE pathogens, together with *E. coli, Proteus mirabilis* and *Salmonella* spp. were included in the study. Only one isolate per species per patient was examined.

Antimicrobial susceptibility testing (AST)

AST was performed on Mueller Hinton agar (MAST Diagnostics, Merseyside, UK) by the EUCAST disc diffusion method[10] and interpreted according to EUCAST clinical breakpoints version 4 (http://www.eucast.org/clinical_breakpoints/). Zone inhibition diameters within the R- and I-categories were defined as reduced susceptibility. Isolates were defined as MDR if they were resistant to at least one agent in three or more distinct antimicrobial classes[11]. Selection of antibiotics (MAST) for each bacterial species was based on a combination of the EUCAST recommendations and the availability of antibiotics in the local hospital pharmacy (Supplementary Table 1). For *Enterobacterales*, the antibiotics were ; ampicillin (AMP), aztreonam (AZT), cefotaxime (CTX), cefoxitin (FOX), ceftazidime (CAZ), cefuroxime (CXM), ciprofloxacin (CIP), gentamicin (GEN), meropenem (MEM), piperacillin-tazobactam (TZP), and trimethoprim-sulfamethoxazole (SXT). The *A. baumannii* panel consisted of CIP, GEN, MEM, SXT and the P. *aeruginosa* panel consisted of AZT, CAZ, CIP, MEM, TZP, tobramycin (TOB). *S. aureus* was tested against penicillin (PEN), clindamycin (CLI), erythromycin (ERY), fusidic acid (FA), GEN, SXT and FOX and enterococci was tested against GEN, and vancomycin (VAN). Enterococci were also examined for high-level aminoglycoside resistance (HLAR) using GEN (30ug) where a zone diameter of ≤ 8mm was deemed as positive for HLAR. *S. aureus* isolates with FOX inhibition zones below 22mm were considered putative MRSA and shipped to Norwegian National Advisory Unit on Detection of Antimicrobial Resistance (K-res), University Hospital of North Norway, Tromsø, Norway, for molecular analyses by an in-house validated *mecA* and *nuc*-gene real-time PCR[12].

Enterobacterales species with reduced susceptibility to 3rd generation cephalosporins (CTX and/or CAZ), FOX, and/or MEM were further analysed for ESBL -, AmpC β-lactamase (AmpC) -, and carbapenemase-production, respectively, as described in EUCAST guidelines[13]. Briefly, the combination disc test was used to detect ESBL and AmpC using ROSCO kits (ROSCO, Taastrup, Denmark)[14]. ESBL production in *E. coli, Klebsiella* spp. and *P. mirabilis* was examined using the ROSCO ESBL confirm kit which contains CTX and CAZ tablets with or without the β-lactamase-inhibitor clavulanic acid. Cefepime with or without clavulanic acid was additionally tested for *Enterobacter* spp. Increased AmpC-production (AmpC-phenotype) was determined by the ROSCO AmpC Confirm ID kit (ROSCO), using CTX and CAZ tablets with and without the AmpC-inhibitor cloxacillin. Interpretation criteria of ESBL- and AmpC detection was ≥5mm increase in the growth inhibition zone diameter between the cephalosporin in combination with the

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inhibitor compared to the cephalosporin alone. *P. aeruginosa* and *A. baumannii* isolates were not examined for ESBL and AmpC. A MEM (10ug) growth inhibition zone diameter of <27mm was used for screening of carbapenemase production in *Enterobacterales* using the ROSCO KPC/ metallo-β-lactamase and OXA-48 Confirm kit (ROSCO)[14]. Molecular verification of the ESBL- and AmpC phenotypes was not performed. They were thus presumed phenotypes.

The KPC-3 producing *K. pneumoniae* NCTC 13438, ESBL-positive K. pneumoniae NCTC 13368/ATCC 700603, S. aureus NCTC 12493- methicillin resistant (mecA), as well as wild-type *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 were used for quality control on a monthly basis and for each new batch of media or reagents.

Table 1. Numbers and proportions of clinical specimens with bacterial growth

Specimen type	Positive n/N (%)
Pus	255/403 (63)
Blood culture	25/153 (16)
Urine	31/88 (35)
Other body fluids	17/37 (46)
CSF	8/13 (62)
Total	336/694 (48)

Table 2. Distribution of ESKAPE pathogens, *E.coli*, *P.mirabilis* and *Salmonella spp.*by specimen type

Species	Pus	Blood	Urine	Other*	CSF	Total
Gram-negative	170/403 (42)	11/153 (7)	31/88 (35)	14/37(38)	3/13 (23)	229/327 (70)
Enterobacterales	128/403 (32)	8/153 (5)	26/88 (30)	10/37(27)	2/13 (15)	174/327 (53)
E. coli	65/403 (16)	3/153 (2)	18/88 (20)	5/37 (14)	1/13 (8)	92/327 (28)
E. cloacae	10 /403 (2)	1/153 (1)	1/88 (1)	1/37 (3)	-	13/327 (4)
K. pneumoniae	18/403 (5)	1/153 (1)	6/88 (7)	3/37 (8)	1/13 (8)	29/327 (9)
P. mirabilis	31/403 (8)	1/153 (1)	1/88 (1)	-	-	33/327 (10)
Salmonella spp.	4/403 (1)	2/153 (1)	-	1/37 (3)	-	7/327 (2)
Non-Enterobacterales	42/403 (10)	3/153 (2)	5/88 (6)	4/37 (11)	1/13 (8)	55/327 (17)
A. baumannii	18/403 (4)	3/153 (2)	2/88 (2)	3/37 (8)	-	26/327 (8)
P. aeruginosa	24/403 (6)	-	3/88 (3)	1/37 (3)	1/13 (8)	29/327 (9)
Gram- positive	85/403 (21)	4/153 (3)	4/88 (5)	5/37 (14)	-	98/327(30)
S. aureus	77/403 (19)	4/153 (3)	2/88 (2)	3/37 (8)	-	86/327(26)
Enterococcus	8/403 (2)	-	2/88 (2)	2/37 (5)	-	12/327 (4)

Data analysis

All data were entered into a Microsoft Excel 2010 © sheet and analysed using EpiInfoTM 7 software (CDC, USA). Data were described using percentages and frequencies.

Ethical Consideration

This study was approved by the Malawian College of Medicine Research and Ethics Committee; (reference no. P.11/17/2308) and by the University of KwaZulu Natal Biomedical Research Ethics Committee (reference no.BE093/16). Voluntary informed consent was sought from every patient prior to inclusion in the study.

Results

Overall culture results

A total of 694 specimens were collected from 367 males and 327 (47%) females aged between 18 – 89 years (mean 36 years). Each patient was entered only once in the study. More than one bacterial species was isolated from some samples. The specimens were received from the departments of surgery (n=370; 53%), medicine (n=230; 33%), obstetrics-gynaecology (n=87; 13%) and other wards (n=7; 1%). Specimens comprised of pus swabs (n=403; 58%), blood cultures (n= 153; 22%), urine (n=88; 13%), CSF (n=13; 2%), and other samples (n=37; 5%) which included ascites fluid (n=8), knee aspirate (n=8), pleural fluid (n=6), peritoneal fluid (n=7), sinus aspirate (n=5), middle ear aspirate (n=2), and a throat swab (n=1).

A total of 336/694 (48%) specimens yielded visible bacterial growth (Table 1): pus (255/403; 63%), urine (31/88; 35%), blood cultures (25/153; 16%), CSF (8/13; 62%), and other samples (17/37; 46%). A total of 411 phenotypically different isolates were recovered from these 336 specimens. A total of 327 isolates (80%) were identified as ESKAPE pathogens (n=195/327; 60%), *E. coli* (n=92/327; 28%), *P. mirabilis* (n=33/327; 10%) or *Salmonella* spp. (n=7/327; 2%). The 327 isolates were recovered from 251 specimens and included for further analysis (Figure 1). Other bacterial species (n=84; 20%) were coagulase negative staphylococci (CNS) (n=25;6%) and non-ESKAPE pathogens (n=59; 14%) including β-haemolytic streptococci (n=23), *Pseudomonas spp.* other than *P. aeruginosa* (n=2), *Kluyvera spp.* (n=1), *Moraxella spp.* (n=1), *Pantoea spp.* (n=3), *Providencia spp.* (n=1), *Raoultella spp.* (n=3), *Aeromonas spp.* (n=2), *Erwinia spp.* (n=1), *K.oxytoca* (n=2), *P.vulgaris* (n=1), *C. sedlakii* (n=4). *E. aerogenes* (n=1), and unidentified oxidase negative Gram-negative rods (n=14; 3%) were excluded from further analysis (Figure 1). The final study sample selected for AST consisted of Gram-negative bacilli (n=229; 70%) and Gram-positive cocci (n=98; 30%) (Figure 2).

Table 2 illustrates the distribution of ESKAPE pathogens, *E. coli, P. mirabilis* and *Salmonella spp.* by specimen type.

Bacterial species distribution

E. coli (n=92; 28%) and *S. aureus* (n=86; 26%) were the most commonly detected species. Pus, blood culture and urine samples yielded the highest number of pathogens (Table

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Table 3. AST results for *Enterobacterales*, *P.aeruginosa* and *A.baumannii* given as numbers and percentages of isolates with reduced susceptibility (I + R category) to the individual antibiotics.

	Enterobacterales						Non-Enterobacterales		
*Antibiotics**	*Enterobacterales*	*E.coli*	*E. cloacae*	*K. pneumoniae*	*P. mirabilis*	*Salmonella spp.*	*Non-Enterobacterales*	*P. aeruginosa*	*A.baumannii*
AMP	146/174 (84)	77/92 (84)	11/13 (85)	29/29 (100)	22/33 (67)	7/7 (100)	NT	NT*	NT
AZT	104/174 (60)	56/92 (61)	10/13 (77)	24/29 (83)	14/33 (42)	0/7 (0)	29/29 (100)	29/29 (100)	NT
CTX	106/174 (61)	56/92 (61)	9/13 (69)	24/29 (83)	17/33 (52)	0/7 (0)	NT	NT	NT
FOX	95/174 (55)	50/92 (54)	13/13 (100)	20/29 (69)	9/33 (27)	3/7 (43)	NT	NT	NT
CAZ	102 /174 (59)	53/92 (58)	8/13 (62)	24/29 (83)	17/33 (52)	0 /7 (0)	5/29 (17)	5/29 (17)	NT
CXM	112/174 (64)	58/92 (63)	8/13 (62)	24/29 (83)	22/33 (67)	0 /7 (0)	NT	NT	NT
CIP	94 /174 (54)	53/92 (58)	7/13 (54)	18/29 (62)	16 /33 (48)	0 /7 (0)	27/55 (49)	5/29 (17)	22/26 (85)
GEN	99 /174 (57)	52/92 (57)	7/13 (54)	24/29 (83)	15/33 (45)	1 /7 (14)	22/26 (85)	NT	22/26 (85)
MEM	8 /174 (5)	3/92 (3)	1/13 (8)	3/29 (10)	1 /33 (3)	0 /7 (0)	23/55 (42)	18/29 (66)#†	4/26 (15)
TZP	45/174 (26)	26/92 (28)	4/13 (31)	13/29 (45)	2/33 (6)	0/7 (0)	2/29 (7)	2/29 (7)	NT
SXT	140/174 (80)	79/92 (86)	9/13 (69)	25/29 (86)	21/33 (64)	6/7 (86)	26/26 (100)	NT	26/26 s(100)
TOB	NT	NT	NT	NT	NT	NT	2/29 (7)	2/29 (7)	NT

*Abbreviations: AMP=ampicillin, AZT= aztreonam, CTX=cefotaxime, FOX=cefoxitin, CAZ=ceftazidime, CXM=cefuroxime, CIP=ciprofloxacin, GEN=gentamicin, MEM=meropenem, TZP=piperacillin-tazobactam, SXT=trimethoprim-sulfamethoxazole, TOB=tobramycin, NT= not tested. # Including R-category (n=10/29; 34%) or I-category (n=8/29; 28%). †negative for carbapenemase production by molecular testing

Table 4. AST results for Gram-positive cocci given as number of isolates and the percentages with reduced susceptibility (I + R category) to the individual antibiotics

Antibiotics*	*S.aureus*	*Enterococcus.*
PEN	81/86 (94)	NT*
ERY	41/86 (48)	NT
CLI	20/86 (23)	NT
FA	7 /86 (8)	NT
VAN	NT	0 /12 (0)
GEN	21/86 (24)	0/12 (0)
SXT	27/86 (31)	NT
FOX	9/86 (10.5)	NT

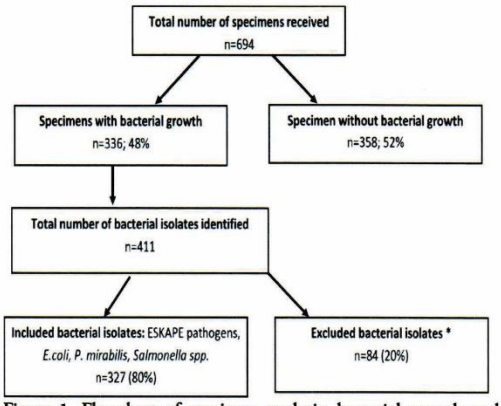

Figure 1. Flowchart of specimen analysis, bacterial growth and selection of clinical isolates for further analysis

*Coagulase negative staphylococci (CNS) (n=25;6%) and non-ESKAPE pathogens (n=59; 14%) including *B-haemolytic streptococci* (n=23), *Pseudomonas spp. other than P. aeruginosa* (n=2), *Kluyvera sp.* (n=1), *Moraxella* sp. (n=1), *Pantoea sp* (n=3), *Providencia sp.* (n=1), *Raoultella sp.* (n=3), *Aeromonas sp.* (n=2), *Erwinia sp.* (n=1), *K.oxytoca* (n=2), *P.vulgaris* (n=1), *C. sedlakii* (n=4). *E. aerogenes* (n=1), and unidentified oxidase negative Gram-negative rods (n=14; 3%).

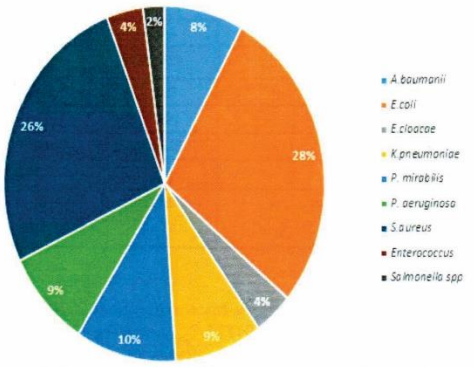

Figure 2. Overall numbers and proportions of ESKAPE pathogens, *E.coli*, *P.mirabilis*, and *Salmonella spp.* (n=327) included for AST.

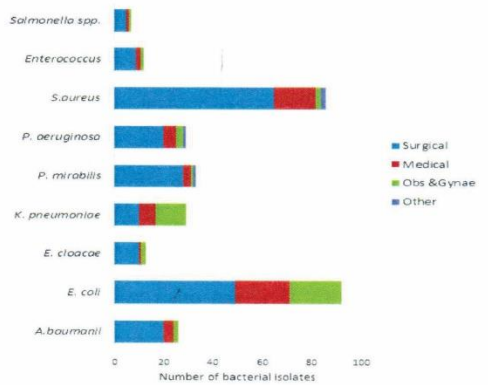

Figure 3. Distribution of ESKAPE pathogens, E.coli, P. mirabilis, and Salmonella spp. by hospital departments

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2): (i) Gram-negative pathogens were recovered from pus (n=170/403; 42%), urine (n= 31/88; 35%), blood cultures (n=11/153; 7%), other body fluids (n=14/37; 35%) and CSF (n=3/13; 23%). (ii) Gram-positive pathogens were recovered from pus (85/403; 21%), urine (4/88; 5%), blood cultures (n= 4/153; 3%), and other body fluids (n=5/37; 14%) (Table 2).

Pathogens were recovered from several different departments (Figure 3). The surgical department dominated in overall numbers with *S. aureus* (n=65/86; 76%), and *E. coli* (n=49/92; 53%). The proportion of *E. coli* (n=22/92; 24%) and *S. aureus* (n=17/86; 20%) were also relatively high in specimens from the medical department. In the obstetrics and gynaecology department, *E. coli* (n=21/92; 23%) and *K. pneumoniae* (n=12/29; 41 %) were the most prevalent bacterial species.

Antimicrobial susceptibility and multidrug resistance

The overall AST-results for Gram-negative bacteria are presented in Table 3. We focused on the prevalence of reduced susceptibility to CTX and/or CAZ and associated co-resistance. *K. pneumoniae* (n=24/29; 83%), *E. cloacae* (n=9/13; 69%), *E. coli* (n=58/92; 63%), and *P. mirabilis* (n=17/33; 52%) showed reduced susceptibility to CTX and/or CAZ, of which the majority were also MDR and resistant to CIP, GEN and SXT. MDR phenotypes were evident in *K. pneumoniae* (n=18/29; 62%), *E. cloacae* (n=5/13; 38%), *E. coli* (n=40/92; 43%), and *P. mirabilis* (n=13/33; 39%). All *Salmonella spp.* were susceptible to CTX and CAZ. ESBL-production was phenotypically confirmed in *K. pneumoniae* (n=20/29; 69%), *E. coli* (n=49/92; 53%), *E. cloacae* (n=6/13; 46%) and *P. mirabilis* (n=11/33; 33%). MDR was observed in a majority of the ESBL-positive isolates, i.e., *K. pneumoniae* (n=16/20; 80%), *E. cloacae* (n=4/6; 67%), *E. coli* (n=30/49; 61%) and P. mirabilis (n=8/11; 73%). In contrast, lower rates of MDR were observed in ESBL-negative isolates; *E. coli* (n=10/43; 23%), *E. cloacae* (n=2/5; 40%), *P. mirabilis* (n=5/22; 23%), *K. pneumoniae* (n=2/9; 22%).

An AmpC-phenotype was most prevalent in *E. cloacae* (n=5/13; 38%), followed by *P. mirabilis* (n=10/33; 30%), *K. pneumoniae* (n=8/29; 28%) and *E. coli* (n=26/92; 28%). The majority of isolates with AmpC phenotypes were also MDR, i.e., *E. coli* (n=10/26; 38%), *E. cloacae* (n=3/5; 60%), *K. pneumoniae* (n=6/8; 75%) and *P. mirabilis* (n=8/10; 80%). In comparison, lower rates of MDR were observed in AmpC negative isolates; *E. cloacae* (n=2/8; 25%), *K. pneumoniae* (n=12/21; 57%) and *P. mirabilis* (n=5/23; 22%) except for *E. coli* (n=30/66; 45%). A combined ESBL- and AmpC-phenotype was observed in all four *Enterobacterales*, i.e., *K. pneumoniae* (n=8/29; 28%), *E. coli* (n=20/92; 22%), *P. mirabilis* (n=6/33; 18%) and *E. cloacae* (3/13; 23%). A large proportion of these isolates were also MDR; *P. mirabilis* (n=5/6; 83%), *K. pneumoniae* (n=6/8; 75%), *E. cloacae* (n=2/3; 67%), *E. coli* (n=7/20; 35%).

The majority of *Enterobacterales E. coli* (66/92; 72%), *P. mirabilis* (31/33; 94%), *E. cloacae* (9/13; 69%) and *Salmonella* spp. (7/7; 100%) showed high levels of susceptibility to TZP except *K. pneumoniae* (n=16/29; 55%). For MEM, high levels of susceptibility were observed across all *Enterobacterales*; E. coli (89/92; 97%), *K. pneumoniae* (26/29; 90%), *P. mirabilis* (32/33; 97%), *E. cloacae* (12/13; 92%) and *Salmonella spp.* (7/7; 100%). *Enterobacterales* isolates with reduced susceptibility to MEM were examined by the ROSCO carbapenemase kit with negative results (data not shown) indicating a combination of reduced permeability and ESBL-/AmpC-production rather than carbapenemases production as an explanation for reduced MEM susceptibility.

For *A. baumannii* and *P. aeruginosa* we focused primarily on reduced susceptibility to MEM. We observed a high rate of reduced susceptibility to MEM in *P. aeruginosa*, R-category (n=10/29; 34%) or I-category (n=8/29; 28%), with evenly distributed growth inhibition zone diameters between 6-23 mm, in contrast to *A. baumannii* (R-category n=3/26; 12% and I-category n=1/26; 4%). Carbapenemase production was not evident in MEM-R *P. aeruginosa* and *A. baumannii* using biochemical and molecular methods (K-res, Norway; data not shown). High levels of resistance was observed in *A. baumannii* against CIP (n=22/26; 85%) and GEN (n=22/26; 85%):

The overall AST results for *S. aureus* and *enterococci* are presented in Table 4. For *S. aureus* we focused on reduced susceptibility to FOX as a marker for presumptive MRSA and associated resistance. Although we observed a high initial rate of reduced susceptibility to FOX (38/86; 44%), MRSA was confirmed in only nine of the 38 isolates re-examined at K-res, resulting in an actual 10.5% MRSA prevalence (n=9/86). Almost all non-confirmed isolates had FOX inhibition zones of 19-21 mm, just below the screening breakpoint of 22 mm. The confirmed MRSA-isolates expressed reduced susceptibility to ERY (n=7/9; 78%) or GEN (n=8/9; 89%) or both ERY and GEN (n=7/9; 78%). In contrast, lower resistance rates were observed in MSSA isolates; ERY (n=33/77; 43%), GEN (n=12/77; 16%) and both GEN and ERY (n=11/77; 14%). For *Enterococcus* we focus on reduced susceptibility to VAN and high-level aminoglycoside resistance (HLAR), neither of which were observed (Table 4).

Discussion

We undertook a prospective observational study of antimicrobial susceptibility in clinically important bacterial pathogens obtained from adult in-patients at a governmental referral hospital for the central region of Malawi during a six months period in 2017. A similar study, also including children, was performed 10 years before our study at the same hospital and could provide comparative hospital-wide baseline data[6]. However, this study only included specimens collected at admission, whereas our study also included specimens during hospitalisation.

During the six-month study period, 22 524 unique admissions were registered in KCH, of which 16 237 were suspected to have a bacterial infection and were prescribed antibiotics. We obtained 694 specimens from unique patients which is relatively low in comparison to the overall number of patient with a potential bacterial infection. This is a typical observation in resource limited settings due to the lack of robust clinical microbiology services (CMS) and inadequate financial resources. However, the number of specimens collected during this study is similar to the number (n=2236) obtained during the 18 months study in 2006-7 when adjusted for study length[6]. This observation implies that most infections are still treated empirically without any microbiology investigations to determine the causative pathogen and its susceptibility profile[15-16]. This could be attributed to clinicians receiving little or no training in microbiology informed antibiotic prescribing, diagnostic stewardship, and availability of the antibiotics in

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the hospital, similar to a recent Egyptian survey[16] finding that 63% of the physicians didn't receive any training on prescribing antibiotics and relied on their seniors or previous experience for prescribing antibiotics[16]. The Egyptian survey also showed that less than half of all antibiotics (44.5%) were prescribed following a microbiology result, 51% were prescribed on patient demand, and 68.2% based on availability of the antibiotics in hospital[16]. Moreover, it may also suggest that sampling is prioritised for the most critically ill patient and/or those not responding to empirical antibiotic treatment. This may create a bias in the overall sampling towards complicated infections and/or diagnostics in treatment failure.

The underuse of CMS may also in part be due to the lack of trust in and knowledge of the importance of local rates of antimicrobial resistance and consequences for therapy as shown in a recent Cambodian survey[17]. The Cambodian study revealed that the use of CMS was facilitated when results and microbiology staff were readily accessible, and if clinicians had trust and confidence in CMS while appreciating the importance of the results in clinical decision-making[17]. During our study we strengthened the interface between the laboratory and the clinicians by building capacity in terms of supplies and consumables, deploying focal contact persons at each ward and providing rapid laboratory results through a WhatsApp group. Caution was taken when using this platform to ensure confidentiality of patients by limiting WhatsApp groups to selected clinicians approved as department focal persons by heads of Department. We believe that these measures were important and allowed us to reach a number of specimens equivalent to the previous KCH study[6].

The overall proportion of specimens supporting bacterial growth was 48%, varying between the highest yield for pus samples (63%) and lowest for blood culture (16%). Low yield of blood cultures may have been attributed to blood cultures primarily collected from patient's already in hospital and on antibiotic therapy supressing bacterial growth. The limited yield of fastidious bacteria such as *S. pneumoniae* support the notion that the majority of blood cultures collected represented hospital infections and not community acquired infections[5]. The proportion of positive blood culture samples is consistent with the previous KCH-study[6]. The lack of visible bacterial growth in a third of pus samples could also be due to ongoing antibiotic treatment suppressing bacterial growth and/or the lack of anaerobic culturing, but the proportion of culture positive samples was comparable to that obtained in pus from burn wounds (74%) in hospitalised patients in Blantyre[18]. Unfortunately, the clinical information provided in the data collection form was limited and did not allow reliable information on the recent use of antibiotics nor any distinction of pus samples between primary abscesses, postoperative wound infections or other wound types.

E. coli and *S. aureus* were the most common species recovered across all specimen types representing 54% of the total isolates. The relative proportions were consistent with observations in the previous KCH-study from 2006-7 although their specimens were dominated by blood culture samples in contrast to our collection consisting of nearly 60% of pus samples[6]. The differences in the relative proportions of different specimen types precludes any further comparisons in bacterial species distributions between the two studies performed at KCH. The relative dominance of *E. coli* and *S. aureus* in similar specimen types has been confirmed in other studies from Sub-Saharan African countries[5,18-22] . The differences in the prevalence of bacterial species between the different hospital departments could be partly explained by their corresponding dominant specimen type, e.g. the predominance of *E. coli* at the medical department from urine samples, and *S. aureus* in the surgical department recovered from pus specimens.

Importantly, we observed an overall high rate of MDR in the most common Gram-negative bacterial pathogens. Reduced susceptibility to most antimicrobials except MEM and TZP was observed in *Enterobacterales*. A high proportion (>50%) of ESBL-producing isolates was observed for all *Enterobacterales* species with reduced susceptibility to third generation cephalosporins except *Salmonella*. A substantial proportion of ESBL-producing isolates also revealed an AmpC-phenotype indicating the presence of plasmid-mediated AmpC, at least for *K. pneumoniae* and *P. mirabilis* species which do not harbour any intrinsic blaAmpC. ESBL- and AmpC phenotypes were categorised as presumed phenotypes as molecular verification was not performed. The clinical importance of co-resistance is illustrated by the high proportion of MDR in ESBL- and AmpC-producing isolates. The high rate of ESBL-producing *Enterobacterales* isolates is consistent with recent observations of MDR invasive isolates of *E. coli* and *K. pneumoniae* at the Queen Elizabeth Hospital in Blantyre[5], and seems to have increased significantly compared to the previous KCH-study[6]. In fact the current resistance pattern in *Enterobacterales* advises against the current use of the recommended first and second line antibiotics (PEN, CIP, GEN, ceftriaxone and amoxicillin-clavulanic acid) as prescribed in Malawi Standard Treatment Guidelines (MSTG) 5th Edition 2015 incorporating the Malawi Essential Medicine List (MEML) 2015[23]. It must be noted that although ceftriaxone was not directly a part of the antibiotic testing panel, representative marker antibiotics (CTX and CAZ) were tested. We did not examine *E. coli*, *Klebsiella spp.* and *P. mirabilis* for amoxicillin-clavulanic acid susceptibility, which still could be useful in the treatment of UTIs caused by ESBL-producing *Enterobacterales*. The antibiotic panels consisted of antibiotics that were available at the hospital pharmacy and actually being used on the ground and ensured that the panels of antibiotics that are tested are in line with international guidelines in particular for tracking ESBL and AmpC phenotypes. The data provided by this study may contribute to further development of national and local guidelines for antibiotic use also allowing antibiotic procurement planning.

The high proportion of resistance to extended-spectrum cephalosporins in clinical isolates of *Enterobacterales* has been reported from several sub-Saharan countries[5,24-26]. These antibiotics are commonly used and readily accessible over the counter without the need for a prescription in LMICs[4,27]. Overuse and misuse often leads to resistance and the findings of this study clearly indicate that the use and prescription of extended spectrum cephalosporins needs to be reviewed and closely monitored.

We observed a high-rate of MEM-resistance in *P. aeruginosa* compared to *A. baumannii*. Extended analyses of selected *P. aeruginosa* and *A. baumannii* isolates did not reveal any carbapenemases-producing isolates (data not shown). Thus, the reduced susceptibility to MEM may be attributed to

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chromosomal mutational mechanisms affecting permeability, efflux mechanisms and hyper-production of AmpC[28]. The rate of confirmed MRSA do not indicate a significant increase compared to the previous KCH study [6]. Fortunately, we did not observe any VAN resistance nor HLAR in the few available clinical *enterococci*.

There are several limitations in this study including a relative short study period and limitations in sample size. The relatively low proportion of samples compared to the overall number of patients with a potential bacterial infection may indicate a sampling bias. Moreover, ESBL- and AmpC-phenotypes have not yet been confirmed by molecular methods and we have not done any phylogenetic typing to disclose clonal relatedness among isolates. On the other hand we have performed a prospective study and managed to mobilize the clinical departments to submit samples in a relative proportion that is comparable to the most recent study at KCH[6]. Moreover, bacterial culture (except anaerobic culturing) and AST has been performed according to international standards.

Conclusion

The overall results have documented a relatively high proportion of clinically important AMR-phenotypes consistent with ESBL-/AmpC-producing Enterobacterales, which strongly limits antimicrobial treatment options. The proportion of these phenotypes have increased since the last study at KCH in contrast to MRSA. These observations have consequences for empirical and targeted antimicrobial treatment as well as CMS and infection control at KCH. Urgent attention is required from local government, and in country public health committees with guidance from global health committees to address the problem. This could be achieved through establishment of local and national antibiotic stewardship programs, development of locally adapted clinical microbiology services, and AMR surveillance.

Declarations

Ethics approval and consent to participate

This study was approved by the Malawian College of Medicine Research and Ethics Committee; (reference no. P.11/17/2308) and by the University of KwaZulu Natal Biomedical Research Ethics Committee (reference no.BE093/16). Voluntary informed consent was sought from every patient prior to inclusion in the study.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed in this study are available from the corresponding author on reasonable request.

Competing interests

Sabiha Essack is chairperson of the Global Respiratory Infection Partnership and member of the Global Hygiene Council, both sponsored by an unrestricted educational grant from Reckitt UK. "The other authors declare that they have no competing interests"

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Authors' contributions

FEC: Study design, sample processing, data collection, data analysis and interpretation, writing the manuscript

BCH: study design, data analysis, technical guidance, review of manuscript

IN: sample processing, data collection

OS: sample processing, data collection

TM: sample processing, data collection

FL: administrative guidance, review of manuscript

GSS: study design and review of manuscript

SYE: study design, review of manuscript

AS: study design, data analysis and interpretation, writing the manuscript

"All authors have read and approved the final manuscript"

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CHAPTER 3

Article 2: Molecular epidemiological characterisation of ESBL- and plasmid mediated AmpC-producing *Escherichia coli and Klebsiella pneumoniae* at Kamuzu Central Hospital, Lilongwe, Malawi[2]

Author Contributions

- Faheema E Choonara: conceptualization , performance and analysis of AST, PCR and bioinformatics analysis and interpretation, writing the manuscript
- Bjorg C Haldorsen: conceptualization, performance and analysis of AST, PCR and bioinformatics analysis and interpretation, review of manuscript
- Jessin Janice: PCR and bioinformatics analysis and interpretation, review of manuscript
- Joshua Mbanga: PCR and bioinformatics analysis and interpretation, review of manuscript
- Isaac Ndhlovu: performance and analysis of AST
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- Tarsizio Maida: performance and analysis of AST
- Fanuel Lampiao: administrative guidance, review of manuscript
- Gunnar S Simonsen: conceptualization and review of manuscript
- Sabiha Y Essack: Co-conceptualized the study, guided the literature review and ethical clearance application, enabled data collection and analysis and undertook the critical revision of the manuscript as co-supervisor
- Arnfinn Sundsfjord: conceptualization, PCR and bioinformatics analysis and interpretation, data analysis and interpretation, funding acquisition, writing the manuscript, supervision

[2] This manuscript has been submitted to the journal of Tropical medicine and infectious disease and is currently under review

Tropical Medicine and Infectious Disease

MDPI

Article

Molecular epidemiological characterisation of ESBL- and plasmid mediated AmpC-producing *Escherichia coli* and *Klebsiella pneumoniae* at Kamuzu Central Hospital, Lilongwe, Malawi

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Abstract: The global rise in infections caused by multidrug resistant (MDR) Enterobacterales poses a public health problem. We have performed a molecular epidemiological characterisation of representative plasmid mediated AmpC (pAmpC) - and ESBL-positive clinical isolates of *Escherichia coli* (n=38) and *Klebsiella pneumoniae* (n=17) from a tertiary hospital in Malawi collected in 2017. *Bla*$_{CTX\text{-}M\text{-}15}$ was the most prevalent ESBL-determinant in *E. coli* (n=30/38) and *K. pneumoniae* (n=17/17), whereas *bla*$_{CMY\text{-}2}$ was detected in nearly all AmpC-phenotype *E. coli* (n=15/17). Whole genome sequencing revealed dominant globally disperses *E. coli* sequence types (STs); ST410 (n=16), ST131 (n=7), and ST617 (n=6). The ST distribution in *K. pneumoniae* was more diverse but included ST101 (n=2), ST14 (n=2), and ST340 (n=2), all considered high-risk MDR clones. The isolates expressed an MDR-profile including resistance against commonly used antibiotics such as fluoroquinolones, aminoglycosides, and/or trimethoprim-sulfamethoxazole and harboured corresponding resistance determinants. Clonal analyses of the major STs of *E. coli* revealed closely related genetic clusters within ST410, ST131 and ST617 supporting within hospital transmission between patients and/or via a common reservoir. The overall findings add to the limited knowledge on the molecular epidemiology of MDR *E. coli* and *K. pneumoniae* in Malawi and may help health policy makers to identify areas to target when addressing this major threat of antibiotic resistance.

Keywords: Escherichia coli; Klebsiella pneumoniae; bla$_{CTX\text{-}M\text{-}15}$; bla$_{CMY}$; clonal spread

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1. Introduction

The global rise in infections caused by multidrug resistant (MDR) *Enterobacterales* poses a public health threat due to delayed effective therapy and poorer clinical outcome [1,2]. The problem is dominated by extended-spectrum β-lactamase (ESBL) and plasmid mediated AmpC (pAmpC) producing *Escherichia coli* and *Klebsiella pneumoniae*, and the dissemination of their MDR clonal lineages [3,4,5]. It is important to detect such isolates to optimize patient treatment and implement infection control measures.

ESBLs and pAmpCs differ in biochemical characteristics which can be used for phenotypic detection. While both enzyme groups, in general, hydrolyse penicillins, narrow and extended spectrum cephalosporins and monobactams, only pAmpCs hydrolyse cephamycins and are inhibited by fourth generation cephalosporins such as cefepime and cefpirome [6]. In contrast to ESBLs, pAmpCs are poorly inhibited by the traditional β-lactamase inhibitors such as clavulanic acid and tazobactam [7].

Differences in amino acid sequence give rise to different families of pAmpCs [6]. CMY-2 enzymes are the most common pAmpC variants worldwide [6]. The distribution of pAmpC in clinical isolates of *Enterobacterales* is underexplored in sub-Saharan Africa. However, a faecal carriage study of university students in Mozambique detected pAmpC alone in 11% and in co-existence with ESBL (CTX-M) in 36% of *E. coli* and *K. pneumoniae* isolates resistant to third generation cephalosporins [8].

ESBLs are more prevalent than pAmpCs in clinical isolates of *Enterobacterales* worldwide (6, 9, 10, 2, 11). CTX-Ms are the most widespread ESBLs followed by subtypes of TEM and SHV, with CTX-M-15 being the most dominant allelic variant in Africa [9, 10]. A worldwide increase in community acquired CTX-M type ESBL-producing *E. coli* has been observed in the last decade, with developing countries being affected the most [12].

A systematic review of ESBL-producing *Enterobacterales* in Africa revealed a diverse prevalence of ESBL-producing *Enterobacterales* dependent upon geographical locations and populations (13). The emergence of ESBL-producing *E. coli* and *K. pneumoniae* and the association with MDR clonal lineages has recently been described in Malawi [14, 15, 16]

It is important to distinguish between pAmpC- from ESBL-producing *Enterobacterales* due to differences in β-lactam susceptibility and propensity for nosocomial dissemination [6]. Moreover, pAmpC can mask phenotypic confirmation of ESBLs resulting in false negative ESBL-tests [17, 18]. In a prospective observational study in 2017 at Kamuzu Central Hospital (KCH), a referral hospital in Lilongwe, Malawi, we phenotypically identified a high prevalence of ESBL- and AmpC-producing *Enterobacterales* [19]. The aim of this study was to perform a molecular epidemiological characterisation of a representative selection of ESBL- and/or AmpC-phenotype positive *Enterobacterales*.

2. Materials and Methods

The bacterial strains were collected from June to December 2017 at KCH, a governmental referral hospital for the central region of Malawi serving a community of 6 million people with approximately 750 beds. KCH has four major hospital departments: medical, obstetrics and gynaecology, surgical and paediatric. Data collection including identification of bacterial isolates and antimicrobial susceptibility testing (AST) was performed as previously described [19](15 submitted).

Gram negative isolates were identified analytical profile index (API) 20E and 20NE systems (BioMerieux, Durham, US) and subjected to antimicrobial susceptibility testing (AST). Briefly, the disk diffusion method as per the EUCAST guidelines was used and zone diameters were interpreted using the EUCAST clinical breakpoints version 4 (http://www.eucast.org/clinical_breakpoints/). Detection of ESBL- and/or AmpC-phenotypes was performed using the combination disk test with clavulanic acid and cloxacillin, respectively, on isolates with reduced susceptibility to cefotaxime and/or ceftazidime [19].

Phenotypic quality control (QC) strains included *E. coli* CCUG 58543, ESBL-positive *K. pneumoniae* NCTC 13368/ATCC 700603, as well as *Escherichia coli* ATCC 25922. Clinical isolates were stored at -80°C in tryptone soy broth with 10% glycerol until further testing.

Selection of ESBL- and/or AmpC-phenotype positive isolates for genetic characterisation

ESBL-production was previously phenotypically confirmed in 86 Enterobacterales isolates: *E. coli* (n=49), *K. pneumoniae* (n=20), *Enterobacter cloacae* (n=6), and *Proteus mirabilis*

(n=11). Of those, 49 isolates also expressed an AmpC- phenotype. A separate AmpC-phenotype was not observed [19].

All those 86 isolates that had reduced susceptibility to cefotaxime and/or ceftazidime ceftazidime and positive for ESBL and/ or pAmpC by combination disk test with clavulanic acid and cloxacillin respectively, as previously described were shipped to the Norwegian Advisory Unit on Detection of Antimicrobial Resistance, Tromsø, Norway, for molecular analyses. Upon arrival, the isolates were re-cultured on lactose agar, re-identified using MALDI-TOF (Bruker Daltonik, Bremen, Germany), as well as retested for an ESBL- or AmpC-phenotype using the ROSCO ESBL and AmpC confirmation kits (ROSCO, Taastrup, Denmark), respectively.

PCR analysis for ESBL and pAmpC β-lactamase genes

DNA was extracted using the bioMerieux NucliSENS-easyMAG (bioMerieux, Marcy l'Étoile, France) and subjected to real time PCR for detection of CTX-M type ESBL and pAmpC using the Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystem Inc, Foster City, CA). Genes encoding CTX-M group 1/2/9, and CTX-M (consensus) as well as CIT, CMY, FOX, MOX, DHA, ACC and EBC type pAmpC were detected as described in (20) and (21).

PCR QC-strains included: For CTX-M RT-PCR; A2-23 *K. pneumoniae* (CTX-M gr1), A2-39 *E. coli* (CTX-M gr.2), A2-37 *K. pneumoniae* (CTX-M gr.9), A2-38 *K. pneumoniae* (CTX-M gr.9) and *E.coli* ATCC 25922. For the pAmpC RT-PCR; A2-57 *Citrobacter freundii* (CIT), A2-20 *P. stuartii* (CMY), A2-24 *K. pneumoniae* (CMY), A2-59 *K. oxytoca* (FOX), A4-27 *E. coli* (MOX), A2-61 *Hafnia alvei* (DHA), A2-60 *Morganella morganii* (ACC), A2-58 *Enterobacter sp.* (EBC) and ATCC 25922 *E. coli.* The A-number strains are all whole genome sequenced internal reference strains at the reference laboratory.

Whole Genome Sequencing (WGS) and Bioinformatics analysis

WGS was performed using the MiSeq platform (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. Briefly, genomic DNA was purified using the EZ1 DNA Tissue kit (Qiagen, Hilden, Germany). DNA libraries were prepared using Nextera/Nextera XT kits (Illumina) followed by paired-end sequencing. Contigs were assembled using SPAdes v3.13.0. The quality control criteria included a minimum of 40x coverage, genomic length not lower than 95% of the smallest and not exceeding 105% of the largest closed species related genome on NCBI, and the total number of contigs below 400. The presence of resistance genes/mutations was determined using Abricate 0.9.8 using NCBI's Bacterial Antimicrobial Resistance Reference Gene Database (PRJNA313047) as the reference and STs were determined from WGS data using MLST 2.16.2 database hosted by the Centre for Genomic Epidemiology (CGE) (http://cge.cbs.dtu.dk/services/MLST/). Acquired antimicrobial resistance genes and chromosomal point mutations including the DNA gyrase *gyr*A, *par*C and *par*E genes (quinolone resistance) were annotated using ResFinder 4.1(https://cge.cbs.dtu.dk/services/ResFinder/). Plasmid replicon types were identified using PlasmidFinder 2.1 on the CGE website (https://cge.cbs.dtu.dk/services/PlasmidFinder/).

Analysis of clonal relatedness

To further distinguish *E. coli* strains within the same sequence type (ST), the SeqSphere+ software (Ridom, Münster, Germany) was used. The *E. coli* cgMLST scheme with reference of K12 (NC_00913.3) with the core genome consisting of 2513 alleles was used to examine clonal relatedness among our *E. coli* genomes with a cluster distance threshold of ≤10 allelic differences.

3. Results

Only 60/86 (70%) of the shipped isolates were available in pure culture for phenotypic confirmation and molecular characterisation due to failure of growth upon arrival in the reference laboratory, mislabeling or mixed culture: *E. coli* (n=38), *K. pneumoniae* (n=17), *P. mirabilis* (n=4) and *E. cloacae* (n=1). All 60 isolates were reconfirmed as ESBL positive. The AmpC phenotype was reconfirmed in 21/60 (35%) isolates: *E. coli* (*n*=17), *P. mirabilis* (*n*=3) and *E. cloacae* (*n*=1). Due to low numbers of other species, we focused only on the molecular characterization of *E. coli* (n=38) and *K. pneumoniae* (n=17) in the following.

PCR analysis for genes encoding CTX-M and pAmpC β-lactamases

The results are summarized in Tables 1 (*E. coli*) and 2 (*K. pneumoniae*). Briefly, *bla*$_{\text{CTX-M Group 1}}$ was present in *E. coli* (n=30/38) and *K. pneumoniae* (n=17/17), while *bla*$_{\text{CTX-M group 9}}$ was found in *E. coli* (n=7). Thus, only one *E. coli* isolate was negative for *bla*$_{\text{CTX-M}}$. The pAmpC-PCR was positive in nearly all AmpC-phenotype *E. coli* (n=15/17), all *bla*$_{\text{CMY}}$, indicating hyperproduction of the chromosomal AmpC-encoding gene in the two isolates which were negative pAmpC. One of the pAmpC-PCR negative isolates was also negative for *bla*$_{\text{CTX-M}}$.

158 **Table 1.** ST-distribution and clinical important antimicrobial resistance determinants in *E.coli* strains (n=37).

	PCR result		β-lactam resistance						Trimethoprim resistance					Sulphonamide resistance		Quinolone mutations			Aminoglycoside
ST	CTX-M +	pAmpC +	CTX-M-15 (n=30)	CTX-M-27 (n=6)	CTX-M-14 (n=1)	CMY-2 (n=15)	TEM-1 (n=32)	OXA-1 (n=23)	dfrA1 (n=2)	dfrA8 (n=1)	dfrA12 (n=2)	dfrA14 (n=1)	dfrA17 (n=31)	sul 1 (n=33)	sul 2 (n=35)	gyrA (n=32)	parC (n= 29)	parE (n=31)	(n=37)
ST410 (n=16)	16	15	16	-	-	15	15	14	-	-	-	-	16	16	15	16	15	15	16
ST131 (n=7)	7	-	2	5		-	4	-	-	-	-	-	7	7	7	7	4	7	7
ST617 (n=6)	6	-	6	-	-	-	6	6			1		5	6	6	5	6	6	6
ST155 (n=1)	1	-	1	-	-	-	1	-	-	-	-	-	-	-	-	1	-	-	1
ST48 (n=1)	1	-	1	-	-	-	1	-	-	-	1	-	-	1	1	1	-	-	1
ST6332 (n=1)	1	-	1	-	-	-	1	1	-	-	-	1	-	-	1	1	1	1	1
ST354 (n=1)	1	-	-	1	-	-	1	-	-	-	-	-	1	1	1	1	1	1	1

ST5824 (n=1)	1	-	-	-	1	-	-	-	1	-	-	-	-	-	1	-	-	-	1
ST38 (n=1)	1	-	1	-	-	-	1	-	1	-	-	-	-	-	1	-	-	-	1
ST44 (n=1)	1	-	1	-	-	-	1	1	-	1	-	-	1	1	1		1	-	1
ST648 (n=1)	1	-	1	-	-	-	1	1	-	-	-	-	1	1	1	1	1	1	1

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161 **Table 2.** ST-distribution and clinical important antimicrobial resistance determinants in K.pneumoniae strains (n=17).

ST	PCR result	β -Lactam Resistance					BLAS	Trimethoprim Resistance						Sulphonamide resistance		Quinolone		Aminoglycoside
	CTX-M (n=17)	CTX-M-15 (n=17)	blaSHV (n=16)	TEM (n=14)	OXA (n=11)	CO-1 (n=1)	dfrA1 (n=1)	dfrA12 (n=1)	dfrA14 (n=12)	dfrA17 (n=1)	dfrA27 (n=1)	dfrA30 (n=1)	Sul1 (n=6)	Sul2 (n=14)	gyrA (n=8)	qnrB1 (n=4)	(n=17)
ST101 (n=2)	2	2	2	-	2	-			2					2	2		2
ST1047 (n=1)	1	1	1	1	1	-			1					1			1
ST14 (n=2)	2	2	2	2	2	-			1			1		2		1	2
ST15 (n=1)	1	1	1	1	1	-			1				1	1	1		1
ST1552 (n=1)	1	1	1	1	-	-	1		1				1	1		1	1
ST231 (n=1)	1	1	1	1	1	-			1						1		1
ST29 (n=1)	1	1	1	1	1	-								1		1	1
ST307 (n=1)	1	1	1	1	-	-			1				1	1			1

ST340 (n=2)	2	2	2	2	1	-			2			1	1	2			2	
ST48 (n=1)	1	1	1	1	1	-		1				1	1	1			1	
ST607 (n=1)	1	1	1	-	-	1					1		1				1	
ST874 (n=1)	1	1	1	1	1	-			1			1	1	1			1	
UNKNOWN (n=2)	2	2	1	1	1	-			1	1			1			1	2	

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Whole genome sequencing (WGS)

E. coli ST-profile and AMR-determinants

All *bla*$_{CTX-M}$ -positive *E. coli* (n=37) were subjected to phylogenetic analyses, potential clonal relatedness, and to verify PCR findings. Detailed information on the individual isolates is given in Supplementary Table 1 (ST1). Table 1 summarizes the ST profiles and distribution of resistance determinants to clinically important classes of antibiotics for which we had phenotypic AST data: β-lactams, fluoroquinolones (FQ), trimethoprim (TMP), sulphonamides (SUL), and aminoglycosides (AG).

ST410 (n=16), ST131 (n=7), and ST 617 (n=6) were the dominant STs among the eleven different STs. Notably, all pAmpC-positive (*bla*$_{CMY-2}$) isolates (n=15) were of ST410, and most *bla*$_{CTX-M\ Group\ 9}$ (*bla*$_{CTX-M-27}$) positive isolates were of ST131. ST410 isolates were prevalent in specimens from the surgical department (n=11) and pus specimens (n=10).

The presence of *bla*$_{CTX-M\ Group\ 1\ and\ 9}$ as well as *bla*$_{CMY}$ as determined by PCR, was confirmed by WGS. *Bla*$_{CTX-M-15}$ (n= 30) and *bla*$_{CTX-M-27}$ (n=6) were the dominant allelic variants in *bla*$_{CTX-M\ Group\ 1}$ and *bla*$_{CTX-M\ Group\ 9}$ positive isolates, respectively. All 37/37 (100%) isolates were resistant to cefotaxime, ceftazidime, and cefuroxime. All *bla*$_{CMY-2}$ isolates were also resistant to cefoxitin. Other major β-lactamase encoding genes included *bla*$_{OXA-1}$ (n=23) and *bla*$_{TEM1-B}$ (n=32).

The dominant FQ-resistance determinants included *aac (6')-Ib-cr* (n=25). Chromosomal mutations affecting the DNA gyrase (*gyr*A; n= 32) and DNA topoisomerase IV (*par*C; n=29 and *par*E; n=31) were also evident. Only two isolates did not contain any quinolone resistance determinants. *Qnr*- resistance determinants were not detected in *E. coli*. We did not observe any inconsistencies between the genotypic presence of quinolone resistance and susceptibility to ciprofloxacin (Table ST1).

TMP-resistance determinants included *dfr*$_{A1}$, *dfr*$_{A8}$, *dfr*$_{A12}$, *dfr*$_{A14}$, and *dfr*$_{A17}$, of which *dfr*$_{A17}$ (n=31) was the dominant subtype. SUL-resistance genes were of subtypes *sul*$_1$ and *sul*$_2$ of which *sul*$_2$ (n=35) was dominant. Nearly all *E. coli* isolates (n=36) contained at least one trimethoprim and one sulphonamide resistance determinant and were associated with phenotypic resistance to trimethoprim-sulfamethoxazole (TMP-SUL). All ST410 and ST131 isolates were *dfr*$_{A17}$ and *sul*$_{1/2}$ positive.

The dominant AG-resistance determinants were *aph (6)-Id* (n=35), *aph (3'')-Ib* (n=33), *aadA5* (n=31) and *aac (3)-IId* (n=23); all of which were predominant in ST410. All isolates of *E. coli* contained at least one resistance determinant encoding AG-modifying enzymes with different substrate profiles. Gentamicin resistance was expressed in 28/37 (76%) of the *E. coli* isolates. Due to the current complexities in AG-resistance determinants and some inconsistencies in published substrate profiles, we did not perform any additional comparison of AG-geno- and phenotype.

Plasmid incompatibility groups

A total of 17 incompatibility groups were identified among the *E. coli* isolates (ST1). IncFIB (n=37/37; 100%), IncFIA (n=34/37; 92%), and IncFII (n=33/38; 89%) were most common. We did not perform any analyses of the association between Inc-groups and pheno- or genotypic characteristics.

Clonal relatedness in the major STs of E. coli

SeqSphere-analyses of the major STs of *E. coli* (ST131, ST410, and ST617) revealed a close genetic relationship between isolates (clusters) within each ST (Figure 1). ST131 isolates (n=7) were genetically diverse with a clonal cluster of three isolates (P31-27, -31, -36). Most of the ST410 isolates (n=16) clustered except three (P31-01, -26, and -44), whereas two ST617 clusters, P30-79 and -81 as well as P31-40 and -58, were observed. The results are consistent with the fact that each cluster has a common origin in the recent past indicating

transmissions between patients or independent infections from a common reservoir (Table ST1, Figure 1).

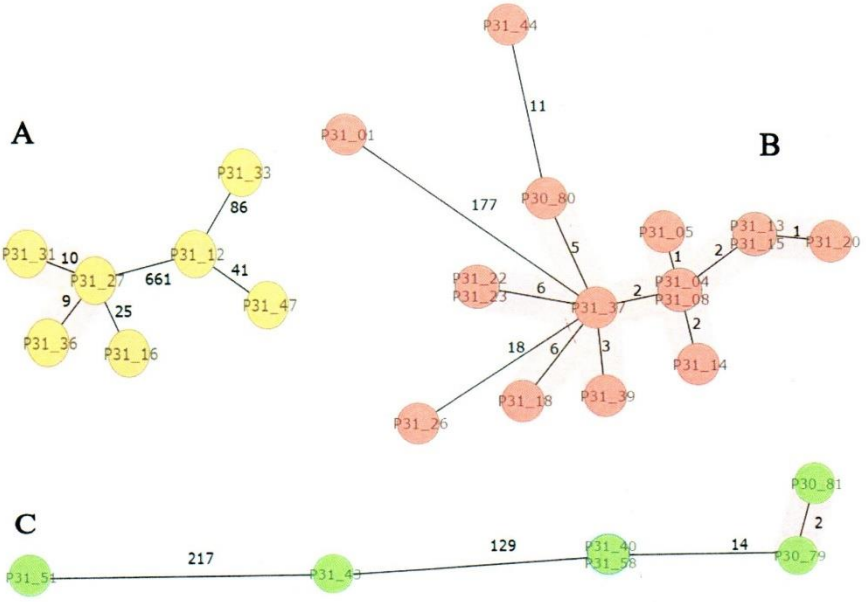

Figure Legend: Minimum spanning three based on cgMLST analysis of ESBL-producing *E.coli* ST131 (A; n=7), ST410 (B; n=16), and ST617 (C; n=6) isolates. The isolates are colored by sequence type (ST) and numbered according to Supplementary Table 1. Clusters are colored in pink and related to the cluster

Figure 1. Seqsphere analysis of *E. coli* high risk clones; ST131, ST410 and ST617.

K. pneumoniae ST-profile and AMR-characteristics

All *bla*$_{\text{CTX-M}}$-positive *K. pneumoniae* (n=17) were subjected to WGS for phylogenetic analyses, to examine potential clonal relatedness, and to verify PCR findings. Detailed information on the individual isolates is given in Supplementary Table 1 (ST1). Table 2 summarizes the ST profile and distribution of resistance determinants to clinically important classes of antibiotics for which we have phenotypic AST data: β-lactams, FQ, TMP, SUL, and AG.

WGS-analyses revealed 12 different STs with two isolates belonging to each of ST101, ST14, and ST340. We did not perform any sequence cluster analyses of *K. pneumoniae* isolates due to low numbers of identical STs. The presence of *bla*$_{\text{CTX-M Group 1}}$ as detected by PCR was confirmed by WGS and *bla*$_{\text{CTX-M-15}}$ was the only allelic variant. All 17 isolates were resistant to cefotaxime, ceftazidime, and cefuroxime. Other major β-lactamase encoding genes included *bla*$_{\text{OXA-1}}$ (n=11) and *bla*$_{\text{TEM1-B}}$ (n=9). SHV-variants except SHV-1 included SHV-28 (n=4), SHV-11 (n=4), SHV-187 (n=1) and SHV-121 (n=1), of which SHV-28 is associated with an ESBL-phenotype.

The dominant FQ-resistance determinants were *oqx*A (n=9) and *qnrB1* (n=4) as well as chromosomal mutations affecting the DNA gyrase (*gyrA*; n=8). The corresponding isolates expressed resistance towards ciprofloxacin.

All *K. pneumoniae* isolates contained TMP- and SUL-resistance determinants. TMP-determinants included dfr_{A12}, dfr_{A14}, dfr_{A17}, dfr_{A27} and dfr_{A30} of which dfr_{A14} (n=13) was the dominant subtype. SUL-resistance genes were of subtypes sul_1 and sul_2 of which sul_2 (n=14) was dominant. All isolates expressed resistance to TMP-SUL.

The dominant AG-resistance determinants were *aac (3)-IIe* (n=13), *aph (6)-Id* (n=12) and *aph (3'')-Ib* (n=11). All *K. pneumoniae* isolates that contained AG-resistance determinants expressed resistance to gentamicin.

Plasmid Incompatibility groups

Sixteen plasmid incompatibility groups were identified from isolates of *K. pneumoniae*. IncF (n=16/17; 94%) (IncFIB (n=13/17; 76%), IncFII (n=10/17; 71%) IncFIA (n=9/17; 53%)) and IncR (n=12/17; 71%) groups were predominant. We did not perform any analyses of association between Inc-groups and pheno- or genotypic characteristics.

4. Discussion

This study adds to the knowledge on the molecular epidemiology of ESBL- and pAmpC-producing clinical isolates of *E. coli* and *K. pneumoniae* in Malawi. ESBLs were predominantly of CTX-M-15 and pAmpC were all CMY-2 type. CTX-M group 9 ($bla_{CTX\text{-}M\text{-}27\ and\ -14}$) were also detected. These observations are consistent with the global distribution of ESBL-[9, 22] and pAmpC-subtypes (18) as well as previous studies from Sub-Saharan Africa (23, 24) and Malawi [14, 15, 16]. CTX-M-15 has previously been shown to be the dominant ESBL type in invasive isolates of *E. coli* and *K. pneumoniae* from hospitalised adults and children in Malawi [14, 15, 16].

CMY-2 was the only detectable pAmpC type. This observation is consistent with recent findings in Ethiopia, where bla_{CMY} was the most frequent pAmpC in *E. coli* bacteraemia isolates [25]. To our knowledge, there is no previous pAmpC-data for comparison in Malawi. Data from the neighbouring country Mozambique have shown the presence of both bla_{CMY}, bla_{DHA}, bla_{FOX} and bla_{MOX} in 3rd generation cephalosporin resistant clinical isolates of *E. coli* [26]. These observations call for more surveillance of pAmpC to address the limited data for Malawi.

Isolates expressed an MDR-profile including resistance against FQ-, AG-, and/or TMP-SUL. These findings are in line with previous observations of MDR clinical isolates of ESBL-producing *E. coli* and *K. pneumoniae* in Malawi [14, 15, 16]. The dominant acquired FQ-resistance determinant for *E. coli* was *aac (6')-Ib-cr* while *qnrb1* and *oxqA* were most prevalent in *K. pneumoniae*. Both of the findings are comparable with previous observations in Sub-Saharan Africa [27] and Malawi [16]. However, *qnrS* in *K.pneumoniae* was previously reported in Malawi [16] but was not found in this study. These differences may be attributed to the fact that the corresponding specimens in this study were largely isolated from surgical specimens versus the study at Queen Elizabeth Central Hospital (QECH) Malawi which investigated *K.pneumoniae* isolated from blood cultures and rectal swabs [16]. To confer high-level FQ-resistance additional chromosomal mutations are required. These chromosomal mutations were observed in our study, of which mutations in *gyr*A, *par* C and *par* E were most prevalent for *E. coli* and mutations in *gyr*A only for *K. pneumoniae*. Mutations in *gyr*A in both *E. coli* and *K. pneumoniae* are comparable to recent findings in similar clinical isolates from Malawi [14, 15, 16], however *par* C and *par* E have not been previously reported in Malawi. Two of the 32 *E. coli* isolates that contained *gyrA* mutations expressed susceptibility to ciprofloxacin. These isolates contained only one codon mutation (S83L). Similar findings have been reported [14] whereby the presence of only one codon mutation did not confer phenotypic resistance to ciprofloxacin.

The dominant TMP-resistance genes were *dfr*$_{A17}$ and *dfr*$_{A14}$, in *E. coli* and *K.pneumoniae*, respectively. *Dfr*$_{A17}$ has been previously described in *E.coli* in Malawi by Thega *et al* (2021) [15] but, to our knowledge, this is the first report of *dfr*$_{A14}$ in *K.pneumoniae* in Malawi. Musicha et al. [16] observed *dfr* in *K.pneumoniae*, however, they did not describe the subtype of *dfr* as such. Though potentially new to Malawi, these findings are common and concurring with WGS-analyses conducted in Ghana on *K. pneumoniae* isolates resistant to 3rd generation cephalosporins [28]. *Sul*$_2$ was the most prevalent SUL-resistance determinant for both *E. coli* and *K. pneumoniae* confirming the previous finding in Malawi [15, 16].

ST410 and ST131 were the most prevalent STs for *E. coli*. These findings are in line with global ST distribution and also with previous studies in Malawi [14, 15]. ST131 and ST410 are classified as high-risk MDR-clones of *E. coli* [29, 30]. They are easily transmitted between patients, have the capability to colonise and persist in hosts, and may cause severe and recurrent infections [29]. Clonal dissemination of ST131 is in particular known to be associated with MDR [4]. MDR ST 410 has also shown clonal expansion over the past decade as evident from isolates of ST410 carrying acquired carbapenamase gene *bla*$_{OXA-181}$ being reported in Italy, China, and a small hospital outbreak in Denmark as described [29].

ST410 isolates were prevalent in specimens from the surgical department (mainly pus) whereas ST131 was prevalent in specimens from the medical department (mainly blood culture and urine). This may indicate local transmission within the wards at the hospital, but the numbers are low. Similar to previous findings in Malawi [15], ST131 was associated with *bla*$_{CTX-M-15}$ and *bla*$_{CTX-M-27}$ in contrast to ST410 which was only associated with *bla*$_{CTX-M-15}$. Clonal cluster analyses of the major STs of *E. coli* in our study strongly indicate transmission between patients and/or to independent patients via a common reservoir. These observations support the need for strengthening infection prevention measures, specifically the use of personal protective equipment (PPE) as well as the need to institute screening programs so that patients' MDR-isolates can be isolated to limit transmission

For *K. pneumoniae*, there was a high ST-variation between the isolates. However, all the identified STs are considered global MDR-clones [5]. These are clones that contribute disproportionately to the global disease burden and are among those clones that commonly cause hospital-acquired infections and outbreaks [5]. The identified STs in our study align with previous findings in Ghana (ST101) [28] and Malawi (ST14, ST340) [31, 16, 32]. ST101 has to our knowledge not previously been identified in Malawi.

Our findings and others [14, 15, 16] strongly suggest that globally distributed MDR-clones of *E. coli* and *K. pneumoniae* are already causing public health threats in hospitals in Malawi. Fortunately, in this study, we did not observe any carbapenem resistance, but the existing clones may acquire and display carbapenemase-encoding determinants [5]. Therefore, there is an urgent need to closely monitor the situation combined with stringent infection control practices while carbapenem resistance is still low in Malawi.

A limitation of this study was that it only examined isolates from a single site and one region of the country. There may be variations in the type of ESBL and AmpC β -lactamases in different regions of the country. Many isolates were also lost in transit to the reference laboratory, thus reducing the number of isolates that could be sequenced. We were also unable to identify potential additional hospital-related sources of the MDR bacteria. ESBL-screening of hospital surfaces, medical devices/equipment, and patients could have added value to the findings of this study.

Infections with ESBLs and AmpC β-lactamase producing MDR *Enterobacterales* are of huge clinical importance. Their increasing rates drive the prescriptions of carbapenems which promotes the spread of potentially untreatable carbapenamase-producing *Enterobacterales*. Due to the limited availability of carbapenems at the study site, the current usage of this antibiotic class is limited. The high transmissibility of MDR-global clones calls for stringent infection prevention practices and an urgent need for molecular and epidemiological studies to inform targeted containment strategies. The spread of these can have

substantial effects on the healthcare systems of Malawi where treatment options are already severely limited. Thus, such infections pose a financial burden on healthcare systems by increasing hospital stays and the cost of drugs which countries like Malawi may find difficult to sustain. Continuous surveillance and early detection are key for limiting their spread.

This study was able to provide insight into the molecular epidemiology of ESBL- and pAmpC-producing *E. coli* and *K. pneumoniae* in a tertiary hospital in Malawi. These findings add to the limited literature that exists for Malawi on the genomic characterisation of pathogens such as *E. coli* and *K. pneumoniae*. Together these data can be used to provide a baseline for purposes of tracking these MDR-clones, and form a basis for policymakers to identify areas to target when addressing this major threat of antibiotic resistance.

5. Conclusions

MDR ESBL (CTX-M-15 type) and/or plasmid-mediated AmpC (CMY-type)-producing *E. coli* and *K. pneumoniae* are prevalent in clinical specimens from KCH. Most of the *E. coli* and *K. pneumoniae* isolates at KCH are representatives of high-risk MDR clones. Close monitoring and early detection for targeted infection control measures are urgently needed.

Author Contributions: Conceptualization: FC, BH, GSS, SE, and AS; Performance and analysis of AST: FC, IN, OS, TM and BH; PCR and bioinformatics analysis and interpretation: FC, BH, JJ, JM and AS; Funding acquisition: SE and AS; Writing – original draft: FC and AS; Writing – review & editing: All authors. All authors have read and approved the final manuscript; Supervision: SE and AS supervised the overall project.

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Institutional Review Board Statement: This study was approved by the Malawian College of Medicine Research and Ethics Committee; (reference no. P.11/17/2308) and by the University of KwaZulu Natal Biomedical Research Ethics Committee (reference no.BE093/16).

Informed Consent Statement: Voluntary informed consent was sought from every patient prior to inclusion in the study.

Data Availability Statement: The datasets used and/or analyzed in this study are available from the corresponding author on reasonable request.

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CHAPTER 4

Short communication: The molecular epidemiology of *S. aureus* isolates from a tertiary referral Hospital in Lilongwe, Malawi

Author contributions:

- Faheema E Choonara: Study design, sample processing, data collection, data analysis and interpretation, writing the manuscript
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- Gunnar S Simonsen: study design and review of manuscript
- Sabiha Y Essack: Co-conceptualized the study, guided the literature review and ethical clearance application, enabled data collection and analysis and undertook the critical revision of the manuscript as co-supervisor
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The molecular epidemiology of *S. aureus* isolates from a tertiary referral Hospital in Lilongwe, Malawi

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Abstract

Staphylococcus aureus is an important opportunistic human pathogen causing a wide variety of infections. The aim of this study was to perform a molecular epidemiological characterization of selected methicillin susceptible (MS) and methicillin resistant (MR) *S. aureus* isolated from different clinical specimens at Kamuzu Central Hospital (KCH) during 2017. Using the cefoxitin disc screening method and *mec*A gene PCR, nine out of 86 (10.5%) *S. aureus* isolates were found to be MRSA. Twenty-four available isolates, four MRSA and 20 MSSA were subjected to *spa* typing. By *spa* typing, 22 isolates were typeable and classified into nine *spa*-types. *Spa*-types t355 (n=12/24; 50%) and t064 (n=3) were dominant among MSSA- and MRSA-isolates, respectively, indicating potential in-hospital dissemination. The data underline the need to strengthen and , ensure adherence to the infection prevention policies and strategies and establish an antibiotic surveillance program at the hospital in order to trace reservoirs, potential transmission lines and to inform evidence-based interventions.to curtail the spread of MRSA.

Key words: Methicillin Resistant *Staphylococcus aureus*, Methicillin sensitive *Staphylococcus aureus*, t064, t355, *mec*A

Introduction

Staphylococcus aureus is an important opportunistic human pathogen causing a wide variety of infections (1)(2). β-lactam antibiotics are commonly used to treat *S. aureus* infections. However, since the introduction of methicillin in 1961, there has been an increase in methicillin resistant *S. aureus* (MRSA) mediating resistance across the β-lactam group of antibiotics (1). Methicillin resistance results from the acquisition of an additional low affinity penicillin binding protein (PBP 2A) which is encoded by the *mec*A gene within the staphylococcal cassette chromosome *mec* (SCC*mec*) elements (1)(3). Detection of the *mec*A gene has been described as the preferred rapid method for confirmation of MRSA (3). Although alternative *mec*-alleles exist, they are seldom detected in human MRSA (4).

MRSA has become a common cause of nosocomial infections worldwide and it is associated with higher morbidity and mortality compared to infections caused by methicillin susceptible *S. aureus* (MSSA) (1) (3). As such, it becomes crucial to distinguish MRSA from MSSA for therapeutic and infection control purposes (1). Depending on the population and study site, data from nine African countries have shown MRSA prevalence ranging from 12 to 80%. In Malawi a study of various clinical specimens at KCH from 2006-2007 showed that 46/147 (31.3 %) of the *S. aureus* were resistant to oxacillin indicating MRSA, but the strains were not molecularly confirmed (6). Another Malawian study at Queen Elizabeth hospital (QECH) found that 107/1118 (9.6%) of *S. aureus* from blood culture between 1998 and 2016 were MRSA (7). Our recent study at KCH showed an overall MRSA-prevalence of 10.5% (9/86) in clinical *S. aureus* isolates (8).

Due to the overall clinical importance of both MSSA and MRSA, it is important to have data on their molecular epidemiology in order to trace reservoirs, identify potential transmission lines and inform evidence-based interventions. The aim of this study was to investigate the spa-types of selected MSSA and MRSA isolated at KCH during 2017. of selected MSSA and MRSA isolated at KCH during 2017.

Material and methods

Study Design

The study was conducted from June –December 2017 at Kamuzu Central Hospital (KCH), a government referral hospital for the central region of Malawi serving a community of six million people with approximately 750 beds. KCH has four major hospital departments: medical, obstetrics and gynaecology, surgical and paediatrics. It is the referral centre for eight districts, six Christian Health Association of Malawi (CHAM) hospitals and 3 level II hospitals (act as referral centres for primary

care). Microbial culture and, antimicrobial susceptibility testing (AST), phenotypic detection of MRSA and data collection was performed as described by Choonara, *et al*. (2022) (8). Quality control for AST was performed on a weekly basis and included strains of *S. aureus* ATCC 25923 and MRSA ATCC 33591. Clinical isolates were stored at -80ºC in tryptone soy broth with 10% glycerol for further testing.

Selection of MRSA-phenotype positive strains for genetic characterization

S. aureus isolates with reduced susceptibility to cefoxitin (38/86; 44%) as described in the primary analyses in Malawi (8) were shipped to the Norwegian Advisory Unit on Detection of Antimicrobial Resistance (K-res), Tromsø, Norway for further analysis including MRSA confirmation and *spa*-typing. Briefly, the isolates were retrieved and re-cultured on blood and chocolate agar (Oxoid, Hampshire, UK). MALDI-TOF-MS (Bruker Daltonik, Bremen, Germany) was used for species identification. All strains were retested using the cefoxitin (30µg) disc using inhibition zones of <22mm as a cut-off.

DNA extraction and PCR analysis for MRSA

The selected strains were subjected to DNA extraction using the Biomerieux NucliSENS-easyMAG kit (Biomerieux, Marcy l'Étoile, France) and subjected to real time PCR for detection of *mecA* gene as described in (1) using the Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystem Inc, Foster City, CA). The PCR parameters were as follows: 95 ºC for 20 sec, followed by 50 cycles of denaturation at 95 ºC for 3 sec and annealing and elongation at 60 ºC for 30 sec.

***Spa*-typing**

DNA was subjected to amplification and sequencing of the SSR region of the *spa* gene as described in (9). Briefly, amplification of the *spa*-gene was performed by conventional PCR using the GeneAmp PCR system 9700 (Applied Biosystem Inc, Foster City, CA). The thermal profile was as follows: initial ramp 96ºC for 5-minutes, denaturing 96ºC for 10 seconds, annealing 50ºC for 10 seconds, elongation 60ºC for 4 minutes, then hold at 4ºC for 4 minutes. The PCR cycle was repeated for 25 cycles. Amplicons were visualized by gel electrophoresis using 2% agarose gel in order to verify that each specimen contained the PCR product, the *spa*-gene.

The spa-amplicons were sequenced. Data were analysed using the databases from Centre for Genomic Epidemiology, DTU, Denmark; http://cge.cbs.dtu.dk/servicdes/spaTyper-1.0/

Results

Upon re-examination only nine of the 38 strains were confirmed as MRSA with cefoxitin inhibition zones below the 22 mm cut-off level (Table 1) and *mecA* PCR analyses. The nine MRSA strains had very small cefoxitin inhibition zones (6-10 mm), while the remaining 29 MSSA strains had cefoxitin inhibition zones around the cut-off value (19-21 mm) in the primary analyses in Malawi showing a high false positive rate. Unfortunately, 14 strains were lost during processing, making only 24 strains available in pure culture for *spa* typing, i.e., 20 MSSA and four MRSA.

Twenty-two out of the 24 *S. aureus* isolates were typeable and classified into nine *spa*-types (Table 2). One MSSA-isolate was negative by *spa*-PCR and one MSSA-isolate was not typable (Table 2). *Spa*-type t355 (n=12/24; 50%) was most prevalent among the MSSA-isolates and was mainly isolated from the surgical department (10/12), all from pus specimens. Three of the four MRSA isolates were *Spa*-type t064, all from the surgical department.

Table 1: Antibiotic resistant profiles of *S. aureus* (n=38)

Antibiotics	**Resistant Isolates n/N (%)**
Cefoxitin	9/38 (24%)
Clindamycin	12/38 (29%)
Erythromycin	20/38 (53%)
Fusidic acid	0
Gentamicin	8/38 (10.5%)
Penicillin	38/38 (100%)
Trimethoprim-sulfamethoxazole	17/38 (45%)

Table 2. *Spa*-typing of MSSA and MRSA-isolates by ward and specimen type

***spa*-type**	**MRSA n (%)**	**MSSA n (%)**	**Pus**	**Blood**	**Surgical**	**Medical**
t355		12 (50)	12		10	2
t064	3 (12)		2	1	3	
t084		1 (4)	1		1	
t12092		1 (4)	1		1	
t1504		1 (4)	1		1	
t186	1 (4)		1		1	
t1973		1 (4)		1		1
t318		1 (4)	1		1	
t6670		1 (4)	1		1	
***spa*-negative**		1 (4)	1			1
Non-typable		1 (4)	1		1	
Total	4 (17)	20 (83)	22	2	20	4

Discussion

To our knowledge this is the first study to report on the molecular typing of MSSA and MRSA in Malawi. This study confirms a relatively low prevalence of MRSA among clinical isolates from KCH during 2017 compared to the phenotypic results in this study as well as that in Musicha *et al*. (2017) (7), who conducted a study on bloodstream infection and antibiotic resistance surveillance on adult and paediatric patients with fever from 1998–2016 at Queen Elizabeth Hospital (QECH) (7). Thus showing the importance of molecular confirmation of phenotypically suspected MRSA and the need for better diagnostic laboratory capacity in low and middle income countries such as Malawi. However, the numbers are low and do not provide strong evidence of the true prevalence of MRSA.

The findings have also shown an over-estimation of MRSA from phenotypic screening; indicating that the expression of methicillin resistance may be a result of other mechanisms other than expression of *mecA* gene such as hyper production of β-lactamase, alterations in different amino acids present in the PBP cascade (PBP 1+2+3), other chromosomally determined components such as FemA and FemB that encode proteins involved in the formation of pentaglycine side of the peptidoglycans and *mec*B and *mec*C (10). Similar findings have been observed in Ghanaian study that investigated the molecular mechanism of resistance in 91 isolates of *S. aureus* collected from hospitals in Ghana from May to September 2015 (10) as well as in a study in Sudan investigating the frequency of *mec*A gene in MRSA isolated from various clinical specimens from different hospitals from 2013-2014 (3).

The *spa*-typing data showed genetic diversity with a dominance of t355 MSSA and t064 MRSA, from various sources with some evidence of clonal spread based on the majority of the predominant SPA types being isolated from one ward-surgical department (11). The dominance of t355 MRSA and t064 MRSA are consistent with previous studies in some African countries (12), such as Zambia (13), Nigeria (14), and Uganda (15). *Spa* type t355 has been known to be commonly recovered from skin and soft tissue infection sites (16), which is comparable with a high prevalence of t355 observed in pus specimens collected from surgical department in this study.

Limitations of this study included the low overall number of *S. aureus* isolates and the single study site design. Moreover, the loss of isolates during processing further limited the sample size for molecular typing. We did not perform *SCCmec*-typing nor whole genome sequencing. Thus, we could not establish a final clonal relationship of the isolates. On the other hand we have been able to contribute to the molecular *S. aureus* data in Malawi which is severely limited.

Conclusions

We confirmed the presence of various *S. aureus spa*-types of MSSA and MRSA at KCH, dominated by t355 and t064, respectively. MRSA significantly limits treatment options for *S. aureus* infections and has consequences on empirical and targeted antibiotic therapy as well as infection control. There is need to strengthen and , ensure adherence to the infection prevention policies and strategies and establish an antibiotic surveillance program at the hospital in order to trace reservoirs, potential transmission lines and inform evidence-based interventions to curtail the spread of MRSA.

Ethics approval and consent to participate

This study was approved by the Malawian College of Medicine Research and Ethics Committee; (reference no. P.11/17/2308) and by the University of KwaZulu Natal Biomedical Research Ethics Committee (reference no.BE093/16). Voluntary informed consent was sought from every patient prior to inclusion in the study.

Availability of data and materials

The datasets used and/or analysed in this study are available from the corresponding author on reasonable request.

Competing interests

Sabiha Essack is chairperson of the Global Respiratory Infection Partnership and member of the Global Hygiene Council, both sponsored by an unrestricted educational grant from Reckitt UK. "The other authors declare that they have no competing interests"

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Authors' contributions

Conceptualization: FC, BH, GSS, SE, FL and AS.

Performance and analysis of AST: FC, IN, OS, TM and BH.

PCR, spa typing and interpretation: FC, BH, JJ, AH and AS.

Funding acquisition: SE and AS.

Writing – original draft: FC and AS.

Writing – review & editing: All authors. All authors have read and approved the final manuscript.

Supervision: SE and AS supervised the overall project,

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CHAPTER 5: CONCLUSION, RECOMMENDATIONS AND LIMITATIONS

Introduction and Key Findings

A total of 327 non-duplicate isolates of ESKAPE bacteria, *E. coli, P. mirabilis* and *Salmonella* spp. collected from a variety of clinical specimens over a 6-month period from June –December 2017 at KCH in Lilongwe Malawi were investigated. Microbial culture and AST was conducted using standard culture methods under aerobic conditions and disk diffusion method using EUCAST guidelines respectively. Resistant isolates were further investigated via PCR, MLST and WGS in order to delineate antibiotic resistance genes, MLST profiles, phylogenetic analysis and clonal relatedness. The following are the main conclusions from the study:

- Out of 411 isolates, the majority (80%) were identified as ESKAPE pathogens (n=195/327; 60%), *E. coli* (n=92/327; 28%), *P. mirabilis* (n=33/327; 10%) and *Salmonella* spp. (n=7/327; 2%).

- *K. pneumoniae* (n=24/29; 83%), *E. cloacae* (n=9/13; 69%), *E. coli* (n=58/92; 63%), and *P. mirabilis* (n=17/33; 52%) showed reduced susceptibility to CTX and/or CAZ, of which the majority were also MDR and resistant to CIP, GEN and SXT

- *E. coli* (n=92; 28%) was one of the most commonly detected species. ESBL- production was phenotypically confirmed in more than half of the *E. coli* (n=49/92; 53%) and AmpC-phenotype was confirmed in 28% of the *E. coli* (n=26/92). Combined ESBL- and AmpC-phenotype was observed in 22% (n=20/92). The majority of ESBL and AmpC positive *E. coli* were of $bla_{\text{CTX-M}}$ (predominantly CTX-M-15) and bla_{CMY} respectively. Other prevalent resistance genes in *E. coli* were *aac (6")-lb-cr*, dfr_{A17} and Sul_2 for fluoroquinolones, trimethoprim and sulphonamide resistance. DNA gyrase and DNA topoisomerase IV were the most common chromosomal mutations identified in *E. coli*. ST410, ST131 and ST617 was the most prevalent ST type in *E.coli*; all of which are classified as high risk clones. ST410 was most frequently associated with surgical specimens whereas ST 131 was frequently isolated from specimens from medical department. Seqsphere analysis showed close genetic relationship between strains (clusters) within each ST. This demonstrates the clusters are of common origin indicating local transmission.

- In *K.pneumoniae*, ESBL- production was phenotypically confirmed in more than half of the isolates (n=20/29; 69%). AmpC production was phenotypically confirmed in 28% (n=8/29) of the isolates; all of which were amongst those that were phenotypically positive for ESBL production. Genotypically, the majority of ESBL positive *K.pneumoniae* were $bla_{\text{CTX-M}}$ type - predominantly CTX-M-15. Other prevalent resistance genes *in K. pneumoniae* were *oqx*A, dfr_{A14} and Sul_2 for fluoroquinolones, trimethoprim and sulphonamide resistance respectively.

- ST101, ST14, and ST340 was the most prevalent ST type in *K. pneumoniae*, all of which are also high risk clones.
- MDR was observed in the majority of the ESBL-positive isolates and AmpC-positive isolates. In contrast, lower rates of MDR were observed in ESBL-negative isolates and AmpC-negative isolates.
- High levels of susceptibility to meropenem were observed across all Enterobacterales except *P. aeruginosa.*
- A high rate of reduced susceptibility to MEM was observed in *P. aeruginosa*, however carbapenemase production was not evident in MEM-R *P. aeruginosa* using biochemical and molecular methods
- All *Salmonella* spp. were susceptible to CTX and CAZ.
- *S. aureus* (n=86; 26%) was also one of the most commonly detected species. MRSA was phenotypically confirmed in 10.5% (n=9/86) of *S.aureus* isolates. These expressed reduced susceptibility to ERY (n=7/9; 78%) or GEN (n=8/9; 89%) or both ERY and GEN (n=7/9; 78%). In contrast, MSSA showed lower resistance rates to ERY (n=33/77; 43%), GEN (n=12/77; 16%) and both GEN and ERY (n=11/77; 14%). All MRSA contained the *mec*A gene. T064 and t355 were the most prevalent *spa* type found in MRSA and MSSA respectively. *Spa*-type t355 (n=12/24; 50%) and *Spa*-type t064 (n=3) were most frequently isolated from the surgical department.
- For *Enterococcus*, reduced susceptibility to VAN and high-level aminoglycoside resistance (HLAR), was not observed

Study significance

This study provides an overview on the prevalence and antibiotic susceptibility patterns of ESKAPE pathogens, *E.coli, P. mirabilis* and *Salmonella* sp. from a variety of clinical specimens for KCH- a referral hospital in the central region of Malawi. This study adds new data for Malawi on the antimicrobial resistance determinants, allelic variants of ESBLS and pAmpCs and sequence types of *E. coli* and *K. pneumoniae*. The study was able to demonstrate the clonal relatedness vis ST and SPA types indicating local transmission of the ESBLs and MRSA within the hospital. This has a major impact on infection control practices of the hospital and can be utilized to plan and strategize for improvements in infection prevention policies and practices. In addition, the major STs identified in this study are classified as high risk clones and pose serious public health threats. These findings can

be utilized by health authorities as evidence and call for urgent action for ABR containment through screening and continuous surveillance of ESKAPE pathogen.

Limitations

There are several limitations in this study including a relative short study period and sample size. Microbiological investigations were not undertaken for all patients presenting with infection, thus indicating a sampling bias. The isolates from this study were from a single site and from one region of the country, hence regional variations could not be accounted for. Many isolates were lost in transit to the reference lab reducing the number of isolates that could be sequenced. Also, there was a lack of availability of isolates from the total population of MRSA for confirmation and *spa* typing. This study was conducted in 2017 and may not be representative of the AMR status at the present time. Despite the limitations, we were still able to provide an overview of the phenotypic and molecular characterisation of ESKAPE pathogens in Malawi.

Recommendations

- Notwithstanding the limitations above, the high prevalence and genetic diversity of ESKAPE pathogens (*K. pneumoniae*, *P. aeruginosa*, and *S. aureus*) and *E. coli* highlights the urgent need to develop and implement infection prevention and control policies, guidelines and interventions to prevent the spread of these pathogens within the hospital, the communities and the country as a whole.
- The high prevalence of ESBL producing MDR Gram-negative ESKAPE pathogens and *E. coli* within the hospital calls for regular screening and surveillance of these pathogens both in the hospital and in the community in order to monitor the epidemiological changes and inform antibiotic treatment.
- Awareness, education and training on AMR, the rational use of antibiotics and the clinical and societal impacts of AMR need to be intensified for health, patients and the community. This is essential to curb the spread and emergence of AMR, and preserve antibiotics so that mortality and morbidity associated with infectious diseases can be controlled in present and future generations
- There is need to set up national and hospital antibiotic stewardship programs with representation from all sectors coupled with strict infection prevention and control programme to combat AMR in Malawi. The antibiotic stewardship program to guide, drive and monitor AMR related activities.

- To advocate and conduct AMR surveillance in the private sector and carriage in communities to understand the true burden of AMR in Malawi
- There is a need to build capacity for microbiology labs across the country. This is to ensure uninterrupted access to microbiology lab supplies which will foster the trust of health professionals and encourage the submission of samples to the microbiology lab. This will allow a shift from empiric to microbiology-informed antibiotic prescriptions. This will also limit unnecessary prescription of antibiotics and reduce AMR associated with inappropriate use of antibiotics.
- To set up whole genome sequencing lab in Malawi and conduct whole genome sequence based epidemiological studies in order to delineate the true burden of antibiotic- resistant ESKAPE and other clinically important pathogens in Malawi.

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APPENDIX: ETHICAL APPROVALS AND PERMISSIONS

Appendix 1: Ethical Approval Certificate COMREC 1

CERTIFICATE OF ETHICS APPROVAL

This is to certify that the College of Medicine Research and Ethics Committee (COMREC) has reviewed and approved a study entitled:

P.04/15/1715 – Prevalence phenotypic and genotypic characterization of resistant clinical isolates of gram negative ESKAPE pathogens by Faheema Choonara

On 15 December 2015

As you proceed with the implementation of your study, we would like you to adhere to international ethical guidelines, national guidelines and all requirements by COMREC as indicated on the next page

[redacted]

Dr. Y. Mlombe- Vice-Chairperson (COMREC)

Approved by College of Medicine
15 DEC 2015
(COMREC) Research and Ethics Committee

15 December 2015
Date

Appendix 2: Ethical Approval Certificate COMREC 2

CERTIFICATE OF ETHICS APPROVAL

This is to certify that the College of Medicine Research and Ethics Committee (COMREC) has reviewed and approved a study entitled:

P.11/17/2308 - Prevalence, Phenotypic and Genotypic Characterization of Resistant clinical isolates of Gram Negative ESKAPE pathogens by Faheema Choonara

On 05-Nov-17

As you proceed with the implementation of your study, we would like you to adhere to international ethical guidelines, national guidelines and all requirements by COMREC as indicated on the next page

[redacted]

Dr. YB. Mlombe - Chairperson (COMREC)

05-Nov-17

Date

Appendix 3: Ethical Approval Letter -BREC

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

13 June 2016

Ms Faheema E Choonara
School of Health Sciences
faheemachoonara@yahoo.co.uk

Dear Ms Choonara

Protocol: Prevalence Phenotypic and Genotypic characterization of Resistant Clinical Gram Negative ESKAPE pathogens (ESKAPE- Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, acinetobacter sp. Pseudomonas aeroginosa, Enterobacter sp.) pathogens
Degree: PhD
***BREC reference* number:** BE093/16

The Biomedical Research Ethics Committee has considered and noted your application received on 23 February 2016.

The conditions have been met and the study is given **full ethics approval.**

This approval is valid for one year from **13 June 2016.** To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date**.**

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

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

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

The sub-committee's decision will be **RATIFIED** by a full Committee at its meeting taking place on **12 July 2016.**

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

Yours sincerely

[redacted]
Professor J Tsoka-Gwegweni
Chair: Biomedical Research Ethics Committee

cc: supervisor: essacks@ukzn.ac.za
Postgraduate office: nenep1@ukzn.ac.za

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
Website: http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

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Appendix 4: Hospital approval letter

Ref. No. KCH/GA/0.01
TELEPHONE NO: (265).1.753 555
TELE FAX NO: (265).1.756380

PLEASE ADDRESS ALL COMMUNICATIONS TO:
THE HOSPITAL DIRECTOR

MINISTRY OF HEALTH
KAMUZU CENTRAL HOSPITAL
P.O BOX 149
LILONGWE
MALAWI

16th March 2015

The Chairman
College of Medicine Research Ethics Committee
(COMREC)
P/Bag 360
BLANTYRE

Dear Sir,

RE: PREVALENCE PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF RESISTANT CLINICAL ISOLATES OF GRAM NEGATIVE ESKAPE PATHOGENS

I am writing this letter of support for the study named above. The proposal is being submitted to the University of Malawi-College of Medicine Research and Ethics Committee (COMREC) for review. The study has been developed with involvement of KCH Laboratory staff and will be conducted at KCH in Lilongwe.

The study is aimed at determining prevalence of resistance strains as well as nosocomial/community linkage of these isolates. It is envisaged that this study shall for the first time provide data of prevalence of resistance bacterial isolates and antibiotic susceptibility patterns at our institution. This will aid in developing local antibiotics prescription guidelines, raising awareness of developing resistance and improve infection prevention practices and policies with the aim to contain antibiotic resistance. This will also encourage the use of microbiology diagnostic services, overall improving prognosis of infectious diseases at KCH.

I support this study and look forward to your favourable review.

Yours sincerely,

[redacted]

Dr. Jonathan Ngoma
HOSPITAL DIRECTOR

KAMUZU CENTRAL HOSPITAL
DIRECTOR
16 MAR 2015
P.O. BOX 149
LILONGWE

Appendix 5: Data collection form

MICROBIOLOGY ANTOBIOTIC RESISTANCE SURVEY QUESTIONNAIRE ABR NO:

*****Please record all information as at time and date of specimen collection*****

CONSULTANT NAME:_____ **E.mail:** _____

Patient Name				**DOB/Age:**		**Gender**	◯ Male ◯ Female
Ward		**Ward Type**		**Location/Area of residence**			
Date of Admission		**Date Spec collected**		**Attending Doctor/s**			

Specimen Source		**Date of Specimen Collection:**	
◯ CSF	◯ Blood culture	◯ Ascitic fluid	◯ Pleural fluid
◯ pus swabs	◯ endotracheal aspirate	◯throat swab	◯sinus aspirate
◯middle ear aspirate	◯stool	◯urine	◯ sputum
◯ secretions from skin lesions		Other (specify)	

Diagnosis			
◯respiratory tract infection	◯gastro-intestinal infection	◯urinary tract infection	◯skin infection
◯nosocomial infection	◯ Other (specify)		

Co-Morbidity					
◯HIV/AIDS	◯Diabetes	◯ Hypertension	◯Cardiac	◯Asthma	◯ Other (specify)

Wounds				
◯Surgical – clean	◯Surgical- contaminated	◯ Surgical - infected	◯Traumatic	◯Atrophic

Invasive Procedures				
◯Urinary catheter	◯IV line	◯Central venous/arterial line	◯Surgical	◯Intrathecal line
◯Endotracheal – ventilator		◯Endotracheal - tracheotomy tube	◯Nasogastric tube	◯Blood transfusion

Previous Hospitalization			
◯No	◯Yes - in the last week	◯Yes - in the last month	◯Yes - in the last 3 months
Reason for previous hospitalization: _____		Details of previous hospital/ward _____	

Previous Antibiotic Treatment (If yes indicate which antibiotic)			
◯No	◯Yes - in the last week	◯Yes - in the last 2 weeks	◯Yes - in the last 3 weeks

Current Antibiotic Treatment			
◯Antibiotic Name	◯Dose	◯Route	◯Duration

Duplicate Isolate Screen			
◯Same patient	◯Identical susceptibility results	◯Within 5 days	◯Same specimen source
◯ Different specimen source			

Facilities/ PPE			
Availability of hand washing facility (hand wash basin/sanitization gel)	◯Yes ◯ No	Use/Availability of Personal Protective Equipment by healthcare worker	◯Yes ◯ No

Discharge Details	
Date of Discharge:	**Duration of Hospitalization:**
Discharged on antibiotics : ◯Yes ◯ No	

Antibiotics received on discharge			
◯Antibiotic Name	◯Dose	◯Route	◯Duration

For comments and enquiries email: kchmicrolab@gmail.com **v01 27.04.16**