MOLECULAR PROFILE OF GRAM-NEGATIVE ESKAPE PATHOGENS FROM KOMFO ANOKYE TEACHING HOSPITAL IN GHANA

BY

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This is a thesis in which the chapters are written as a set of discrete research publications, with an overall introduction and final summary.

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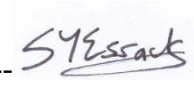
Signed: --  ------- Name: Prof Sabiha Y. Essack. Date: -17/09/2018---

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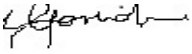
Signed: --  ---- Name: Dr Usha Govinden. Date: ----11/09/2018

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DEDICATION

This thesis is dedicated to my late grandparents, Nana Odei Ntow and Maafio Beatrice.

Thank you for your love and kindness

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Abstract

Gram-negative ESKAPE (*Enterococcus* spp., *Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter* spp) pathogens are a major healthcare concern globally due to their increasing multidrug resistance and ability to cause debilitating infections. Phenotypic and genotypic characteristics of multidrug resistant Gram-negative ESKAPE pathogens from Komfo Anokye Teaching Hospital in Ghana were investigated. Two hundred (200) clinical, non-duplicate Gram-negative bacterial pathogens were randomly selected from various human specimens routinely processed by the diagnostic microbiological laboratory in the hospital. Multidrug resistant (isolates resistant to at least one agent in three or more antibiotic class) isolates selected from each group of Gram-negative ESKAPE pathogens constituted the final sample. Identification and antibiotic susceptibility profiles were carried out using Vitek-2. Identity of isolates for whole genome sequencing was further confirmed by MALDI-TOF MS. Four *P. aeruginosa* and 10 *K. pneumoniae* were subjected to whole genome sequencing based on their extensively drug resistant profiles and resistance to third-generation cephalosporins respectively using Illumina MiSeq, after genomic DNA extraction using the NucliSens easyMAG®. Antibiotic resistance genes and plasmids were identified by mapping the sequence data to an online database using ResFinder and plasmidFinder respectively. MLST was also determined from the WGS data. The raw read sequences and assembled whole genome contigs have been deposited in GenBank under project number PRJNA411997. An average multidrug resistance of 89.5% was observed, ranging from 53.8% in *Enterobacter* spp to 100.0% in *Acinetobacter* spp and *P. aeruginosa.* Gram-negative ESKAPE bacteria constituted 48.5% (97) of the 200 isolates. *P. aeruginosa* (n=4) belonging to ST234 harboured *bla*$_{\text{DIM-1}}$, *bla*$_{\text{IMP-34}}$, *bla*$_{\text{OXA-10}}$, *bla*$_{\text{OXA-129}}$, *bla*$_{\text{OXA-50}}$, *bla*$_{\text{PAO}}$ *aadA1, aac4 aph(3')-IIb, fosA, sul1, dfrB5, catB7*, *arr-2* conferring resistance to β-lactams, aminoglycosides, fosfomycin, sulphonamides, trimethoprim

phenicols and rifampin respectively. *qnrVC* was detected in two of the four isolates. Both *bla*$_{\text{DIM-1}}$ and *bla*$_{\text{IMP-34}}$-like positive contigs showed identical DNA sequences and were linked to type 1 integron structures. *Bla*$_{\text{DIM-1}}$ was 100% identical to the *bla*$_{\text{DIM-1}}$ prototype gene, while *bla*$_{\text{IMP-34-like}}$ had two base pair (bp) differences T190C and C314G respectively compared to *bla*$_{\text{IMP-34,}}$ leading to one amino acid substitution in IMP-34-like indicating that, the gene may have independently evolved, perhaps due to selection pressure. Blast analysis did not reveal identical genetic structures deposited in NCBI, neither among the nucleotide collection, completed genomes nor among the completed plasmids. β-lactamases (*bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{SHV-11}}$, *bla*$_{\text{TEM-1B}}$) and resistance genes for aminoglycosides (*aac(3)-IIa-like,aph(3')-Ia*) quinolones/fluoroquinolones (*oqxA-like,oqxB-like,qnrB10-like,qnrB2*) and others including fosfomycin (*fosA*), trimethoprim (*dfrA14*), and sulphonamide (*sul2*) were found in the *K. pneumoniae* (n=10). Multiple and diverse mutations of the quinolone resistance-determining regions *gyrA*, *gyrB* and *parC* genes were detected in the *K. pneumoniae* (n=4), which were clonally distinct. The diversity of resistance genes expressed by Gram-negative ESKAPE pathogens conferring resistance to multiple antibiotics is problematic in a resource-constrained country like Ghana, necessitating urgent antibiotic stewardship and infection prevention and control interventions.

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Supplementary Material

A compact disc (CD) with the electronic copy of this thesis in portable digital format (PDF)

List of abbreviations

aac	aminoglycoside acetyltransferase
Bla	β-lactamase
BLAST	basic local alignment tool
CLSI	Clinical Laboratory Standards Institute
CMY	Cephamycinase
CPE	Carbapenemase-producing Enterobacteriaceae
CRE	Carbapenem-resistant Enterobacteriaceae
CTX-M	Cefotaximase-München
DHA	Dhahran AmpC β-lactamase
DNA	Deoxy-ribonucleic acid
EDTA	Ethylene Diamine Tetra Acetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
ESKAPE	*Enterococcus* spp., *Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter* spp.
ESBL	Extended Spectrum β-lactamase
GES	Guiana extended-spectrum
GIM	Guiana Imipenemase
HGT	Horizontal Gene Transfer
IMP	Imipenem metallo-β-lactamase
IS	insertion sequence
KATH	Komfo Anokye Teaching Hospital
KPC	*Klebsiella pneumoniae* carbapenemase

MALDI-TOF MS	Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry
MBL	Metallo-β-lactamase
MIC	Minimum Inhibitory Concentration
MLST	Multi-locus sequence typing
MDR	Multidrug resistant
NDM	New Delhi Metallo-β-lactamase
OXA	Oxacillinase
PCR	Polymerase Chain Reaction
PER	Pseudomonas extended resistant
SHV	sulfhydryl variable
SPM	Sao-Paolo Metallo-β-lactamase
SIM	Seoul Imipenemase
TEM	Temoneira
Tn	Transposon
ST	sequence type
VEB	Vietnam extended-spectrum β-lactamase
VGT	Vertical Gene Transfer
VIM	Verona Integron-Encoded Metallo- β-lactamase
WGS	Whole Genome Sequencing

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CHAPTER ONE

INTRODUCTION

The escalating rate of bacterial strains acquiring resistance to many clinically relevant antibiotics is currently a major global health concern. Antibiotic resistance has an impact on morbidity and mortality that is increasingly attributable to infections caused by multi-drug resistant bacteria worldwide (Bassetti & Righi, 2015; Brown & Wright, 2016). The mortalities associated with drug resistant infections are estimated to increase from the current 700 000 to about 10 million annually, and projected to cost the global economy as much as one hundred trillion US dollars by the next two decades, if effective measures are not put in place to combat resistance (O'Neill, 2014). The emergence and spread of antibiotic resistance is both a community and healthcare-associated burden, in the developed and developing world, with serious consequences for infection prevention and treatment (Carlet & Pittet, 2013; Spellberg & Gilbert, 2014; Ventola, 2015). Developing countries are considered as key role players, with the burden escalating particularly in sub-Saharan African countries such as Ghana, due to sub-optimal enforcement of antibiotic control policies and limited logistics for infection surveillance (Holloway *et al*., 2011; Reader, 2015).

Bacterial pathogens of particular concern and associated with outbreaks of multi-drug resistance include the 'ESKAPE' pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp), an acronym designated by the Infectious Diseases Society of America. ESKAPE pathogens are the leading cause of hospital-acquired infections globally (Navidinia, 2016;Pendleton, Gorman, & Gilmore, 2013; Rice, 2010; Santajit & Indrawattana, 2016). Infections caused by multi-drug resistant Gram-negative bacteria including those belonging to the ESKAPE group, are of

particular significance in both community and hospital settings worldwide, as they are extremely challenging to treat, leaving physicians with limited therapeutic options (Cerceo *et al*., 2016; Laxminarayan *et al.*, 2013; Rice, 2010). In Africa, several studies have indicated high antibiotic resistance among bacteria including Gram-negative ESKAPE pathogens to mainstay antibiotics (Dada-Adegbola & Muili, 2010; Hackman, 2015; Sonda *et al*., 2016) and resistance is rapidly spreading, particularly in sub-Saharan regions where resources are limited for surveillance and newly effective antibiotics tend to be unavailable or unaffordable (Baiden *et al*., 2010; Holloway, Mathai, & Gray, 2011; Jasovský *et al.*, 2016; Laxminarayan *et al*., 2016).

Clinical Gram-negative 'ESKAPE' (*K. pneumoniae*, *A. baumannii*, *P. aeruginosa* and *Enterobacter* spp) bacteria are associated with high morbidity and mortality and are particularly implicated in nosocomial infections (Flynn *et al.*, 2015;Paramythiotou & Routsi, 2016; Pendleton *et al*., 2013). These pathogens are reported to be increasingly resistant to all clinically available antibiotic classes including β-lactams (particularly carbapenems and the third- and fourth-generation cephalosporins), fluoroquinolones, aminoglycosides and to some extent polymixin B (colistin), often used as a last resort antibiotic (Navidinia, 2016; Lim *et al.*, 2011). These bacteria thus feature as critical or high priority pathogens in the World Health Organization's "Global Priority List of Antibiotic-Resistant Bacteria to Guide Research, Discovery and Development of New Antibiotics" (WHO, 2017).

The therapeutic options for infections caused by the multi-drug resistant pathogens are very limited. Colistin, an old drug with significant toxic side effects and for which there is lack of robust data to guide dosage regimens and duration of therapy, is the last resort antibiotic for treatment of infections caused by these resistant organisms (Gallardo-Godoy *et al.*, 2016;

Ozsurekci *et al.,* 2016). In addition, some multidrug-resistant Gram-negative isolates including *P. aeruginosa*, *A. baumanii, K. pneumoniae* and *Enterobacter* spp have developed resistance to colistin (Kaye *et al*., 2016). A study by Falagas and colleagues evaluating the effectiveness of antibiotic treatment commonly administered to patients with infections due to carbapenem-resistant Enterobacteriaceae reported increasing spread of resistance with mortality rates ranging from 26% to 46% (Falagas *et al.*, 2013). A review by Martirosov and Lodise (2016) on emerging trends in epidemiology and management of infections due to Enterobacteriaceae highlighted increasing resistance among Gram-negative bacteria. They indicated about 64% and 67% deaths in patients with extensively drug-resistant *Klebsiella* spp. and *Acinetobacter* spp. infections despite combination treatment with tigecycline-colistin and carbapenem-colistin respectively (Martirosov & Lodise, 2016). Extensively drug-resistant (XDR) strains of *Acinetobacter* spp. and *P. aeruginosa* have also been implicated in resistance to colistin (Lim *et al.*, 2011;Bae *et al*., 2016; Tseng *et al*., 2016).

A number of studies in Ghana on antibiotic resistance among Gram-negative bacteria including some ESKAPE pathogens have been undertaken in hospitals, but many of the studies were centred at Korle-Bu Teaching Hospital in the Greater Accra region of the country (Hackman *et al.*, 2014; Obeng-Nkrumah *et al*., 2013). Apart from the research being in the form of point prevalence studies, it has also focused mainly on *P. aeruginosa* and *K. pneumoniae* describing resistance phenotypes in the main. Two such examples are the 'phenotypic determination and antimicrobial resistance profile of extended spectrum β-lactamases (ESBLs) in *E. coli* and *K. pneumoniae'* and 'antimicrobial sensitivity pattern of urine isolates from large Ghanaian hospital by Hackman *et al.* (2013) and Odonkor *et al.* (2011) respectively. Literature on the prevalence and underlying molecular epidemiology of antibiotic resistance is sparse. To date, there are no

published studies on the resistance genotypes of ESKAPE pathogens from Komfo Anokye Teaching Hospital, the only referral and tertiary care hospital in the Ashanti region of Ghana. This study is thus necessary to expound the underlying molecular mechanisms of resistance of Gram-negative ESKAPE pathogens in this setting in order to inform strategies for their containment. The results of this study in Ghana are expected to provide information to guide policy makers on rational antibiotic use, infection prevention and control measures to combat antibiotic resistance in the country and the sub region. They are additionally expected to yield evidence to inform treatment guidelines and associated essential medicines lists.

1.2 LITERATURE REVIEW

1.2.1 Advent of Antibiotics and Resistance

The introduction of antibiotics modernized the treatment of bacterial infections. The penicillins and sulphonamides were the early antimicrobial drugs to be discovered and developed between 1920s and 1930s (Aminov, 2017; Marinho *et al.*, 2016). Decades of intense antibiotic drug research and technological advances led to the discovery of many new antibiotic classes and derivatives of antibiotics among which were the aminoglycosides, tetracyclines, cephalosporins, macrolides, glycopeptides, quinolones and carbapenems. These antibiotics became a "wonder weapon" for the prevention and treatment of bacterial infections which reduced mortality with a marked shift in patient care (Marinho *et al*., 2016; Wright, 2014). Among the molecules that were successful against a wide-range of bacterial pathogens were the β-lactams, aminoglycosides and fluoroquinolones. The emergence and spread of resistance to antibiotics due to abuse or misuse has led to a loss in clinical efficacy (Qin, Panunzio, & Biondi, 2014). The high cost of discovering novel compounds or chemicals with bacterial selectivity, the increased regulatory requirements for safety and efficacy with low tax incentives for pharmaceutical companies and the fact that similar investments made in the development of non-anti-infective drugs could yield higher financial returns are major setbacks to the development of new antibiotics (Boucher *et al*., 2013; Fernandes, 2015). Thus, evolution of resistance and the waning interest in the development of new efficacious antibiotics have resulted in bacterial resistance becoming a global public health concern.

1.2.2 Mechanisms of Action of antibiotics

The mode of action of antibiotics involves four major mechanisms including interference of bacterial cell wall biosynthesis, inhibition of protein synthesis, inhibition of nucleic acid

metabolism and repair, and, disruption of membrane structure or permeability of the bacteria (Zhou *et al*., 2015). The enzymes mainly targeted for inhibition in the bacterial cell include transpeptidases, transglycosylases, topoisomerases, ribonucleic acid (RNA) polymerase and peptidyl transferases. A major component of bacteria cell wall is peptidoglycan layer which provides integrity to the cell structure. β-lactam antibiotics such as penicillins, cephalosporins, carbapenems, and monobactams interfere with the cell wall biosynthesis by inhibiting the penicillin-binding proteins or transpeptidase enzymes involved in assembly and cross-wall linking of the peptidoglycan (Giedraitienė *et al.*, 2011; Sauvage & Terrak, 2016). These agents bind to the acyl-D-alanyl-D-alanine amino acid thereby inhibiting the addition of new units to the peptidoglycan, thus triggering murein hydrolases or transpeptidases to lyse the cell (Lupoli *et al*., 2011; Münch *et al*., 2015). The glycopeptides vancomycin and teicoplanin are related to the β-lactams that interfere at different stages in cell wall synthesis. These antibiotics bind to peptidoglycan units and interfere with the transglycosylase and transpeptidase activity in the growing bacteria cell leading to cell inhibition or death (Kahne *et al.*, 2005).

Protein synthesis is a vital metabolic process necessary for the replication and survival of all bacterial cells. Aminoglycosides, tetracyclines, macrolides and chloramphenicol are examples of antibiotic classes that inhibit protein synthesis by binding to either 30S or 50S units of the intracellular ribosome. Tetracycline and aminocyclitol (aminoglycosides and spectinomycin) classes of antibiotics are 30S ribosome inhibitors that prevent the entry of aminoacyl tRNA to the ribosome or bind to 16S rRNA component of the 30S ribosome units in protein synthesis (Chellat, Raguž, & Riedl, 2016). Other classes including lincosamides, streptogramins, amphenicol and oxazolidinones also inhibit the 50S ribosome. These antibiotics inhibit either initiation of protein translations or translocation of peptidyl-tRNAs thus preventing the

elongation reaction of the peptide chain by peptidyl-transferase. By mechanisms of action, these interfere with the normal cellular metabolism of the bacteria, leading to the inhibition of growth or death of the organism (Giedraitienė *et al*., 2011; Huang, Zhu, & Melançon, 2015).

Deoxyribonucleic acid (DNA) replication and repair are crucial to any cell survival including bacteria. Antibiotics such as the quinolones, fluoroquinolones and sulphonamides inhibit enzymes involved in the DNA or RNA synthesis. For instance DNA gyrase (DNA type II topoisomerase) and RNA polymerase are inhibited by quinolones and rifampicin respectively, by preventing the supercoiling of DNA which interferes with the cellular processes of DNA synthesis, thereby compromising bacterial cell replication and survival (Giedraitienė *et al.*, 2011; Zhou *et al*., 2015). Polymixin B or colistin, a cyclic peptide antibiotic disrupts the bacterial cell membrane by interacting with phospholipids thereby increasing the permeability of the membrane causing the cell to uptake excess water to induce bacteria death (Velkov, Roberts, Nation, Thompson, & Li, 2013).

1.2.3 Causes of bacterial resistance to antibiotics

Antibiotics have been effective in curing bacterial infections over decades since their introduction. However, misuse for both medical and non-medical purposes, such as failure to complete drug courses or overuse by infected individuals as well as the extensive usage in animals for therapeutic purposes and as growth promoters, have led to resistance in many clinical bacteria strains by selection pressure (Cabello *et al*., 2013; Caruso, 2016; Kiguba, Karamagi, & Bird, 2016). Consequently, common antibiotics that were previously used to treat infections are no longer efficacious. The resistance to currently available antibiotics has led to poor treatment outcomes of bacterial infections especially those caused by the Gram-negative bacterial pathogens, in many parts of the world particularly the developing countries including Ghana

(Hackman, Brown, & Twum-Danso, 2014; Kiguba *et al.*, 2016; Opintan *et al*., 2015). A review of the clinical relevance of ESKAPE pathogens by Pendleton and co-workers (2013) indicated that resistant Gram-negative ESKAPE bacteria account for about 15.5% of all bacterial infections in Africa compared to 5.0% and 7.1% in America and Europe respectively (Pendleton *et al.*, 2013). The high prevalence of antibiotic resistance in Africa, is largely due to indiscriminate and inappropriate use of antibiotics in healthcare settings and communities (Donkor *et al.*, 2012; Kiguba *et al*., 2016).

The indiscriminate use of antibiotics to treat any infection, whether severe or minor, and for symptoms in many cases that may not be caused by bacteria have contributed immensely to the emergence of resistance (Read & Woods, 2014). In healthcare settings, clinicians are often faced with a challenge to address an immediate need of patients, especially in critical conditions for treatment, over the consideration of concepts of antibiotic resistance. Particularly, in the out-patient setting, patients' expectations for antibiotics has been reported as a major driving factor for over-prescription by clinicians (Huttner *et al*., 2013). Commentary from the 2013 world healthcare-associated infections forum and a non-systematic review on antibiotics stewardship in the intensive care unit have indicated that, about 30% to 50% of antibiotics prescribed by clinicians are inappropriate and 30% to 60% are unnecessarily prescribed in the ICU (Huttner *et al.*, 2013; Luyt *et al.*, 2014; Ventola, 2015).

In many parts of the world, self-medication with antibiotics typically occurs outside the health care system with over 50% purchased and used over-the-counter (Ocan *et al*., 2015). This is particularly serious in the developing countries, including sub-Saharan Africa, where inadequacies in healthcare systems such as poor enforcement of legislation policy on antibiotics restrictions, have resulted in easy availability of antibiotics without prescriptions (Esimone,

Nworu, & Udeogaranya, 2007; Michael, Dominey-Howes, & Labbate, 2014; Ocan *et al.*, 2015). This has resulted in many cases in the abuse of antibiotics such as incomplete treatment course and inadequate dosing (Ocan *et al.*, 2015), which are major driving factors for evolution of resistance.

The frequency of abuse and inappropriate use of antibacterial agents was reported by Vialle-Valentin et al. (2012) in their study on predictors of antibiotic use in African communities, evidence from medicines household survey in five countries including Gambia, Nigeria, Ghana, Kenya and Uganda. The study revealed that of the 95% of individuals with acute illness who took medicines, 90% sought healthcare outside homes, with 36% prescribed antibiotics. Of the 36% antibiotic prescriptions, 31.7% were either given antibiotics based on prescriber's experience or discretion guided by clinical presentation or from untrained personnel (Vialle, Valentin, Lecates, Zhang, Desta, & Ross, Degnan, 2012). Many healthcare facilities in sub Saharan region, have antibiotic regimen of first-line drugs, mostly limited to ampicillin, chloramphenicol, erythromycin, gentamicin, penicillin, tetracycline and trimethoprim-sulfamethoxazole with second-line antibiotics usually consisting of amikacin, amoxicillin-clavulanic acid, cefuroxime, ciprofloxacin and nalidixic acid in accordance with their clinical guidelines (Howard *et al*., 2015; Mathur, 2016). These treatment protocols are often used without adequate microbiological investigations to guide the treatment of infections, usually resulting in inappropriate antibiotic choice (Ab-Rahman, Teng, & Sivasampu, 2016; Ocan *et al*., 2015) and consequently leading to high selection pressure favouring antibiotic resistance.

Antibiotics are also widely used in livestock to treat or prevent infections as well as in animal feed stock as growth supplements in both developed and developing countries (Gross, 2013; Spellberg & Gilbert, 2014; Ventola, 2015). It is estimated that 25-50% of all antibiotics used in

the US are for non-curative purposes such as metaphylaxis, prophylaxis and growth promotion in livestock (Fair & Tor, 2014). The unrestrained use has exacerbated the spread of resistance as it creates a reservoir of bacteria that become resistant, and can be transmitted to humans through the food chain (Ventola, 2015). For example, Gram-negative ESKAPE bacterium such as *A. baumanni* has been implicated in the dissemination of clinically important resistance determinants in poultry (Wilharm *et al*., 2017). Also a study on antibiotic resistance and phylogenetic characterisation of *A. baumannii* isolates from commercial raw meat in Switzerland reported contamination with *A. baumannii* in 48% of poultry meat and 25% of raw retailed meat overall , thus serving as a reservoir of resistance (Lupo *et al*., 2014). In China, *E. coli* clones, harbouring a plasmid mediated *mcr-1* colistin resistance gene, were isolated from both animals (swine) and humans (Liu *et al.*, 2016). Further, it has also been reported that, about 90% of antibiotics used in livestock are excreted in urine and faeces, and widely spread into the environment through surface runoff and water bodies (Bartlett, Gilbert, & Spellberg, 2013). This affects the environmental microbiome (Bartlett *et al.*, 2013) where bacteria are exposed to sub inhibitory or sub lethal doses of antibiotics, the susceptible strains are killed, leaving the resistant ones to thrive by natural selection (Chee-Sanford *et al*., 2009). A study in the UK indicated substantial levels of resistance genes to clinically relevant antibiotics including tetracyclines, sulfonamides and trimethoprim isolated from water bodies that are fed with runoff effluents from livestock farms where antibiotics were used (Rowe *et al.*, 2016). The resistance gene (s) is/are passed on to the daughter cells by vertical gene transfer or disseminated among different bacteria species on mobile genetic elements such as plasmids, transposons or integrons, by horizontal gene transfer (Read & Woods, 2014), and ultimately multiply to form a population that becomes completely resistant to the agent.

Bacterial resistance to multiple antibiotic classes is currently a common phenomenon in sub-Saharan region including Ghana, in both community and hospital settings mainly due to inappropriate use of antibiotics and increasing spread due to lack of strict infection prevention and control and hygiene and sanitation measures (Obeng-Nkrumah *et al*., 2013; Oduro-Mensah *et al.*, 2016). Although the growing public health crisis of antibiotic resistance seems overwhelming, it is an issue that has long been anticipated and many key elements for its effective management have already been identified. However development of a holistic approach, by incorporating especially intense research into the underlying molecular mechanisms or factors involved in the resistance is crucial.

1.2.4 Resistance Mechanisms of Gram-negative ESKAPE pathogens

The Gram-negative bacteria of the 'ESKAPE' group have particularly emerged as multidrug-resistant and implicated commonly in hospital-associated infections globally (Navidinia, 2016; Santajit & Indrawattana, 2016). Similar to other bacteria, these Gram-negative pathogens have evolved with several mechanisms of resistance to overcome the effects of antibiotics in their surrounding environment. These include the production of hydrolytic enzymes such as ESBLs and carbapenemases to modify the chemical composition of the antibiotics (β-lactams), rendering the agents ineffective (Wilson, 2014). β-lactamases capable of hydrolysing the penicillins as well as the first-, second- and third generation cephalosporins and aztreonam are the extended spectrum β-lactamases (ESBLs) (Maina, Revathi, & Whitelaw, 2017; Sutton, 2014). The production of ESBLs and carbapenemase hydrolytic enzymes among the Gram-negative bacteria has especially become a public health threat, as few antibiotics are effective against them. Other mechanisms involve the overexpression of efflux proteins pumps that extrude or regulate the concentration of antibiotics such as fluoroquinolones, macrolide-lincosamide-streptogramin (MLS), aminoglycosides and tetracyclines from or within the cell and alterations in outer membrane proteins (porins) to decreased uptake or cell wall permeability to antibiotics. In multi-drug resistant bacteria,

antibiotic resistance genes may be mobilized on plasmids, transposons or integrons. Antibiotic resistance gene(s) may be transferred by conjugation, transformation or transduction to another bacterial species (Jansen, Barbosa, & Schulenburg, 2013; Karisetty *et al*., 2013; Seitz & Blokesch, 2013). During conjugation, plasmids carrying resistance are transferred to other bacteria by cell-to-cell transfer using pili (Ali, 2018; Davies & Davies, 2010). Bacteria also acquire resistance by transduction via mediation of bacteriophages or transformation in which naked DNA is acquired from the environment. The acquisition of resistance via conjugation, transformation and transduction together accounts for more mechanisms of resistance than mutations or natural selection (Chee-Sanford et al., 2009; Davies & Davies, 2010). Bacteria express different mechanisms of resistance to antibiotics, however, in Gram-negative bacteria including members of ESKAPE pathogens, production of β-lactamases that are hydrolytic enzymes are most widely described (Bush, 2013; Soto, 2013; Navidinia, 2016; Santajit & Indrawattana, 2016).

1.2.4.1 β-Lactamases

β-lactamases are enzymes produced by bacteria that mediate resistance to β-lactam antibiotics such as penicillins, cephalosporins and carbapenems which are among the safest and commonly prescribed antibiotics worldwide (Sekyere, Govinden, & Essack, 2016). The production of β-lactamases is the most common and important mechanism of resistance in Gram-negative bacteria. The β-lactamase genes may be encoded chromosomally or by a diversity of mobile genetic elements (plasmid-mediated) such as integrons and transposons (Bush, 2010; Poirel, Naas, & Nordmann, 2010). The enzymes catalyse the inactivation of the antibiotics by splitting the amide bond of the β-lactam's ring (acetylation reaction) rendering it ineffective in the bacteria cell (King, Sobhanifar, & Strynadka, 2017; Thenmozhi *et al.*, 2014)..

- **Classification**

The β-lactamases are commonly classified by molecular structure (Ambler classification) or functional properties (Bush-Jacoby-Medeiros classification) based on the hydrolysis and inhibition characteristics of the enzyme (Bush & Jacoby, 2010). Though, the functional classification helps to relate the diverse enzymes to their clinical role, it can be more subjective compared to the structural classification which is easier and less complicated. Notwithstanding, the molecular or: Ambler classification is widely used currently due to an increasing molecular analysis of enzymes (Bush & Jacoby, 2010). The Ambler or structural classification based on amino acid sequence and hydrolytic activity is further grouped into four molecular classes A, C and D, which are serine β-lactamases and utilise serine for β-lactam hydrolysis whereas class B are metallo-β-lactamases which require divalent zinc ions for hydrolytic activity (Jeon *et al.*, 2015).

- ***Class A***

Group A consists of penicillinases, cephalosporinases, extended-spectrum β-lactamases (ESBLs) and carbapenemases including *Klebsiella pneumoniae* carbapenemase (KPC) which hydrolyses penicillins, cephalosporins as well as carbapenems (Jasper *et al*., 2015; Jeon *et al.*, 2015; Miller & Humphries, 2016; Partridge, 2015). Carbapenemases comprise chromosomal (IMI-1, NmcA, SFC-1, SME-1) and plasmid encoded (KPC-2, GES, IMI-2, derivatives) carbapenemase genes and are able to hydrolyse all β-lactams including monobactams (aztreonam) but are inhibited or partially inhibited by β-lactamase inhibitors such as sulbactam, tazobactam, or clavulanic acid but not ethylene diamine tetra acetic acid [EDTA] (Giedraitienė *et al*., 2011; Rice, 2010). ESBLs have emerged as a significant cause of antibiotic resistance in Gram-negative bacteria. Apart from resistance to penicillins, cephalosporins and aztreonam, ESBL-producing bacteria are

also associated with resistance to other classes of non-β-lactams including aminoglycosides, trimethoprim sulfamethoxazole, fluoroquinolones as well as β-lactams/lactamase-inhibitor combinations (Thenmozhi *et al.*, 2014). ESBLs consist of three major classes including Temoneira (TEM), sulfhydryl variable (SHV) and Cefotaximase-München (CTX-M) types. Widely disseminated among Enterobacteriaceae including *K. pneumoniae, Enterobacter* spp and in non-fermenting bacteria such as *P. aeruginosa* are TEM-1, TEM-2 and SHV-1 type derivatives, which confer resistance to penicillins but not broad-spectrum cephalosporins, and are composed of single or multiple code gene mutations (Thenmozhi *et al.*, 2014). The high mutation dynamism of TEM and SHV encoded genes have resulted in high level of diversity in enzyme types thus increasing spread of antibiotic resistance (Džidić, Šušković, & Kos, 2008). Among the Gram-negative ESKAPE pathogens, CTX-Ms have been commonly reported (Zhao & Hu, 2013).

A non-systematic review by Storberg (2014) on ESBL-producing Enterobacteriaceae in Africa, reported 10% to 40% and 10% to 96% ESBL-producing isolates sampled from hospital and community settings respectively. The study further indicated CTX-M-15, TEM-1 and SHV-1 type derivatives commonly among Gram-negative bacteria including *K. pneumoniae, Enterobacter* spp. and *P. aeruginosa* of ESKAPE pathogens. Several studies have also been conducted in sub Saharan African countries, including the prevalence and multi-drug resistance from community-acquired infections in Nigeria (Adenipekun *et al*., 2016) and extended-spectrum β-lactamase producing Enterobacteriaceae among clinical isolates in Burkina Faso (Ouedraogo *et al*., 2016). Reports on resistance patterns of ESBL-producing *Klebsiella* and *E. coli* isolates in tertiary hospitals in Ghana (Feglo & Adu-Sarkodie, 2016; Hackman *et al*., 2013), have all indicated high prevalence of ESBLs, posing challenges to antibiotic therapy.

- ***Class B***

Group B or metallo-β-lactamases (MBLs) exhibit broad spectrum hydrolytic activity with ability to virtually hydrolyse all classes of β-lactam antibiotics including expanded-spectrum cephalosporins and carbapenems but not aztreonam. The enzymes are however inhibited by dipicolinic acid and EDTA but not β-lactamase inhibitors (Mohamed & Al-Ahmady, 2015). The MBLs commonly consist of imipenemase metallo-β-lactamases (IMP), German imipenemase (GIM), Seoul imipenemase (SIM), Verona integron encoded metallo-β-lactamases (VIM), and the New Delhi metallo-β-lactamase-1 (NDM-1) enzymes with encoding genes located on the plasmid or transposons and therefore easily disseminated among the bacteria (Dahiya *et al.*, 2015; Rice, 2010). The IMP-type MBLs have widely been described in Gram-negative ESKAPE pathogens, whiles VIM-type enzymes have been commonly detected in *P. aeruginosa* and *A. baumannii*. The NDM-1-type enzymes have been widely isolated from *K. pneumoniae* and *Enterobacter* spp. (Kim *et al.*, 2016; Yong *et al*., 2009).

A number of studies conducted in Africa have reported on IMP-, VIM-, and NDM-type as common metallo-β-lactamases among Enterobacteriaceae including some Gram-negative ESKAPE bacteria mainly *K. pneumoniae, P. aeruginosa* and *A, baumanii* as shown in Table 1.

Table 1 showing studies reporting on metallo-β-lactamases among Gram-negative ESKAPE bacteria in Africa

Country of isolation	Organism(s)	MBL-type(s)	Reference
Algeria	*P. aeruginosa*	VIM-4	Mellouk *et al*., 2017
	A. baumannii	NDM-1	
	K. pneumoniae	VIM-19	Rodriguez-Martinez *et al.*, 2010
Ethiopia	*A. baumannii*	NDM-1	Pritsch *et al.*, 2017
Nigeria	*K. pneumoniae*	NDM-1	Ogbolu & Webber, 2014
Tanzania	*A. baumannii*		
	P. aeruginosa	IMP, VIM	Mushi *et al.*, 2014
	K. pneumoniae		
Morocco	*K. pneumoniae*	NDM-1, VIM-1	Barguigua *et al*., 2013
	K. pneumoniae	IMP-1	Barguigua *et al*., 2012
	E. cloacae	IMP-1	
South Africa	*E. cloacae*	NDM-1	Govind *et al*., 2013
	K.pneumoniae	IMP, VIM	Sekyere, Govinden, & Essack, 2016
	K.pneumoniae	VIM-1	Peirano *et al*., 2012
Kenya	*K. pneumoniae*	NDM-1	Poirel *et al.*, 2011
Tunisia	*P. aeruginosa*	VIM-2	Mansour *et al*., 2009
Sierra Leone	*E. cloacae*	VIM, DIM-1	Leski *et al*., 2013
	K. pneumoniae	VIM	
Egypt	*A. baumanni*	VIM	Fouad *et al.*, 2013
	P. aeruginosa	VIM-2, NDM, IMP	Zafer *at al*., 2014

In Ghana, there is no published study on MBL production among Gram-negative ESKAPE pathogen, however the first MBL (VIM-2) with TniC-transposons in *P. aeruginosa* identified in Norway, was isolated from a transferred patient after protracted hospitalization in Ghana (Samuelsen *et al.*, 2009). The strain is likely to have imported from Ghana, suggesting MBL production among some Gram-negative ESKAPE pathogens in the country.

- ***Class C***

Group C comprises of penicillinases and cephalosporinases such as AmpC β-lactamase which exhibit higher hydrolytic activity against early cephalosporins than benzylpenicillin (Jeon *et al*., 2015). These enzymes are chromosomally encoded and commonly consist of ACT-1, FOX-1, CMY-2, CMY-10, CMY-19, CMY-37, MIR-1, GC1and DHA that are predominantly expressed by *P. aeruginosa* and Enterobacteriaceae, particularly in *Enterobacter* spp (Peymani *et al*., 2016). The AmpC β-lactamases are distinct from ESBLs, hydrolyse aztreonam, all penicillins and most cephalosporins (cephamycins and oxyimino-β-lactams). The enzymes are commonly resistant to inhibition by EDTA and most β-lactamases inhibitors except avibactam, a current non-β-lactam β-lactamase inhibitor (Bush & Jacoby, 2010). The AmpC expression in many Gram-negative bacteria including *Enterobacter* spp. and *P. aeruginosa* is low, but inducible when exposed to certain β-lactams such as ampicillin, amoxicillin, imipenem and clavulanate inhibitors (Jacoby, 2009). Carbapenems are mainly stable to AmpC β-lactamases (Thenmozhi *et al*., 2014), however in some bacteria including *A. baumanii*, one or more components of the induction system are lost, and hyper-production of the enzymes with reduced β-lactam accumulation, inactivate carbapenems, particularly ertapenem (Bush & Jacoby, 2010). In *P. aeruginosa* hyper-expression of the intrinsically occurring AmpC confers resistance to extended-spectrum cephalosporins such as ceftazidime (Rodríguez-Martínez, Poirel, & Nordmann, 2009). In Ghana, reports on AmpC β-lactamases among Gram-negative ESKAPE bacteria are sparse, however high prevalence of AmpC production in *P. aeruginosa* has been detected in a teaching hospital resulting in high resistance to commonly used β-lactam antibiotics (Feglo & Opoku, 2014).

- ***Class D***

The group D or oxacillinases (OXA-48) type carbapenemases have a higher hydrolysis affinity for cloxacillin or oxacillin than other penicillins (Sekyere, Govinden, & Essack, 2016). The enzymes are plasmid encoded consisting of OXA-11 and OXA-15 as significant members, and exhibit ESBL activities which are commonly detected in *P. aeruginosa* (Bakthavatchalam, Anandan, & Veeraraghavan, 2016). The enzymes are variably affected by β-lactamase inhibitors, but not inhibited by EDTA (Bush & Jacoby, 2010). The Bush-Jacoby scheme classified the OXA enzymes as group 2d, which are all resistant to β-lactamase inhibitors except OXA-18 (Bakthavatchalam, Anandan, & Veeraraghavan, 2016; Džidić *et al.*, 2008; Sgrignani, Grazioso, & De Amici, 2016), while OXA-17 confers high resistance to cefotaxime and cefepime (Thenmozhi *et al*., 2014). OXA carbapenemases tend to have low catalytic activity towards penicillins and cephalosporins, but together with porin mutation and other resistance mechanisms in *A. baumanii* can provide resistance to almost all antibiotics including carbapenems (Thenmozhi *et al.*, 2014). In Africa, production of oxacillinases (OXA-23) have been commonly identified in *A. baumanii* in studies from Senegal, Libya, South Africa, Tunisia and Nigeria (Mathlouthi et al., 2017; Mugnier, Poirel, Naas & Nordmann, 2010; Olaitan et al., 2013). In Nigeria, detection of OXA-10 conferring resistance to carbapenems and most broad--spectrum β-lactam antibiotics in clinical isolates of *P. aeruginosa* from various clinical specimens has been reported (Odumosu, Adeniyi, & Chandra, 2016).

1.2.4.2 Efflux pumps

The efflux pumps are transport proteins that control the concentration of antibiotics within the bacteria or export the drug molecules from within the cells into the external environment. In antibiotic-resistant Gram-negative bacteria, the membrane proteins function as exporters or

efflux pumps that expel a broad array of antibiotics from the cell, thus contributing to multiple drug resistance. The efflux pumps are a key mechanism employed by Gram-negative bacteria against fluoroquinolones, macrolide-lincosamide-streptogramin (MLS), aminoglycosides and tetracyclines conferring multiple resistance to various antibiotics across the bacterial species (Lau, Hughes, & Poole, 2014; Nikaido & Pagès, 2012). The efflux pump enzymes may be chromosomal or plasmid encoded and generally grouped into two classes, the Adenosine Triphosphate (ATP) binding cassette (ABC) and secondary multidrug transporters based on the source of energy required for the transport. While the ABC-type utilize energy resulting from ATP hydrolysis, secondary transporters require membrane energy in the form of the proton motive force (Fernández & Hancock, 2012).

Secondary multidrug transporters are widely described, and subdivided into four super groups, including the small multi-drug resistance, the major facilitator, the resistance nodulation-division (RND) and multi-drug and toxic compound extrusion family based on primary homology and secondary structures (Sun, Deng, & Yan, 2014). The poly-selective efflux pump, belonging to the RND super family is mainly responsible for multidrug resistance in Gram-negative bacteria. This pump exports wide range of antibiotics and other chemicals commonly used in the practice of medicine (Nikaido & Pagès, 2012). The expression of chromosomal encoded AcrAB-TolC and MexAB-OprM efflux pumps of RND super family in especially *Enterobacter, P. aeruginosa* and *K. pneumoniae* confers resistance to fluoroquinolones and also enhances bacterial survival against other toxic compounds (Kocsis & Szabó, 2013). *mexAB-oprM* and *mexCD-oprJ* encoded efflux pumps are widely characterized and mainly associated with resistance to carbapenems, fluoroquinolones and aminoglycosides, commonly identified in *P. aeruginosa* (Vaez *et al.,* 2014). The expression of efflux pump *oqxAB* encoded by the *oqxA* and *oqxB* genes is commonly

identified in ESBL-producing *K. pneumoniae* as both chromosomal and plasmid borne contribute to reduce activity of quinolones and fluoroquinolones (Rodríguez-Martínez et al., 2012). The expression of RND-type efflux pumps AdeABC, AdeDE, AdeFGH, and AdeIJK among Gram-negative members of ESKAPE, like in other Gram-negative bacteria confers resistance to tetracyclines, fluoroquinolones, aminoglycosides, erythromycin and chloramphenicol (Nikaido & Pagès, 2012).

1.2.4.3 Porin Alteration or Reduced Permeability

The outer membrane of Gram-negative bacteria is an important barrier, protecting the cell against damage by toxic compounds. It contains several specific β-barrel protein channels called 'porins' that are regulated to confer resistance to toxic compounds including antibiotics, especially the β-lactams. The porins are classified into several groups including, the general porins, which are responsible for determining the permeability barrier, the specific porins involved in an uptake of specific compounds and the iron-regulated porins for engagement of cytoplasmic membrane energization system for the uptake of special iron complexes with bacterial siderophores (Fernández & Hancock, 2012). In Gram-negative bacteria, multi-component pumps combined with a periplasmic membrane fusion (synthesis) protein (MFP) and outer membrane protein (OMP) components transfer substrates across the cell envelope. Hydrophobic antibiotics including chloramphenicol and aminoglycosides diffuse into the cell through the lipid components of the outer membrane while hydrophilic antibiotics such as β-lactams pass through water-filled channel (selective porins) in the outer membrane proteins. The resistance to antibiotics is acquired through the alteration of the barrier by changing the hydrophobic properties of the membrane by either reduced permeability or loss of porins (Miller, 2016). The reduction of OMP channels or loss of porins is as a result of mutations due to the

expression of *oprD, carO* and *ompF* in *P. aeruginosa, Acinetobacter* and *Enterobacter* spp respectively conferring resistance to β-lactams such as imipenem and meropenem (Sun *et al*., 2014). In *K. pneumoniae* loss or reduced expression of the major porins, o*mpK35* and *ompK36* together with other β-lactamases has been reported to confer resistance to fluoroquinolones, chloramphenicol and β-lactams including cephalosporins and carbapenems (Doumith *et al.*, 2009; Sun *et al*., 2014).

1.2.5 Clinical importance of Gram-negative ESKAPE pathogens

1.2.5.1 *Klebsiella pneumoniae*

K. pneumoniae is a Gram-negative, non-motile, oxidase negative and encapsulated bacterium belonging to the family Enterobacteriaceae. The polysaccharide capsule is important for pathogenicity and virulence determination that protects the bacterium from phagocytosis as well as the host's antibodies. The bacterium is a nosocomial pathogen that causes various forms of infections such as pneumonia, blood stream infection or sepsis, urinary tract infections (UTIs), wound infections and gastro-intestinal infections in humans, with increasing display of antibiotic resistance which usually result in prolonged hospitalization and high mortality (Shon, Bajwa, & Russo, 2013; Viale *et al.*, 2013). The pathogen is a major cause of community-acquired infections such as pneumonia and meningitis in infants, elderly and immuno-compromised patients resulting in high mortality (Giovane & Brooks, 2015; Nordmann & Poirel, 2014; Russo *et al.*, 2011). It is among the Gram-negative bacteria that are commonly involved in central line-associated bloodstream infections, catheter-associated urinary tract infections, ventilator-associated pneumonia and surgical site infections commonly reported in many hospitals in developing countries such as India (Mathur *et al*., 2016) and Egypt (See *et al*., 2013). The therapeutic choice for treatment of their infections include cephalosporins, amino and carboxy-

penicillins as well as monobactams or combination therapy with β-lactamases inhibitors such as clavulanate or tazobactam depending on the bacterial susceptibility and patient risk profile (Breurec *et al*., 2013; Vaara, 2010).

The mechanism of resistance employed by *K. pneumoniae,* like other bacteria mainly include antibiotic target modification, reduced cell permeability, increased efflux activity and enzymatic deactivation (Kumar *et al*., 2011). However enzymatic deactivation mediated by β-lactamases is widely reported. Most significant enzymes include ESBLs and carbapenemases hydrolysing penicillins, cephalosporins and carbapenems (King *et al*., 2017; Thenmozhi *et al.*, 2014). The ESBLs are commonly identified as transmissible β-lactamases in the pathogen and inhibited by clavulanic acid, tazobactam or sulbactam (Shaikh *et al.*, 2015; Swain & Padhy, 2016). The emergence of the New Delhi metallo-β-lactamase-1 'super-enzyme' in *K. pneumoniae* confers resistance to carbapenems and other broad spectrum β-lactam antibiotics, posing a challenge to β-lactam chemotherapy (Baraniak *et al*., 2016). The enzymatic inactivation of DNA gyrase or topoisomerase (IV) encoded by *gyrA* and *parC* genes in the bacterium combined with the expression of efflux pumps confer resistance to fluoroquinolones and quinolones (Guillard *et al*., 2015).

In sub-Saharan regions including Ghana, several studies have indicated high resistance in *K. pneumoniae* to commonly used antibiotics in health care practice. These include *inter alia* Hackman and co-workers in their studies on phenotypic determination and antibiotic resistance profile of extended spectrum β-lactamases among some bacterial pathogens, which recorded high resistance in *K. pneumoniae* to most commonly used antibiotics including penicillins, cephalosporins and some non-β-lactam antibiotics in Ghana (Hackman, Brown, & Twum-Danso,

2014). The high prevalence of antibiotic resistance was reiterated by other studies including Opintan et al. (2015), Feglo and Adu-Sarkodie (2016), indicating widespread antibiotic resistance in the bacterium which has consequently become a serious public health concern.

1.2.5.2 *Acinetobacter baumannii*

A. baumanii is non-fermentative Gram-negative coccobacillus that is strictly aerobic, catalase-positive and oxidase-negative. It is a short rod non-motile opportunistic pathogen, mostly isolated from intensive care units and surgical wards in hospitals, where widespread use of antibiotics has enabled selection for resistance (Behnia *et al.*, 2014; Higgins *et al*., 2013). They are ubiquitous microbes and grow across a range of temperatures, pH and nutrient levels, making them highly adaptable for survival in both human and environmental vectors (Al Atrouni *et al*., 2016; Vila, Martí, & Sánchez-Céspedes, 2007).

The bacterium is commonly implicated in various forms of severe hospital-acquired infections including bacteraemia, UTIs, post-neurosurgical meningitis, wounds and burn infections and most importantly ventilator-associated pneumonia, particularly in immune compromised patients in ICUs (Abdallah *et al*., 2015; Al Mobarak *et al.*, 2014; Chiang *et al*., 2015). In the last decade *A. baumanii*, combined with *P. aeruginosa* and *K. pneumoniae*, have emerged as the most significant Gram-negative nosocomial pathogens, with *A. baumanii* mostly implicated in infections and hospital outbreaks (Abdallah *et al.*, 2015; Sengstock *et al*., 2010). Crude mortality rate of 53% associated with ICU infections have been reported from a teaching hospital in Turkey in a study to investigate risk factors associated with mortality of MDR *A. baumannii* infections among hospitalized patients (Gulen *et al.*, 2015). Major surgery, trauma, burns, premature birth, prolonged hospitalization in hospital ICUs, mechanical ventilation, indwelling foreign devices and previous antimicrobial therapy have all been identified as the predisposing

risk factors for the *A. baumanii* infections (Visca, Seifert, & Towner, 2011). The pathogen's renowned environmental persistence, broad spectrum of antibiotic resistance and ability to withstand dry conditions by widening of the periplasmic space and thickening the cell wall, provides it with survival advantages over the other Gram-negatives (Otter, Yezli, & French, 2014; Trivedi *et al.*, 2015; Visca *et al.*, 2011).

The resistance of *A. baumanii* to a broad array of antibiotic classes is a challenge to antibiotic therapy in clinical practice. The production of β-lactamases (carbapenemases and ESBLs) confers resistance to penicillins, cephalosporins and carbapenems. Imipenem metallo-β-lactamases and oxacillin (OXA) serine β-lactamases were the first to be isolated from *A. baumannii* resistant to carbapenems (Bush *et al*., 2013; El Salabi, Walsh, & Chouchani, 2013). The swift acquisition of resistance genes to different and multiple classes of antibiotics, such as the β-lactams, aminoglycosides, quinolones and tetracyclines has led to the exclusion of these antibiotics as treatment options for *A. baumannii* infections (Sharaf & Gerges, 2016). β-lactam/lactamases inhibitors combination such as ampicillin-sulbactam possess relatively high bactericidal action against *A. baumanii* isolates, and thus 9 out of 10 (90%) patients with severe infections is reported to be effectively treated with the combination therapy in certain clinical settings (Fishbain & Peleg, 2010; Peleg, Seifert, & Paterson, 2008). Although with significant side effects, colistin, may be used as viable option due to its high activity against *A. baumanii,* (Mardani, 2011).

Other mechanisms such as up-regulation of existing genes of antibiotic-hydrolytic enzymes, expression of efflux pumps and reduced outer membrane permeability contribute to multi-drug resistance (Mardani, 2011; Miller, 2016). Multi-resistant *A. baumannii* strains possess a resistance island with genes encoding efflux pumps and conferring resistance to ammonium-

based disinfectants (Miller *et al*., 2016). In addition to the resistance island, epidemic strains have been shown to possess open reading frames for all known efflux pump families and super families acquired from other species (Pendleton *et al.*, 2013). Clinicians and healthcare providers are currently faced with a challenge to manage infections caused by multidrug resistant *Acinetobacter* spp in many hospitals worldwide especially the developing countries such as Ghana. In a study conducted by Acquah and co-workers on the susceptibility of bacterial etiological agents to commonly used antimicrobial agents in children with sepsis from a teaching hospital in Ghana, 100% *A. baumannii* were resistant to ampicillin, tetracycline and cotrimoxazole. Multidrug resistant *A. baumannii* is a public healthcare threat requiring urgent interventions for containment of resistance (Acquah *et al*., 2013).

1.2.5.3 *Pseudomonas aeruginosa*

P. aeruginosa is a Gram-negative, rod-shaped bacterium, classified as a facultative anaerobe that grows on a wide range of substrates and can quickly respond to environmental alterations. It is a common opportunistic nosocomial pathogen responsible for various infections, exhibits high antimicrobial resistance and is isolated mostly from patients hospitalised longer than one week, with UTIs, wound infections, severe burns and from immuno-compromised patients (De Angelis *et al.,* 2014; Nanvazadeh *et al.*, 2013; Tumbarello *et al*., 2013). Apart from the hospital acquired infections it can also cause other infections including respiratory tract infection, bacteraemia, endocarditis, meningitis, osteomyelitis, enterocolitis, diarrhoea and ecthyma with about 50% of the infections particularly associated with pneumonia being fatal (Hirsch & Tam, 2010; Micek *et al*., 2015). Aminoglycoside combination with antipseudomonal β-lactams such as penicillins and cephalosporins are often preferred treatment for *P. aeruginosa* infections (Kaye & Pogue, 2015).

P. aeruginosa is one of the candidates of ESKAPE pathogens that exhibits high resistance to multiple antibiotic classes including fluoroquinolones, carbapenems, aminoglycosides and polymyxins with more than 10% of extended drug resistance isolates resistant to carbapenems in many European countries under the European Antimicrobial Resistance Surveillance Network (Magiorakos *et al*., 2012). The most common factor involved in the resistance is MexAB-OprM, component of efflux overexpression (Labarca *et al.*, 2016; Morales *et al.*, 2012; Vaez *et al*., 2014). The lipopolysaccharide which is the major component of the outer membrane of *P. aeruginosa* serves as a barrier that prevents the passage of large hydrophilic molecules. The passage of aminoglycosides and colistin is through interaction with the lipopolysaccharide to change the permeability of the membrane while β-lactams and quinolones need to diffuse through porin channels (Tomás *et al*., 2010). The reduction in permeability through the loss of outer membrane porins (OprD) is associated with resistance to imipenem and reduced susceptibility to meropenem (Fowler & Hanson, 2014; Li *et al.*, 2012; Sun *et al.*, 2016). The OprD is also co-regulated with an efflux pump system, MexEF-OprN, which results in highly impermeable mutants with up-regulated efflux (Poole, 2011). The occurrence of double mutations is less frequent but results in significantly higher rates of antibiotic resistance. The hyper mutant strains of *P. aeruginosa* are often implicated in chronic infections as reported in the study on *in-vivo* evolution of resistance of *P. aeruginosa* strains isolated from patients admitted to an intensive care in Barcelona, Spain hospital (Solé *et al*., 2015).

The acquisition of resistance to multiple antibiotic classes, especially aminoglycosides and fluoroquinolones is mediated by transferable aminoglycoside modifying enzymes, rRNA methylases and expression of endogenous efflux systems. The genes encoding aminoglycosides modifying enzymes (AMEs) are located on integrons with other genes that are responsible for

conferring resistance to other classes of antibiotics, a major cause of the dissemination of resistance genes within and between bacterial species (Bush *et al*., 2013; Garneau-Tsodikova & Labby, 2016; Ramirez, Nikolaidis, & Tolmasky, 2013). Studies on laboratory-based nationwide surveillance of antimicrobial resistance conducted in Ghana, indicated *P. aeruginosa* as the second most prevalent pathogen implicated in various forms of infections, resulting in the use of broad array of antibiotics for treatment (Opintan *et al.*, 2015) resulting in high emergence of multidrug resistance by selection pressure.

1.2.5.4 *Enterobacter* spp.

Enterobacter spp. are motile aerobic Gram-negative, facultative anaerobic, non-spore forming bacilli that form part of normal intestinal flora belonging to the family Enterobacteriaceae (Davin-Regli, 2015). It is an opportunistic and ubiquitous bacterium in the environment and can survive on skin and dry surfaces as well as replicate in contaminated fluids, and therefore has a high propensity to cause nosocomial infections. The high pathogenicity of the bacteria is mainly due to the formation of biofilm and secretion of various forms of cytotoxins such as hemolysins, pore-forming toxins and enterotoxins. The relevant species include *E. cloacae*, *E. aerogenes*, and *E. agglomerans* most commonly isolated from human clinical specimens (Davin-Regli, 2015; Mezzatesta, Gona, & Stefani, 2012). Sydnor and Pearl (2011) in their review on hospital epidemiology and infection control in acute care settings reported high incidence of *Enterobacter* spp implicated in hospital-acquired infections in hospitals, particularly in ICUs with the risk of spread among patients due to inadequate adherence to infection control measures in some health care centres. The bacteria is a common cause of MDR infections in hospitalised patients, including cerebral abscess, pneumonia, meningitis, septicaemia, and wound, UTIs, particularly catheter-related UTIs and abdominal cavity or intestinal infections with high morbidity and

mortality. *Enterobacter* spp has also been implicated in various community infections and display high resistance to broad spectrum of antibiotics (Davin-Regli & Pagès, 2015).

The species has intrinsic resistance to amoxicillin, ampicillin, first-generation cephalosporins, and cefoxitin due to low level production of chromosomal AmpC β-lactamases and resistance to third generation cephalosporins mostly caused by overexpression. The species displays high resistance to broad spectrum-β-lactam antibiotics such as carbapenems through plasmid-encoded ESBLs and carbapenemases, including KPC, VIM, MBLS and OXA-types (Castanheira *et al*., 2011; Deshpande *et al.*, 2014). *Enterobacter* spp have EefABC and AcrAB–TolC efflux genes that export antibiotics from the cytosol or periplasmic region into extracellular environment, which contribute to the resistance against fluoroquinolones and aminoglycosides (Martins *et al*., 2010). Besides colistin and tigecycline, only few antibiotics are effective against these resistant strains due to the multi-drug resistant characteristics and hence there are little or no drugs in the 'pipeline' that are known to be capable of effectively addressing its mounting health crisis (Bergen *et al.*, 2015; Yarlagadda *et al*., 2015). In Ghana, information on *Enterobacter* spp resistance is sparse, however few studies on antibiotic resistance prevalence including that by Obeng-Nkrumah *et al*. (2013) which sought to investigate the monitoring and evaluation of antibiotic resistance in a major teaching hospital, and Newman et al. (2011) in their research on antimicrobial resistance drugs in Ghana, have indicated high resistance among the species to most first line antibiotics used in the country which gives cause for concern.

1.2.6 Challenges to resistance control strategies

The development of novel antibiotics over the past decade has slowed down, failing to keep pace with the increasing pandemic of multidrug resistant bacteria, thus putting life at risk in treatment

of bacterial infections worldwide (Harbarth *et al.*, 2015). The rapid loss of antibiotic efficacy together with increasing multiple drug resistant bacterial infections is worrisome, and demands urgent global concerted effort to contain the crisis (Cole, 2014). The situation is particularly threatening in the developing countries including Ghana, where resource constraints impede surveillance that can yield reliable data for interventions to combat resistance (Baker, 2015). Thus, strengthening the research base and making funding available for antibiotic resistance studies should be a priority for the problem of resistance to be effectively tackled in the sub Saharan African countries.

Another problem challenging the control of bacterial resistance may be weak health care systems and challenges such as inadequate funds, limited infrastructure and logistics, limited diagnostic capabilities, lack of qualified medical and laboratory personnel, poor implementation of infection control policies and especially lack of surveillance records for continuous update of prevalence in many parts of the world (Perez & Villegas, 2015). Although antibiotic resistance is a current global public health concern, there is marginal awareness of the magnitude of the problem, particularly in middle and low-income countries due to lack of surveillance systems to inform strategies and interventions that can detect prevalence, emerging resistance and inform treatment guidelines (Perez & Villegas, 2015). These are major setbacks for effective management and control of antibiotic resistance particularly in sub Saharan African countries including Ghana (Gandra, Merchant, & Laxminarayan, 2016; Ndihokubwayo *et al.*, 2010; WHO, 2014). Strengthening surveillance through implementation of infection control and antibiotic stewardship programs as well as improvement of laboratory capacity as recommended by the World Health Assembly, 2014 resolution 67.25 on Global Action Plan to tackle antibiotic

resistance, is crucial to mitigate the alarming rate of bacteria resistance (Shallcross & Davies, 2014).

The continuous irrational use of antibiotics is one of the major causes of antibiotic resistance, which has been a common practice in Ghana. Studies conducted by Donkor and co-workers on self-medication practices with antibiotics among tertiary level students in Accra, 2012, reported 70% (population size of 600) prevalence of self-medication among tertiary students in Accra, the capital of Ghana and projected higher percentages across the country especially in the rural communities where illiteracy may be comparatively high (Donkor *et al*., 2012). Selection pressure from antibiotic abuse contributes significantly to the emergence of bacterial resistance (Michael *et al*., 2014). To combat the spread of antibiotic resistance, public education on rational use of antibiotics and monitoring through the establishment of national surveillance system for tracking drug resistance is necessary. Consequently, intensifying research on antibiotic resistance in Ghana and the Sub-Saharan regions is paramount if the current burden of resistance among bacterial pathogens including the Gram-negative ESKAPE bacteria presently identified as problematic resistance pathogens is to be adequately tackled. Therefore a study to determine the resistance factors or molecular profile among the clinical isolates in a teaching hospital in Ghana is critical to aid in health care policies, antibiotic protocols and prescription regulations. This will also provide quality or improve data on the nature and prevalence of the various resistance genes or mechanisms involved in antibiotics resistance in Ghana. Such data can become a baseline for further research and comparative studies.

1.3 AIM OF STUDY

The aim of this study is to ascertain the phenotypic and genotypic characteristics of multi-drug resistant Gram-negative ESKAPE pathogens from Komfo Anokye Teaching Hospital in Kumasi in the Ashanti region of Ghana.

1.3.1 SPECIFIC OBJECTIVES

- To verify the identity and delineate the antibiotic susceptibility profiles of the Gram-negative ESKAPE pathogens from the bacteriology laboratory at KATH in Ghana over a 6-month period by using the Vitek-2 automated system.
- To phenotypically determine the expression of ESBLs, AmpC, inducible AmpC and metallo-β-lactamases by the double disc synergy test for ESBLs, the cefoxitin disc sensitivity test for AmpC beta-lactamases, the disk antagonism test for inducible AmpC β-lactamase production and the imipenem-EDTA combined disk test respectively.
- To undertake whole genome sequencing of selected isolates with a view to delineating: antibiotic resistance genes and their associated mobile genetic elements as well as their multi-locus sequence typing (MLST) profiles

1.4 STUDY DESIGN AND METHODOLOGY

1.4.1 Study design

This was an observational, descriptive study where the phenotypic and genotypic characteristics of antibiotic resistance was delineated in Gram-negative ESKAPE pathogens collected over a 6-month period in the Komfo Anokye Teaching Hospital in Ghana.

1.4.2 Ethical Considerations

Ethical clearance was obtained from the Joint Committee of Human Research Publications and Ethics, School of Medical Sciences, Kwame Nkrumah University of Technology, Research and Development Unit of the Hospital Administration (ref: CHRPE/AP/015/15) and the Biomedical Research Ethics Committee of University of Kwa-Zulu Natal (ref: BE 494/14). Voluntary, informed consent was obtained from all participants in writing or in the form of a thumb print. The document was also read, interpreted and further explained in the~~ir~~ local dialect to the illiterate participants. Parents or guardians for minors provided consent after explanation of the procedure and the purpose of the study.

1.4.3 Methodology

- **Study setting**

The study was conducted between February and August 2015, in Komfo Anokye Teaching Hospital (KATH) in Kumasi, in the Ashanti region of Ghana. The region covers a total land area of 24,389 square kilometres representing 10.2% of the total land area of Ghana. The population of the region is concentrated in a few districts, with the Kumasi metropolis accounting for nearly one-third of the region's population of 4,780,380 (Owusu *et al*., 2015). The facility is a 1000-bed tertiary care government hospital. The average daily primary care and specialist outpatient attendance was 169 and 954 patients respectively, during the period of study. KATH is the only regional and referral hospital that takes care of about 80% of both emergencies and regular medical cases in the region and also serves as referral hospital for part of Brong Ahafo, Western, Eastern and the Northern regions of Ghana.

- **Sampling**

Two hundred (200) clinical, non-duplicate Gram-negative bacterial pathogens were randomly selected from various human specimens routinely processed by the diagnostic microbiological laboratory in the hospital from both in-patients and out-patients. Multidrug resistant (isolates resistant to at least one agent in three or more antibiotic class) isolates selected from each group of Gram-negative ESKAPE pathogens constituted the final sample. The specimens were from urine (94), wound swabs (45), sputum (24), blood (11), ear swabs (6), pus (5) gastric lavage (4), tracheal aspirate (4) urethral swabs (2), pleural fluid (2), ascetic fluid (1), nasal swab (1) and bronchial lavage (1). Information on diagnosis, sex, age and ward type was obtained from patients' records. The isolates were maintained on nutrient agar slants and subsequently transported by Dalsey Hillblom Lynn (DHL) Express Logistic Company ensuring adequate health and safety precautions to South Africa for further investigations as described in the objectives.

- **Sample Analysis**

Bacterial identification and antibiotic susceptibilities were determined by the Vitek-2 (Biomerieux, France) automated system. Identity of isolates selected for whole genome sequencing was further confirmed by MALDI-TOF MS. The genomic DNA extraction and libraries were generated using the NucliSens easyMAG® (BioMérieux) and Nextera® kit (Illumina) respectively, followed by whole genome sequencing on an Illumina MiSeq platform for selected *P. aeruginosa* and *K. pneumoniae* isolates based on their extensively drug resistant profiles and resistance to second- and third-generation cephalosporins respectively. Antibiotic resistance genes and plasmids were identified by mapping the sequence data to an online

database using ResFinder and plasmidFinder respectively. MLST was also determined from the WGS data. The raw read sequences and assembled whole genome contigs have been deposited in GenBank under project number PRJNA411997

CHAPTER TWO

SCIENTIFIC PAPER/PUBLISHED ARTICLE: Multidrug-Resistant Gram-Negative Bacterial Infections in a Teaching Hospital in Ghana.

Author's contributions

- N Agyepong, as the principal investigator, co-conceptualized the study, undertook the laboratory work and drafting of manuscript
- U. Govinden, as co-supervisor, supervised the laboratory analysis and undertook critical revision of the manuscript
- A. Owusu-Ofori as co-supervisor, co-conceptualized the study, supervised the preliminary laboratory work, undertook critical revision of the manuscript, submission to the journal and led the response and rebuttal to the journal during the publication process
- S.Y Essack, as principal supervisor, co-conceptualized the study and undertook critical revision of the manuscript.

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Antimicrobial Resistance and Infection Control

RESEARCH Open Access

Multidrug-resistant gram-negative bacterial infections in a teaching hospital in Ghana

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Abstract

Background: Multidrug-resistant Gram-negative bacteria have emerged as major clinical and therapeutic dilemma in hospitals in Ghana.
To describe the prevalence and profile of infections attributable to multidrug-resistant Gram-negative bacteria among patients at the Komfo Anokye Teaching Hospital in the Ashanti region of Ghana.

Methods: Bacterial cultures were randomly selected from the microbiology laboratory from February to August, 2015. Bacterial identification and minimum inhibitory concentrations were conducted using standard microbiological techniques and the Vitek-2 automated system. Patient information was retrieved from the hospital data.

Results: Of the 200 isolates, consisting of *K. pneumoniae, A. baumannii, P. aeruginosa, Enterobacter spp., E. coli, Yersinia spp., Proteus mirabilis, Pasteurella spp., Chromobacterium violaceum, Salmonella enterica, Vibrio spp., Citrobacter koseri, Pantoea spp., Serratia spp., Providencia rettgeri Burkholderia cepacia, Aeromonas spp., Cadecea lapagei* and *Sphingomonas paucimobilis*, 101 (50.5%) and 99 (49.5%) recovered from male and female patients respectively The largest proportion of patients were from age-group ≥60 years (24.5%) followed by < 10 years (24.0%) and least 10–19 years (9.5%) with a mean patient age of 35.95 ± 27.11 (0.2–91) years. The decreasing order of specimen source was urine 97 (48.5%), wound swabs 47 (23.5%), sputum 22 (11.0%) bronchial lavage, nasal and pleural swabs 1 (0.50%). Urinary tract infection was diagnosed in 34.5% of patients, sepsis in 14.5%, wound infections (surgical and chronic wounds) in 11.0%, pulmonary tuberculosis in 9.0% and appendicitis, bacteremia and cystitis in 0.50%. The isolates showed high resistance to ampicillin (94.4%), trimethoprim/sulfamethoxazole (84.5%), cefuroxime (79.0%) and cefotaxime (71.3%) but low resistance to ertapenem (1.5%), meropenem (3%) and amikacin (11%). The average multi-drug resistance was 89.5%, and ranged from 53.8% in *Enterobacter* spp. to 100.0% in *Acinetobacter* spp. and *P. aeruginosa*.

Conclusion: Bacterial infections caused by multi-drug resistant (isolates resistant to at least one agent in three or more antibiotic classes) Gram-negative pathogens among patients at Komfo Anokye Teaching Hospital in Kumasi, Ghana are rife and interventions are necessary for their containment.

Keywords: Antibiotic resistance, Infections, Multidrug resistance, Pathogens

Background

The emergence of multidrug-resistant Gram-negative bacteria is a major concern in hospital settings in many parts of the world. Infections caused by these pathogens have become significantly challenging over the past two decades, particularly in the developing countries, and are associated with high morbidity and mortality rates as well as protracted hospital stay [1]. Enterobacteriaceae including *Klebsiella pneumoniae, Escherichia coli* as well as *Enterobacter spp.* and non-lactose fermenting bacteria such as *Pseudomonas aeruginosa* and *Acinetobacter spp.* have been identified as major cause of multi-drug resistant bacterial infections [2–4].

Studies conducted in many developing countries including Africa, have indicated high antibiotic resistance among Gram-negative bacteria to commonly used antibiotics, leading to a loss of efficacy for treatment of common infections [5–7]. These resistant bacterial pathogens are a major cause of both community and hospital-acquired infections. Respiratory tract, urinary tract, bloodstream (septic), post-surgical (wound) infections and pneumonia are among

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most commonly reported infections attributable to these pathogens in many hospitals [8].

Although, the impact of antibiotic resistance caused by multidrug resistant Gram-negative bacteria has been recognised in hospitals in Ghana, measures such as surveillance studies that provide reliable data to mitigate the problem are not in place. Therefore studies to establish the prevalence and extent of resistance are necessary to bridge the information gap and provide the basis to guide empiric therapy. This study aimed to assess multidrug-resistance among Gram-negative bacteria in Komfo Anokye Teaching Hospital in Ghana to guide treatment protocols. The data further provides a baseline for future comparative studies.

Materials

Setting of study

The study was conducted between February and August 2015, in Komfo Anokye Teaching Hospital (KATH) in Kumasi, in the Ashanti region of Ghana. The facility is a 1000-bed tertiary care government hospital. The average daily primary care and specialist outpatient attendance was 169 and 954 patients respectively, during the period of study. The population of the region is concentrated in a few districts, with the Kumasi metropolis accounting for nearly one-third of the region's population of 4,780,380 [9]. KATH is the only regional and referral hospital that takes care of about 80% of both emergencies and regular medical cases in the region and serves as referral hospital for part of Brong Ahafo, Western, Eastern and the Northern regions of Ghana.

Bacterial collection and identification

Two hundred (200) clinical, non-duplicate Gram-negative bacteria were randomly selected from urine, pus, wound swab, pleural fluid endotracheal tubes, gastric lavage and blood specimens processed by the diagnostic microbiological laboratory in the hospital from both in-patients and out-patients. Information on diagnosis, sex, age and ward type were obtained from patients records. The isolates were maintained on nutrient agar slants, frozen in lyophilizing medium at −70 °C and subsequently transported to National Health Laboratory Services in South Africa to confirm identification and ascertain antibiotic susceptibility profiles using Vitek-2 (Biomerieux, France) Automated Systems with *P. aeruginosa* ATCC27853 and *E.coli* ATCC35218 as control strains. Multidrug-resistance in this study was defined as isolates that were resistant to at least one agent in three or more antibiotic classes.

Results

Of the 200 resistant Gram-negative bacterial isolates obtained, *E. coli* was most frequent pathogen 49 (24.5%), followed by *P. aeruginosa* 39 (19.5%), *K. pneumoniae* 38 (19.0%), *Enterobacter spp.* 12 (6.0%) *Serratia spp.* 8 (4.0%), *Sphingimonas spp.* 10 (5.0%) and *Acinetobacter spp.* 8 (4.0%). The remaining 36 (18%) (Table 1) consisted of *Yersinia spp.* (5) *Proteus mirabilis* (1), *Salmonella enterica* (1), *Vibrio spp.* (5), *Citrobacter koseri* (3), *Pantoea* spp. (5), *Providencia rettgeri* (4), *Pasteurella spp.* (2) *Chromobacterium violaceum* (2), *Burkholderia cepacia* (2), *Aeromonas spp.* (5), *Cadecea lapagei* (1). The distribution of the specimen types showed highest proportion of isolates, were from urine specimens 94 (47.0%), followed by wound swabs 45 (22.5%) and sputum 24 (12.0%) with just 1 (0.5%) from each of bronchial lavage, nasal and pleural swabs respectively. A higher proportion of isolates were recovered from in-patients 161 (80.5%) compared to the out-patients 39 (19.5%). The number of isolates from the medical intensive care unit (ICU) 78 (39.0%) was highest, followed by Child Health 39 (19.5%) and the Obstetrics and Gynecology wards 17 (8.5%) with the smallest number of 10 (5.0%) isolated in the Accident and Emergency Unit. *E. coli* 49 (24.5%), *P. aeruginosa* 39 (19.5%) and *K. pneumoniae* 38 (19.0%) (Table 1) were the most predominant pathogens implicated in 63.0% of all infections. The distribution of bacterial pathogens among clinical diagnosis showed urinary tract infection (UTI) 69 (34.5%) as the most prevalent, followed by sepsis 29 (14.5%), tuberculosis 18 (9.0%) and wound infections 12 (6.0%) with the least prevalence in appendicitis, bacteremia, cystitis and prostitis 1 (0.5%) (Table 1). *E. coli* 24 (34.8) was identified as most common cause of UTI, followed by *K. pneumoniae* 17 (24.6%) and *P. aeruginosa* 9 (13.04%) implicated in 72.4% of all UTI pathogens.

Demographic characteristics of patients with bacterial infections

The patients' ages ranged between 2 months to 90 years and mean age was 35.95 ± 27.11 years with the gender distribution of 101 (50.5%) males and 99 (49.5%) females. Samples from outpatients (OPD) made up 19.5% of the total samples. All the other samples (80.5%) from Accident and Emergency, Child Health, Medical ICU, Surgery and Obstetrics and Gynaecology departments were inpatient samples (Table 1). The prevalence of infections were highest among the patients of age-group ≥60 years 49 (24.5%) followed by < 10 years 48 (24.0%), 20–29 years 26 (13.0), 30–39 years and least in 50–59 years 17 (8.5%) (Table 2). UTI was highest among the age-group < 10 years 23 (33.3%) followed by 30–39 years 12 (17.4%) and least 50–59 years 4 (5.8%). Septic infection was highest in patients within the age-group <10 years 8 (40.0%), followed by ≥60 years 5 (25.0%) least in 10–19 years 3 (15.0%) (Table 2). Among the different types of wound infections, diabetic foot ulcer showed high proportion within age-groups 50–59 and ≥60 years of patients.

Table 1 Distribution of isolates among clinical specimen, wards and diagnosis

	Isolates (N)								
	Acinetobacter spp	*E. coli*	*Enterobacter spp*	*K. pneumonia*	*P. aeruginosa*	*Serretia spp*	*Sphingimonas spp*	Other*	Total (%)
Specimen									
Ascitic fluid	–	–	–	–	–	–	–	1	1 (0.5)
Aspirate	–	2	1	1	–	–	–	–	4 (2.0)
Blood	–	1	2	2	–	–	–	6	11 (5.5)
Bronchial Lavage	–	–	1	–	–	–	–	–	1 (0.5)
Ear Swab	–	1	–	1	1	1	–	2	6 (3.0)
Gastric Lavage	–	–	–	1	1	–	–	2	4 (2.0)
Nasal Swab	–	–	–	–	1	–	–	–	1 (0.5)
Pleural Fluid	–	–	–	1	–	–	1	–	2 (1.0)
Pus	–	3	–	–	–	–	1	1	5 (2.5)
Sputum	–	3	3	3	8	3	2	2	24 (12.0)
Urethral Swab	2	–	–	–	–	–	–	–	2 (1.0)
Urine	6	35	5	23	12	4	4	5	94 (47.0)
Wound Swab	–	4		6	16	–	3	16	45 (22.5)
WARD/DEPT									
A &E	1	2	–	2	2	1	1	1	10 (5.0)
Child Health	1	12	3	11	3	–	1	8	39 (19.5)
Medical ICU	2	14	6	10	21	5	4	16	78 (39.0)
OBS &GYN	2	3	2	4	2	1	1	2	17 (8.5)
OPD	2	12	1	9	5	1	3	6	39 (19.5)
Surgery	–	6	–	2	6	–	–	3	17 (8.5)
Diagnosis									
Abscess	–	1	1	1	1	1	–	2	7 (3.5)
Appendicitis	–	1	–	–	–	–	–	–	1 (0.5)
Bacteremia	–	1	–	–	–	–	–	–	1 (0.5)
Bronchitis	–	–	–	1	1	–	–	–	2 (1.0)
Cellulitis	–	–	–	–	–	–	–	1	1 (0.5)
Cirrhosis	–	1	1	–	–	–	–	–	2 (1.0)
Cystitis	–	–	–	1	–	–	–	–	1 (0.5)
Diabetic foot ulcer	–	1	1	–	4	–	1	3	10 (5.0)
Gastroenteritis	–	4	–	1	–	–	–	–	5 (2.5)
Nephritis	–	6	–	3	–	1	–	1	11 (5.5)
Otitis	–	1	–	–	–	–	–	1	2 (1.0)
Pericarditis	–	1	–	–	2	–	–	–	3 (1.5)
Peritonitis	–	1	–	–	1	–	–	–	2 (1.0)
Pneumonise	–	–	–	1	–	2	1	–	4 (2.0)
Prostitis	–	–	–	–	–	1	–	–	1 (0.5)
RTI	–	–	–	1	1	–	2	1	5 (2.5)
Sepsis	1	6	1	4	4	2	3	8	29 (14.5)
Surg. site Infection	–	1	–	4	4	–	–	1	10 (5.0)
Tuberculosis	–	1	4	2	7	1	1	2	18 (9.0)

Table 1 Distribution of isolates among clinical specimen, wards and diagnosis *(Continued)*

	Isolates (N)								
	Acinetobacter spp	*E. coli*	*Enterobacter spp*	*K. pneumonia*	*P. aeruginosa*	*Serretia spp*	*Sphingimonas spp*	*Other**	Total (%)
UTI	7	24	4	17	9	–	2	6	69 (34.5)
Ulcer	–	–	–	–	1	–	–	1	2 (1.0)
Wound infection	–	–	–	1	4	–	–	7	12 (6.0)
Frequency (%)	**8 (4.0)**	**49 (24.5)**	**12 (6.0)**	**38 (19.0)**	**39 (19.5)**	**8 (4.0)**	**10 (5.0)**	**36 (18.0)**	**200 (100)**

Abbreviation: *A&E* Accident and Emergency, *OPD* Out-Patient Department, *OBS&GYN* Obstetrics and Gynecology, *Other** Other Gram-negative bacteria. The In–Patient Department comprises of A&E, Medical ICU, OBS&GYN and Surgical ward, –: Non-detected

Table 2 Distribution of infections among gender and patients' age groups

	Age (Years) Number of Cases (N)							
	< 10	10–19	20–29	30–39	40–49	50–59	≥60	Total (%)
Gender								
Female	21	8	20	13	11	9	17	99 (49.5)
Male	27	11	6	8	9	8	32	101 (50.1)
Infections								
Abscess	2	1	2	1	–	1	–	7 (3.5)
Appendicitis	–	1	–	–	–	–	–	1 (0.5)
Bacteremia	1	–	–	–	–	–	–	1 (0.5)
Bronchitis	1	–	–	–	–	–	1	2 (1.0)
Cellulitis	–	–	–	–	–	–	1	1 (0.5)
Cirrhosis	–	1	1	–	–	–	–	21.0)
Cystitis	1	–	–	–	–	–	–	1 (0.5)
Diabetic foot ulcer	–	–	1	–	1	1	7	10 (5.0)
Gastroenteritis	4	3	–	–	–	–	–	7 (3.5)
Nephritis	–	1	1	1	1	1	6	11 (3.5)
Otitis	1	–	1	–	–	–	–	2 (1.0)
Pericarditis	–	–	–	–	2	–	1	3 (1.5)
Peritonitis	2	–	–	–	–	–	–	2 (1.0)
Pneumoniae	–	1	–	–	–	–	3	4 (2.0)
Prostitis	–	–	–	–	–	–	1	1 (1.0)
RTI	2	1	–	1	1	–	–	5 (2.5)
Sepsis	8	3	1	–	1	2	5	20 (10.0)
Surgical wound infection	1	1	2	2	–	2	2	10 (5.0)
Tuberculosis	1	1	3	–	6	3	4	18 (9.0)
UTI	23	4	10	12	7	4	9	69 (34.5)
Ulcer	–	1	–	–	1	–	–	2 (1.0)
Wound infection	1	–	2	2	1	2	2	10 (5.0)
Frequency (%)	**48 (24.0)**	**19 (9.5)**	**26 (13.0)**	**21 (10.5)**	**20 (10.0)**	**17 (8.5)**	**49 (24.4)**	**200 (100)**

Abbreviation: *UTI* Urinary tract infection, *RTI* Respiratory tract infection, –: Non-detected

Susceptibility profile

The antibiotic susceptibility profile showed that the isolates were most resistant to ampicillin (94.4%), trimethoprim/sulfamethoxazole (84.5%), cefuroxime/Axetil (80.0%), cefuroxime (79.0%), cefotaxime (71.3%), cefoxitin (57.5%) and were least resistant to ertapenem (1.5%) (Table 3).

Multi-drug resistance

Multidrug resistance was observed in 89.5% of the bacterial isolates, ranging from 53.8% in *Enterobacter* spp. to 100.0% in *Acinetobacter* spp. and *P. aeruginosa* (Table 4).

Discussion

Epidemiological surveillance of bacterial infection and resistance to antibiotics are essential for awareness creation, implementation of control measures and effective management of infections. This is important in developing countries particularly in sub-Saharan Africa where studies have indicated that many hospitals have rudimentary and poor enforcement of infection control measures and marginal awareness on the extent of infections caused by multi-drug resistant bacteria which have resulted in increased morbidity and mortality [10, 11].

Our study observed higher prevalence of infections among in-patients (80.5%) compared to the out-patients (19.5%), with ICU accounted for highest incidence. The frequency of bacterial pathogens isolated from male (50.5%) and female (49.5%) patients, showed no appreciable difference. The high infections of in-patients is consistent with the finding from nationwide surveillance on antimicrobial resistant pathogens from patients' blood cultures, which recorded prevalence of >70% of bacterial infections among in-patients in Ghanaian hospitals [12]. Several predisposing factors are associated with the higher infection rates among hospitalized patients such as the use of invasive procedures like catheterization, central lines and mechanical ventilation [13]. A number of risk factors account for the high ICU infections including non-compliance of care professionals (physicians and nurses) to hand-hygiene practices which has been identified in a study conducted in a neonatal ICU in a Ghanaian tertiary care hospital as a major factor contributing to healthcare infections in ICU [14]. The ICU houses critically ill patients and are often exposed to extensive use of antibiotics causing selection pressure for the emergence of resistance. These factors coupled with interventional instrumentations such as mechanical ventilation and invasive procedures, commonly used in ICU [15], exposes the patients to high risk of infections. KATH is the only tertiary and referral facility receiving patients into the ICU from many healthcare facilities within the region and other parts of the country, often resulting in congestion or overcrowding, thus increasing chances of transmitting infections among patients.

This study indicated high infections among advancing and infant age-groups with UTI and sepsis recorded as most frequent. The geriatric and pediatric patients are

Table 3 Antibiotic susceptibility profile of isolates

Antibiotics	Total number of isolates (*N*)	Susceptible *N* (%)	Intermediate *N* (%)	Resistant *N* (%)
Ampicillin	162	7 (4.3)	2 (1.2)	153 (94.4)
Amox/clav	200	51 (25.5)	47 (23.5)	102 (51.5)
Piperacillin-tazobactam	198	109 (54.9)	62 (31.0)	27 (13.5)
Cefuroxime	200	35 (17.5)	7 (3.5)	158 (79.0)
Cefoxitin	200	74 (37.0)	11 (5.5)	115 (57.5)
Cefotaxime	199	44 (22.1)	12 (6.0)	143 (71.3)
Ceftazidime	200	104 (52.0)	15 (7.5)	81 (40.5)
Cefepime	200	119 (59.5)	64 (32.0)	17 (8.5)
Ertapenam	132	130 (98.5)	1 (0.8)	1 (0.8)
Imipenem	199	191 (95.5)	0 (0.0)	8 (4.0)
Meropenem	198	192 (97.0)	1 (0.5)	5 (2.5)
Amikacin	199	178 (89.4)	14 (7.0)	7 (3.5)
Gentamicin	198	105 (53.0)	5 (2.5)	88 (44.4)
Ciprofloxacin	200	116 (58.0)	2 (1.0)	82 (41.0)
Tetracycline	199	124 (62.3)	14 (7.0)	61 (30.7)
Nitrofurantoin	200	84 (42.0)	17 (8.5)	99 (49.5)
Colistin	198	164 (82.8)	2 (1.0)	32 (16.2)
Trim/Sulfamethoxazole	200	31 (15.5)	0 (0.0)	169 (84.5)

Not all the antibiotics were tested for all 200 isolates and not all *N*-values added up to 200
Abbreviation: *amox/clav* amoxicillin-clavulanate, *trim/sulfamethoxazole* trimethoprim-sulfamethoxazole

Table 4 MDR among isolates

Bacterial isolates	Number of Isolates (*N*)	MDR *N* (%)
Acinetobacter spp	8	8 (100.0)
E. coli	49	44 (89.9)
Enterobacter spp	12	7 (53.8)
K. pneumoniae	38	36 (94.7)
P. aeruginosa	39	39 (100.0)
Serratia spp	8	7 (87.5)
Sphingomonas paucimobilis	10	9 (90.0)
Other Gram negative	36	29 (80.6)
Frequency	**200**	**179 (89.5)**

usually more disposed to infections due to their immune status. The advancing age are commonly associated with risk factors including reduced immunity, co-morbid diseases such as diabetes mellitus, chronic heart diseases, neurogenic bladder [13, 16] whilst in infants, lack of fully developed immunity, malnutrition as well as inadequate hygiene [17] put them at greater risk of infections. Urinary tract infection (34.5%) was most prevalent within the period of our study, and is comparable to 31.5% reported from a study on prevalence and antibiotic susceptibility pattern of uropathogens conducted in secondary hospital in Ghana [18]. Our study found, *E. coli* and *K. pneumoniae* as most predominant pathogens implicated in UTI. Several studies conducted in the region and other parts of the country have reported UTI as most common infections frequently caused by *E.coli* and *K. pneumoniae* with high resistance to broad spectrum antibiotics, that remains a major clinical problem in health care system in Ghana [19, 20]. Among the isolates, *E. coli*, *K. pneumoniae Proteus mirabilis*, *P. aeruginosa*, *Enterobacter*, *Acinetobacter* and *Serratia* spp. in the present study have been reported as clinically important urine pathogens [18, 21], associated with about 90% of both community and hospital acquired UTIs [22, 23]. Urinary tract infection prevalence was high among infants and the middle aged with incidence higher in females (10.8%) than the males (6.6%) in the middle age group. In infants predisposing factors such as lack of personal hygiene, incomplete emptying of the bladder with residual urine and severe acute malnutrition have been reported [24], whilst the middle aged, in particular females, high parity coupled with increased frequency of sexual activities have been identified, contributing to the high incidence [25]. The prevalence of sepsis among infants, 40.0% (Table 2), was higher compared to the 25.9% previously reported in the country [26]. The higher prevalence is attributed to higher patients' intake in the study site and also situated in most populous region, thus receiving higher numbers of patients (infants) with complications compared to other regions of Ghana. This study is however limited in its representativeness of the entire hospital because we did not systematically collect data on how much of each specimen type was sent to the lab and which types of isolates were obtained from each specimen type.

Among the isolates, *E. coli*, *P. aeruginosa* and *K. pneumoniae* were most prevalent pathogens implicated in 63.0% (Table 1) of the infections with high resistance to antibiotics commonly used in Ghana. The finding that *E coli*, *P. aeruginosa* and *K. pneumoniae* were most prevalent Gram-negative pathogens is consistent with previous studies conducted in Ashanti region of Ghana [20] and other parts of the country [26, 27]. A nationwide surveillance on antimicrobial resistant pathogens study, conducted by Opintan et al. also indicated *E.coli* and *P. aeruginosa*, *Enterobacter*, *Citrobacter spp.* and *K. pneumoniae* as most common gram-negative bacterial pathogens in Ghana [28]. The current prioritized lists of bacterial pathogens by World Health Organization (WHO), categorized *E. coli*, *P. aeruginosa*, *K. pneumoniae*, *Acinetobacter*, *Enterobacter*, *Serratia*, *Proteus* and *Providencia* spp. identified in our study, as critical or most life-threatening Gram-negative pathogens under surveillance due to their high antibiotic resistance especially to carbapenems and third generation cephalosporins, associated with attributable mortality [29]. In particular, *K. pneumoniae*, *P. aeruginosa*, *Acinetobacter* and *Enterobacter* spp. have further been described by the Infectious Diseases Society of America as Gram-negative ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp) pathogens, frequently associated with multidrug resistance [30, 31]. These ESKAPE Gram-negative pathogens were implicated in 48.5% (Table 1) of the infections which is higher than 35.6% previously reported [27]. The pathogens were also implicated in 50% (Table 1) of the ICU infections, and therefore posing a major threat to public healthcare in Ghana.

The susceptibility profile of the isolates displayed high level multidrug resistance of 89.5% with high resistance to ampicillin (94.4%), trimethoprim sulfamethoxazole (84.5%), cefuroxime (79.0%) cefotaxime (71.3%), cefoxitin (57.5%) and amoxacillin-clavulanate (51.5%) observed, was consistent with other studies conducted in Ghana [27, 32]. The degree of resistance among the isolates to ampicillin, trimethoprim/sulfamethoxazole and amoxacillin-clavulanate showed in the study, also agreed with other studies conducted in the sub-Sahara African countries such as Tanzania [7], Nigeria [33], Ethiopia [34], Zimbabwe [35] and Rwanda [6]. The high resistance trend in the sub region is indicative of high antibiotic selection pressure largely due to relatively cheap and easy availability of these agents, mostly used as first line or common choice of treatment in many healthcare settings in the sub region [36–38].

The decreased susceptibility of isolates to the beta-lactam/beta-lactamases inhibitor combination antibiotic therapy and fluoroquinolones (ciprofloxacin) is comparable to other studies in Ghana [20, 27], which poses a challenge to treatment of common infections as these agents are readily available therapeutic options [39]. The high resistance to second and third-generation cephalosporins (cefuroxime [79.0%], cefoxitin [57.5%] and cefotaxime [71.3%]), may suggest high expression or production of extended spectrum beta-lactamases among Gram-negative bacteria as previously reported in Ghana [40]. The carbapenems (imipenem, ertapenem and meropenem), amikacin and colistin were sensitive, as these antibiotics are used as last resort in treatment of serious infections. In addition the carbapenems have been introduced into Ghanaian market for relatively short span of time and is comparatively more expensive than the mainstay antibiotics and therefore, not commonly used.

This has possibly led to relatively low natural selection and hence low development of antibiotic resistance among the isolates.

Conclusion

The study demonstrated high multidrug resistant Gram-negative bacteria implicated in the infections, with UTI as most frequently diagnosed among patients. Infections were common among the elderly and infants, and predominantly caused by *E. coli*, *K. pneumoniae* and *P. aeruginosa* during the period of our study. These results should inform the empirical treatment of infections in Komfo Anokye Teaching Hospital of Ghana as appropriate.

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Availability of data and materials
Data and materials have been provided in the main manuscript. Where necessary additional information of the study can be made available from the corresponding author on request.

Authors' contributions
The study was co-conceptualized and jointly designed by NA, AO and SE. NA collected the data and undertook laboratory analysis with the help from UG. NA analyzed and interpreted the data with assistance from AO & SE. All the authors contributed in preparation and submission of manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Ethical clearance was approved by the joint Committee of Human Research Publications and Ethics, School of Medical Sciences, Kwame Nkrumah University of Technology, Research and Development Unit of the Hospital Administration (ref: CHRPE/AP/01 5/15) and the Biomedical Research Ethics Committee of University of Kwa-Zulu Natal (ref: BE 494/14). Informed consent was obtained from all participants, and from parents or guardians for minors in written form either signed or by a thumb print after explanation of the procedure and the purpose of the study.

Consent for publication
Not applicable

Competing interests
Professor Essack is a member of the Global Respiratory Infection Partnership sponsored by an unrestricted educational grant from Reckitt and Benckiser, UK. The other authors have no competing interest to declare.

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CHAPTER THREE

SCIENTIFIC MANUSCRIPT TWO: Genotypic characterization of multidrug resistant ESBL-producing *Klebsiella pneumoniae* isolated from a Ghanaian teaching hospital

Author's contributions

- N Agyepong, as the principal investigator, co-conceptualized the study, undertook the preliminary laboratory work, analyzed the whole genome sequencing results and drafted the manuscript
- A. Owusu-Ofori as co-supervisor, co-conceptualized the study, supervised the sampling and preliminary laboratory work and undertook critical revision of the manuscript
- U. Govinden, as co-supervisor, co-conceptualized the study facilitated analysis of the whole genome sequencing results and undertook critical revision of the manuscript
- A.G. Daniel, contributed to data analyses and undertook the critical revision of the manuscript
- A. Mushal, contributed to data analyses and undertook critical revision of the manuscript
- T. Pedersen, contributed to data analyses and writing process, focusing on the results and discussion
- A. Sundsfjord, contributed to data analyses and writing process, assuring the quality of the final manuscript
- S. Y. Essack, as principal supervisor, co-conceptualized the study and undertook critical revision of the manuscript

Genotypic characterization of multidrug resistant ESBL-producing *Klebsiella pneumoniae* isolated from a Ghanaian teaching hospital

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Abstract

The resistance of bacteria to multiple classes of antibiotics is a major global health concern. Extended spectrum-β-lactamase-producing Enterobacterales including *Klebsiella pneumoniae* are among the multidrug-resistant Gram-negative bacteria classified as critical on the World Health Organization's list of priority pathogens for the research and development of new antibiotics. ESBL encoding plasmids are commonly associated with genes mediating resistance to other antibiotic classes including fluoroquinolones and aminoglycosides, facilitating the dissemination of multidrug-resistant bacteria in hospital settings. This study delineated the multi-locus sequence types, antibiotic resistance genes and associated mobile genetic elements in multidrug-resistant *K. pneumoniae* from a teaching hospital in Ghana. Identification and MIC-determinations were done using the Vitek-2 automated system and confirmed by MALDI-TOF MS. DNA extraction was carried out using the NucliSens easyMAG® (BioMérieux) kits and the DNA was subjected to Illumina WGS. The isolates were characterised by the presence of diverse sequence types (STs) include (ST2171, ST1887, ST2816, ST17, ST152, ST397, ST1788, ST798 and ST101) and multiple genes encoding resistance to β-lactams (*bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{SHV-11}}$, *bla*$_{\text{TEM-1B}}$, *bla*$_{\text{OXA-1}}$), aminoglycosides (*aac(3)-IIa, strB, StrA, aadA16*), fluoroquinolones/quinolones (*qnrB49, qnrB10, oqxA, oqxB*) and other antibiotic classes. Resistance genes were associated with plasmids, predominantly IncFIB(K) and ColRNAI. We found multiple and diverse mutations in quinolone resistance-determining regions of *gyrA, gyrB* and *parC* in isolates resistant to ciprofloxacin, but no mutation was detected in *gyrB* among isolates with a ciprofloxacin (MIC ≥4mg/mL). None of the isolates susceptible to ciprofloxacin presented mutations. The diverse resistance genes identified in multidrug-resistant *K. pneumoniae* is a major threat to the management of infections in Ghana. The molecular characterization of antibiotic resistance is thus imperative to inform strategies for containment.

Introduction

Increasing resistance to multiple antibiotic classes among Gram-negative bacteria is a major challenge to physicians and health-care providers in the treatment of infections. Gram-negative bacteria are commonly associated with hospital-acquired infections such as ventilator associated pneumoniae, bloodstream infections, surgical site infections and urinary tract infections (UTI) particularly among immune-compromised patients in intensive care units (ICU) (1). *Enterobacteriaceae* including *Klebsiella pneumoniae* are prominent hospital-acquired pathogens and are listed among the six multidrug-resistant ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp.) bacteria described by Infectious Disease Society of America (2). Third-generation cephalosporin and carbapenem-resistant Enterobacteriaceae have also been ranked as critical priority pathogens for the research, discovery and development of new antibiotics by the World Health Organization (3, 4).

The mechanisms of β-lactam antibiotic resistance employed by Gram-negative bacteria including *K. pneumoniae* involve the expression of β-lactamases with/without other non-enzymatic resistance mechanisms such as efflux and/or, outer protein membrane or porin reduction rendering the agents ineffective (5-7). The resistance may be intrinsically expressed or acquired. Ruppé and colleagues (2015) reported high rates of resistance to β-lactam antibiotics, particularly second and third generation cephalosporins among Enterobacteriaceae, ranging from 10% to 70% in a systematic review on mechanism of antibiotic resistance in Gram-negative bacilli in the last decade. The swift acquisition of plasmid-borne extended-spectrum β-lactamases (ESBLs), especially those belonging to the TEM, SHV and CTX-M β-lactamase families produced by Enterobacteriaceae including *K. pneumoniae* with high preference for oxyimino-

cephalosporin hydrolysis is increasing globally (8-11). Plasmids of the IncF group represent one of the most common plasmid types contributing to the spread of antibiotic resistance genes in Enterobacteriaceae with CTX-M-15-positive IncFIIK plasmids commonly characterized in *K. pneumoniae* (12, 13). The spread of these resistant bacteria has compromised the use of β-lactams, considered as the safest and most easily available antibiotics for treatment of infections in many parts of the world, including Ghana.

Studies conducted in Ghana have reported *K. pneumoniae* as a major pathogen responsible for UTI (14). A laboratory-based nationwide surveillance of antimicrobial resistance in Ghana by Opintan and co-workers reported that *K. pneumoniae* represented 1.06% of all bacterial infections and 1.4% of Gram-negative bacilli (15). Agyepong et al (2018) indicated an increased *K. pneumoniae* resistance of 19% (38/200) of Gram-negative bacteria in their study on multidrug bacterial infections in a teaching hospital in Ghana. In spite of the threat posed by multidrug resistant Gram-negative bacteria in health care settings in Ghana, there is paucity of molecular epidemiology studies. This study, which forms part of a broader study on the molecular profile of Gram-negative ESKAPE pathogens in a Ghanaian teaching hospital, delineates the resistance genotypes of a sub-set of *K. pneumoniae* with resistance to the third-generation cephalosporins.

Materials and Methods

Ethical Approval and Voluntary Informed Consent

Ethical clearance was granted by the Joint Committee of Human Research Publications and Ethics, School of Medical Sciences, Kwame Nkrumah University of Technology, Research and Development Unit of the Hospital Administration (ref: CHRPE/AP/015/15) and the Biomedical Research Ethics Committee of University of Kwa-Zulu Natal (ref: BE 494/14). Voluntary, informed consent was obtained from all participants and from parents or guardians for minors in written form either signed or by a thumb print after explaining the procedure and purpose of the study, using an interpreter as appropriate

Study Setting

The study was conducted between February and August 2015 in Komfo Anokye Teaching Hospital (KATH) in Kumasi, in the Ashanti region of Ghana. The facility is a 1000-bed tertiary care government hospital. The average daily primary care and specialist outpatient attendance was 169 and 954 patients respectively during the period of study. The population of the region is concentrated in a few districts, with the Kumasi metropolis accounting for nearly one-third of the region's population of 4,780,380 (16). KATH is the only regional and referral hospital that takes care of about 80% of both emergencies and regular medical cases in the region and serves as referral hospital for parts of Brong Ahafo, Western, Eastern and the Northern regions of Ghana**.**

Bacterial Selection and Identification

The *K. pneumoniae* sample used in this study was a subset of isolates from a larger study of 200 clinical, non-duplicate Gram-negative bacterial samples (17). Of the 200 isolates, 38 were *K. pneumoniae* and of these, 10 multi drug resistant isolates resistant to all second- and third-generation cephalosporins mainly used as last resort antibiotic in Ghana, were selected for genotypic characterization by whole genome sequencing. Bacterial identification and antibiotic

susceptibilities were determined by the Vitek-2 (Biomerieux, France) automated system. Identity and MICs were further confirmed by using MALDI-TOF MS (Bruker Daltonic Gmbh, Bremen, Germany) broth micro-dilution in accordance with European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines respectively (18). *K. pneumoniae* ATCC700603 was used as the control strain.

DNA Extraction and Genome Sequencing

DNA extraction was carried out using the NucliSens easyMAG® (BioMérieux) kits according to the manufacturers' instructions. The genomic DNA libraries were generated using the Nextera® kit (Illumina) followed by sequencing on an Illumina MiSeq platform at the Genomics Resource Center at the University of Tromso, the Arctic University of Norway. Raw sequence reads were adaptor and quality-trimmed using Trimmomatic (19). After assembly by using SPAdes 3.11.10 (20) assembly quality was assessed by QUAST 4.6.0 (21). The assembled reads were annotated using the Bacterial Analysis Pipeline of software revision 4.2 and National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) searches (https://www.ncbi.nlm.nih.gov/genome/annotation_prok/). The antibiotic resistance genes and plasmids were identified by mapping the sequence data to an online database using ResFinder (22) and plasmidFinder (23) respectively. Multi-locus Sequence Typing (MLST) was also determined from the assembled genomes (https://github.com/tseemann/mlst). Comparative genomic analysis was further performed using *K. pneumoniae* ATCC 13883 (PRJNA244567) as reference strain to elucidate the chromosomal mutation resulting in quinolone resistance.

Phylogenetic Analyses

To investigate the global phylogeny of the *K. pneumoniae* isolates, genome assembly datasets including metadata were downloaded from the Pathosystems Resource Integration Center

(PATRIC) database (https://www.patricbrc.org/). Genomes with less than 400 contigs and with MLST and isolation country available were selected and run through parsnp (software designed for intraspecific or core genome alignment for high quality assemblies) v.1.2 (24) with "–c "-flags enabled to include all the selected genomes in the phylogenetic tree, and random reference selection among the included samples. FigTree (http://tree.bio.ed.ac.uk/software/figtree/) and R-ape package (v5.1**)** were used to visualize and edit the phylogenetic trees.

Accession numbers: The raw read sequences and the assembled whole genome contigs have been deposited in GenBank. The data is available under project number PRJNA411997.

Results

The clinical data indicated that *K. pneumoniae* was frequently implicated in UTI. Antibiotic susceptibility profiles showed that all the isolates were resistant to cefuroxime, cefotaxime and ceftazidime (second- and third-generation cephalosporin) but sensitive to imipenem, ertapenem, meropenem, amikacin and colistin. Eight of the ten isolates were additionally resistant to gentamicin, nitrofurantoin and sulfamethoxazole-trimethoprim and six were sensitive to ciprofloxacin (Table 1). WGS analysis revealed that all the isolates were predominantly characterized by the presence of multiple resistance genes encoding for resistance within and between antibiotic classes. The isolates carried 3-5 β-lactamase genes, 3-6 aminoglycoside resistance genes, 2-5 fluoroquinolone resistance genes in different permutations and combinations (Table 2). *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{SHV-11,}}$ *and bla*$_{\text{TEM-1B}}$), (*aac(3)-IIa-like, aph(3')-Ia* and *aac(6')Ib-cr)* and (*oqxA-like, oqxB-like, qnrB10-like* and *qnrB2*) were the most common β-lactam aminoglycoside and fluoroquinolone resistance genes observed respectively. The isolates were also characterised with resistance genes for other antibiotic classes including *sul2, fosA, dfrA14 and catB7-like* encoding resistance for sulphonamide, fosfomycin trimethoprim and phenicol

respectively with one of the isolates harbouring the *mph(A)* resistance gene conferring to macrolides. MLST analysis identified ST2171, ST1887, ST2816, ST17, ST152, ST397, ST1788, ST798 and ST101 *K. pneumoniae* strains indicating the circulation of multiple *K. pneumoniae* sequence type in a single hospital. IncFIB(K) and ColRNAI were the most prevalent plasmid replicon types among *K. pneumoniae* isolates (Table 2).

Phylogenetic analyses including the isolate genomes from this study (n=10) and from a global strain collection (n=1158, including 20 South African isolates) show that the isolates from our study are widely distributed in the global tree (Figure 1). As visualized, the two ST101-isolates from Ghana (P26-75 and P26-81) are closely related to each other and belong to the same distinct phylogenetic cluster as the South African lineage of ST101. Moreover, isolates P26-71 (ST397) and P26-66 (ST152) are closely related to the South African ST14 and South African ST323 isolates, respectively. As shown, most branches constitute isolates from diverse geographic origin. In **A**, isolate P27-02 clusters with isolates from China, Thailand, Malaysia, UK and USA (diverse STs). The branch **B** isolates, P27-01 and P26-63 are closely related to each other and to ST17-isolates from the USA. In branch **C**, P26-71 clusters with isolates (mainly ST14) from eight countries, including South Africa. In branch **D**, P26-79 relates to isolates from UK and Norway (diverse STs), and in branch **E**, P26-78 is most closely related to isolates of ST493 from the Netherlands and the USA. In branch **F**, P26-66 is located together with isolates of diverse origin and STs, while isolates from four countries (diverse STs) collocate with P26-62 in branch **G**. The ST101-cluster situates on branch **H,** which in addition to the Ghanaian and South African isolates includes isolates from UK and Pakistan.

Table 1. Antibiotic susceptibility profiles of the collected multidrug resistant *K. pneumoniae* (n=10)

Isolate code	Date	SPM	Diagnosis	WT	Susceptibility profile - MICs (mg/L)																
					AMC	TZP	CXM	FOX	CTX	CAZ	CFP	ETP	IMP	MEM	AMK	GEN	CIP	TET	NIT	COL	SXT
P27-01 [20]	06/06/2015	Urine	UTI	O&G	≥32	≥64	≥64	4	≥64	16	8	≤0.5	≤0.25	≤0.25	≤2	≥16	≤0.25	1	64	≤0.5	≥320
P27-02 [46]	22/07/2015	Urine	UTI	Med	≥32	16	≥64	≤4	≥64	4	2	-	≤0.25	≤0.25	≤2	≥16	2	1	64	≤0.5	≥320
P26-62 [70]	13/06/2015	Urine	UTI	Surg	≥32	16	≥64	16	≥64	16	16	≤0.5	≤0.25	≤0.25	≤2	≥16	≤0.25	2	64	≤0.5	≥320
P26-63 [76]	02/06/2015	Urine	UTI	CH	≥32	32	≥64	≤4	32	16	2	≤0.5	≤0.25	≤0.25	≤2	≥16	≤0.25	1	128	≤0.5	≥320
P26-66 [117]	11/03/2015	Urine	UTI	O&G	16	32	≥64	≤4	≥64	16	2	≤0.5	≤0.25	≤0.25	≤2	≥16	≥4	4	≥128	≤0.5	≥320
P26-71 [155]	14/09/2015	Gastric lavage	Gastritis	CH	16	≥64	≥64	≥64	16	16	2	≤0.5	≤0.25	≤0.25	≤2	≥16	≤0.25	1	≤16	≤0.5	≥320
P26-75 [183]	01/07/2015	Urine	UTI	CH	16	≥64	≥64	16	≥64	16	2	≤0.5	≤0.25	≤0.25	≤2	≥16	≥4	4	>256	≤0.5	≥320
P26-78 [201]	19/03/2015	Aspirate	Sinusc itis	ICU	≥32	32	≥64	≤4	≥64	16	2	≤.5	≤.25	≤.25	≤2	≥16	≤0.25	≤1	≤16	≤0.5	≥320
P26-79 [202]	11/03/2015	Urine	UTI	OPD	≥32	128	≥64	≤4	≥64	16	2	≤0.5	≤0.25	≤0.25	≤2	≥16	≤0.25	≤0.5	32	≤0.5	≥320
P26-81 [206]	14/03/2015	Urine	UTI	Med	16	32	≥64	16	≥6	16	2	≤0.5	≤0.25	≤0.2	≤2	≥16	≥4	≤0.5	≥512	≤0.5	≥320
Resistant MICs breakpoint (EUCAST, 2017)					**>8**	**>16**	**>8**	**NA**	**>2**	**>4**	**>4**	**>1**	**>8**	**>8**	**>16**	**>4**	**>0.5**	**>2**	**>64**	**>2**	**>4**

AMC- Amoxicillin-Clavulanate, TZP- Piperacillin Tazobactam, CXM-Cefuroxime, FOX-Cefoxitin CTX- Cefotaxime , CAZ- Ceftazidime, CFP- Cefepime, ETP- Ertapenem, IMP- Imipenem, MRP-Meropenem, AMK- Amikacin, GEN-Gentamicin, CIP-Ciprofloxacin, TET- Tetracycline, NIT- Nitrofurantoin, COL-Colistin, SXT- Trimethoprim-sulfamethoxazole, CH- Child Health, O&G-Obstetrics and Gynaecology, Med-Medicine, Surg- Surgery, WT-Ward type, SPM-Specimen

Table. 2 Genotypic characterizations of multidrug-resistant *K. pneumoniae* isolates from WGS Analysis

Isolate code	MLST	Plasmid replicons	Genotypic Resistance Mechanisms				Chromosomal mutation		
			β-lactamases	Aminoglycosides	Fluoroquinolones/ Quinolones	Other resistance	*gyrA*	*gyrB*	*parC*
P27-01 [20]	ST2171	IncFIA(HI1),IncFIB(K), IncFII(K),IncR	*blaTEM-1B, blaCTX-M-15, blaSHV-11*	*aac(3)-IIa, strB, StrA, ,*	*oqxA, oqxB*	*fosA, sul2, tet(D), dfrA14*			
P27-02 [46]	ST1887	IncHI1B,IncFIB(K),IncFII(K), IncFIB(Mar),ColRNAI	*blaTEM-1B, blaCTX-M-15, blaOXA-1, blaOKP-B-8*	*aac(6')Ib-cr, aac(3)-IIa, aph(3')-Ia*	*qnrB49, qnrB10, oqxA, oqxB,*	*fosA, mph(A), catB4, sul1, dfrA14, dfrA15*	T408A D445E	S558A A692T	S440N, S673N
P26-62 [70]	ST2816	IncFIA(HI1),IncFIB(K), IncFII(K),IncR,ColRNAI	*blaTEM-1B, blaCTX-M-15, blaSHV-11*	*strA, aac(3)-IIa, strB, ,*	*oqxA, oqxB*	*fosA, catA2, sul2, tet(D), dfrA14*			
P26-63 [76]	ST17	IncFIA(HI1),IncFIB(K),IncFII(K), Col(MGD2),IncR,ColRNAI	*blaCTX-M-15, blaSHV-11, blaTEM-1B*	*strA, aac(3)-IIa, strB,*	*oqxA, oqxB,*	*fosA, catA2, sul2, tet(D), dfrA14,*			
P26-66 [117]	ST152	IncFIB(K),IncFII(K),ColRNAI	*blaCTX-M-15, blaOXA-1, blaSHV-1*	*aac(6')Ib-cr, aac(3)-IIa, aac(6')Ib-cr, ,*	*oqxB, oqxA, qnrB66*	*fosA, catB4, sul2, tet(A), dfrA14*	S83F D87A D87G	NM	S80I
P26-71 [155]	ST397	IncFIB(pKPHS1),IncFIB(K), IncFII(K),ColRNAI	*blaTEM-1B, blaSHV-1, blaCTX-M-15*	*aac(6')Ib-cr, aac(3)-IIa, strB, strA,*	*oqxB, oqxA*	*fosA, catB4, sul2, dfrA14*			
P26-75 [183]	ST101	IncFIA(HI1), IncFII,IncFIB(K),ColRNAI	*blaCTX-M-15, blaSHV-1, blaOXA-1*	*aac(6')Ib-cr, aac(3)-IIa, strA, strB*	*qnrB66, oqxA, oqxB,*	*fosA, catB4, sul1, sul2, dfrA14, dfrA5*	S83Y D87A	NM	S80I, N304S
P26-78 [201	ST-1788	IncFIB(pKPHS1),IncFIB(K), IncFII(K),ColRNAI	*blaCTX-M-15,blaSHV-11-like,blaTEM-1B*	*aac(3)-IIa,strA,strB*	*oqxA,oqxB*	*FosA sul2 dfrA14*			
P26-79 [202]	ST789	IncFIA(HI1),IncFIB(K),IncFII(K), Col(MGD2),IncR,ColRNAI	*blaCTX-M-15,blaSHV-25,blaTEM-1B*	*aac(3)-IIa,aadA16-strA,strB*	*oqxA,oqxB*	*fosA sul1,sul2 tet(D) dfrA14 catA2*			
P26-81 [206]	ST-101	IncFIA(HI1),IncFII,IncFIB(K), ColRNAI	*blaCTX-M-15,blaOXA-1,blaSHV-1,blaTEM-1B*	*aac(3)-IIa-strA,strB*	*oqxA-like,oqxB-like,qnrB66 aac(6')Ib-cr*	*fosA sul1,sul2 dfrA14 dfrA5*	S83Y D87A	NM	S80I, N304S

Unless otherwise stated in the footnote, *K. pneumoniae* ATCC 13883 (PRJNA244567) was used as reference strain in the comparative genomic analysis. MLST-mullti locus sequence typing, NM-No mutation.

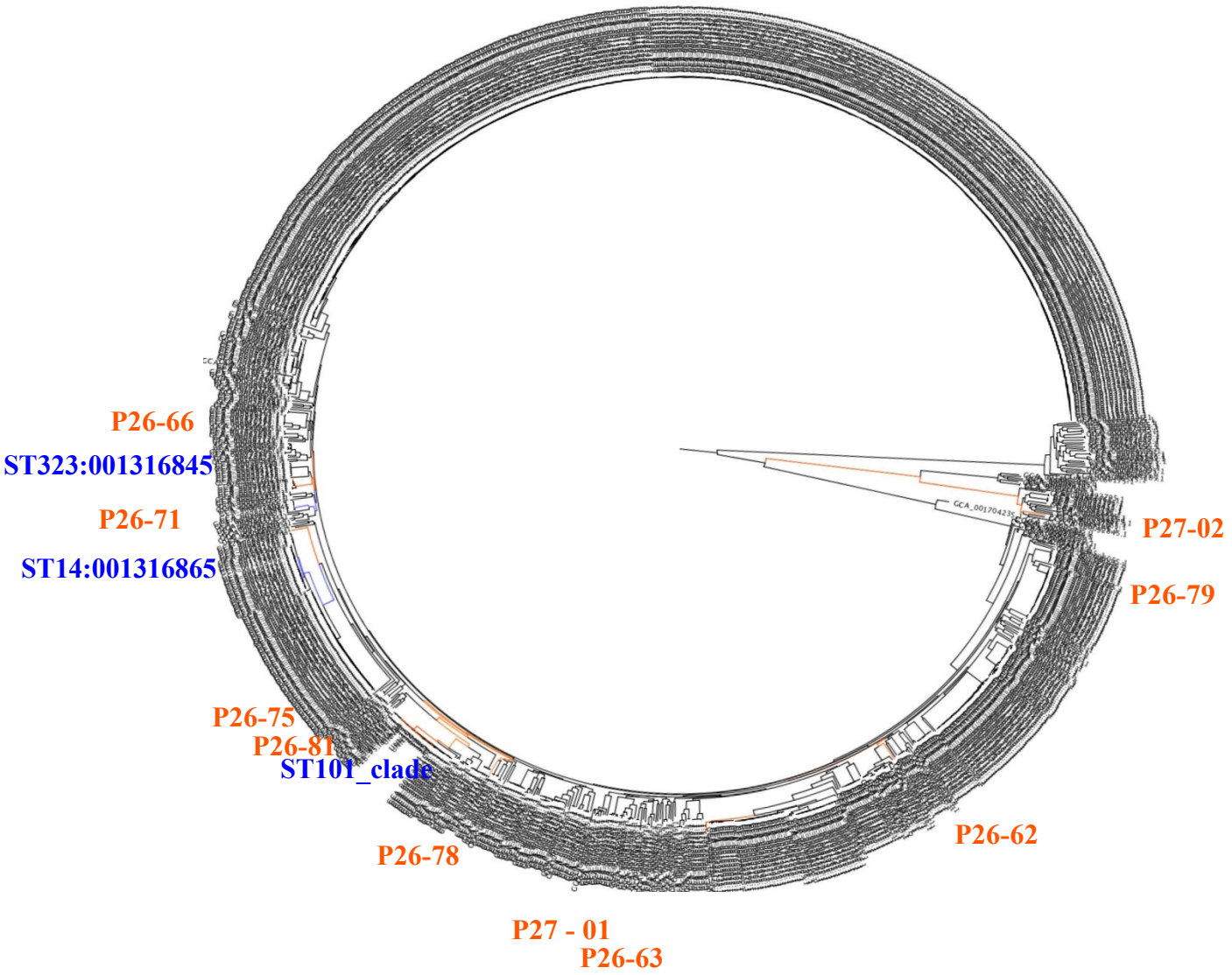

Figure 1a *Klebsiella pneumoniae* global phylogeny as revealed by rapid core genome multi-alignment (https://github.com/marbl/parsnp). Assembly dataset from this study was analyzed together with datasets from the PATRIC database (*n*=1158) and from South Africa (ref. 35). The circular tree was mid-rooted and depicted using Figtree (http://tree.bio.ed.ac.uk/software/figtree/). Assembly ID for the downloaded samples and isolate ID for the samples from Ghana (*n*=10; orange) and South Africa (*n*=20; blue) are given and position of the ST101-cluster indicated.

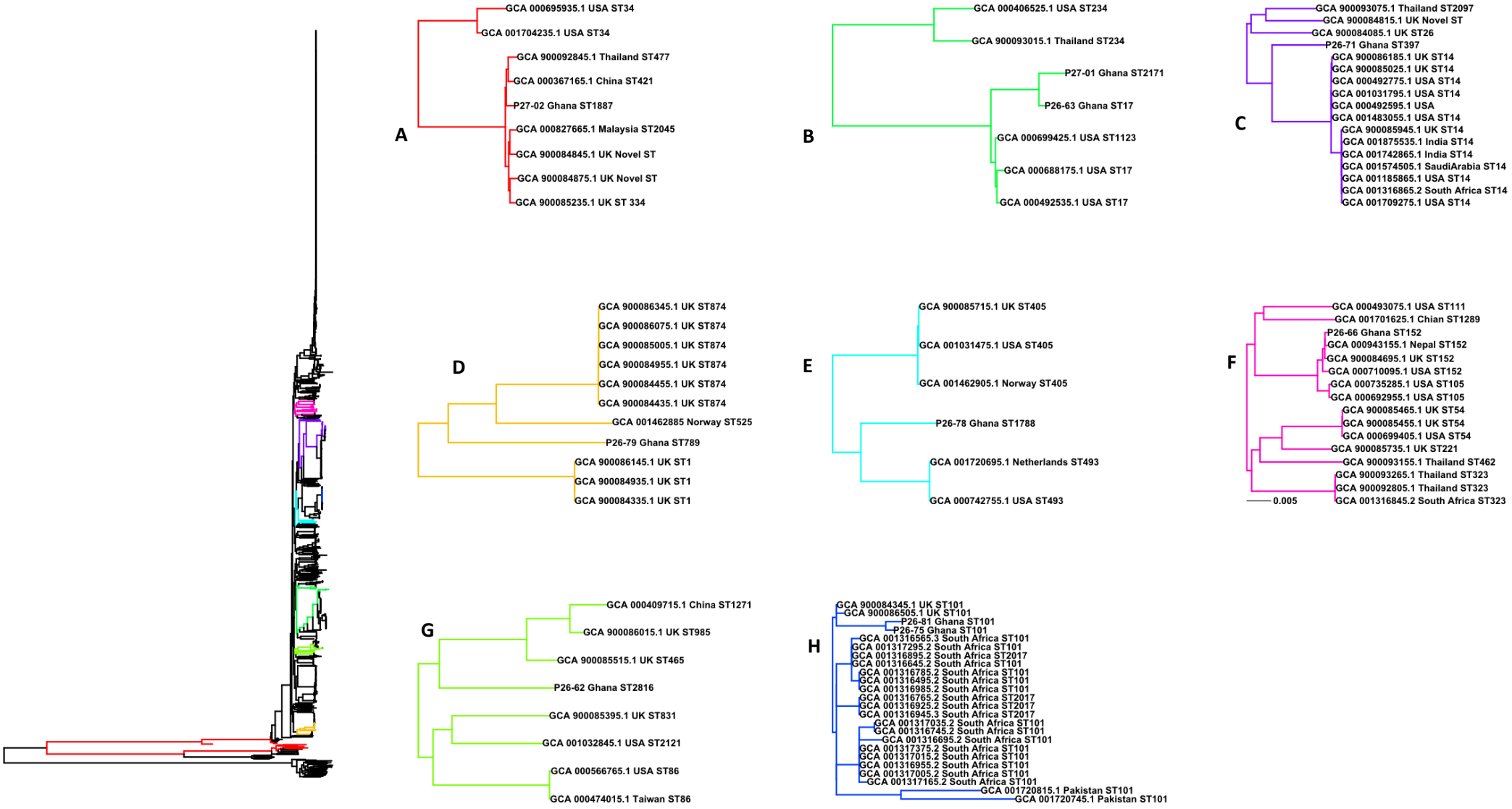
A
GCA 000695935.1 USA ST34
GCA 001704235.1 USA ST34
GCA 900092845.1 Thailand ST477
GCA 000367165.1 China ST421
P27-02 Ghana ST1887
GCA 000827665.1 Malaysia ST2045
GCA 900084845.1 UK Novel ST
GCA 900084875.1 UK Novel ST
GCA 900085235.1 UK ST 334
B
GCA 000406525.1 USA ST234
GCA 900093015.1 Thailand ST234
P27-01 Ghana ST2171
P26-63 Ghana ST17
GCA 000699425.1 USA ST1123
GCA 000688175.1 USA ST17
GCA 000492535.1 USA ST17
C
GCA 900093075.1 Thailand ST2097
GCA 900084815.1 UK Novel ST
GCA 900084085.1 UK ST26
P26-71 Ghana ST397
GCA 900086185.1 UK ST14
GCA 900085025.1 UK ST14
GCA 000492775.1 USA ST14
GCA 001031795.1 USA ST14
GCA 000492595.1 USA
GCA 001483055.1 USA ST14
GCA 900085945.1 UK ST14
GCA 001875535.1 India ST14
GCA 001742865.1 India ST14
GCA 001574505.1 SaudiArabia ST14
GCA 001185865.1 USA ST14
GCA 001316865.2 South Africa ST14
GCA 001709275.1 USA ST14
D
GCA 900086345.1 UK ST874
GCA 900086075.1 UK ST874
GCA 900085005.1 UK ST874
GCA 900084955.1 UK ST874
GCA 900084455.1 UK ST874
GCA 900084435.1 UK ST874
GCA 001462885 Norway ST525
P26-79 Ghana ST789
GCA 900086145.1 UK ST1
GCA 900084935.1 UK ST1
GCA 900084335.1 UK ST1
E
GCA 900085715.1 UK ST405
GCA 001031475.1 USA ST405
GCA 001462905.1 Norway ST405
P26-78 Ghana ST1788
GCA 001720695.1 Netherlands ST493
GCA 000742755.1 USA ST493
F
GCA 000493075.1 USA ST111
GCA 001701625.1 Chian ST1289
P26-66 Ghana ST152
GCA 000943155.1 Nepal ST152
GCA 900084695.1 UK ST152
GCA 000710095.1 USA ST152
GCA 000735285.1 USA ST105
GCA 000692955.1 USA ST105
GCA 900085465.1 UK ST54
GCA 900085455.1 UK ST54
GCA 000699405.1 USA ST54
GCA 900085735.1 UK ST221
GCA 900093155.1 Thailand ST462
GCA 900093265.1 Thailand ST323
GCA 900092805.1 Thailand ST323
GCA 001316845.2 South Africa ST323
0.005
G
GCA 000409715.1 China ST1271
GCA 900086015.1 UK ST985
GCA 900085515.1 UK ST465
P26-62 Ghana ST2816
GCA 900085395.1 UK ST831
GCA 001032845.1 USA ST2121
GCA 000566765.1 USA ST86
GCA 000474015.1 Taiwan ST86
H
GCA 900084345.1 UK ST101
GCA 900086505.1 UK ST101
P26-81 Ghana ST101
P26-75 Ghana ST101
GCA 001316565.3 South Africa ST101
GCA 001317295.2 South Africa ST101
GCA 001316895.2 South Africa ST2017
GCA 001316645.2 South Africa ST101
GCA 001316785.2 South Africa ST101
GCA 001316495.2 South Africa ST101
GCA 001316985.2 South Africa ST101
GCA 001316765.2 South Africa ST2017
GCA 001316925.2 South Africa ST2017
GCA 001316945.3 South Africa ST2017
GCA 001317035.2 South Africa ST101
GCA 001316745.2 South Africa ST101
GCA 001316695.2 South Africa ST101
GCA 001317375.2 South Africa ST101
GCA 001317015.2 South Africa ST101
GCA 001316955.2 South Africa ST101
GCA 001317005.2 South Africa ST101
GCA 001317165.2 South Africa ST101
GCA 001720815.1 Pakistan ST101
GCA 001720745.1 Pakistan ST101

Figure 1b. Phylogenetic tree of *K. pneumoniae* strains from Ghana, South Africa and a global strain collection drawn from the PATRIC database. Subtrees showing the distribution of the Ghanaian isolates in eight in distinct phylogenetic branches (A-H) as indicated by the color codes in the global tree. The subtrees rooted (includes branch lengths) and scale of 0.005 (for all the trees) using (https://cran.r-project.org/web/packages/ape/ape.pdf). For the isolates included in each of these branches, assembly ID, isolation country and MLST are shown.

Discussion

We report on the complexity of multidrug resistance in ESBL-producing *K. pneumoniae* isolates from a referral hospital in Ghana. The isolates were phylogenetically diverse and clustered with geographically distant isolates. They were characterised with diverse and multiple permutations and combinations of antibiotic resistance genes. A high prevalence of CTX-M-15 β-lactamases was observed, which mediated high-level phenotypic resistance to the second- and third-generation cephalosporins as indicated in the MICs profile (Table 1 and 2). The resistance genes were mainly associated with IncFIB(K) plasmids, with ColRNAI also being common among the isolates.

The isolates were resistant to cefuroxime, cefotaxime ceftazidime, amoxicillin-clavulanate, piperacillin-tazobactam, gentamicin, nitrofurantoin and trimethoprim-sulfamethoxazole. This poses a serious challenge to antibiotic therapy as these agents are commonly used as empirical treatment in Ghana (25). The phenotypic profile was corroborated by the whole genome sequencing results as evident from Tables 1 and 2. This is comparable to studies from many parts of the world, which reported CTX-M class of β-lactamases as a major resistance mechanism among Gram-negative bacteria to oxyimino-cephalosporins, particularly cefotaxime (9, 26), with CTX-M-15 being the most common allele in *Enterobacteriaceae* in Africa (27, 28), including Gram-negative ESKAPE bacteria and (28-30), particularly in *K. pneumoniae* (31).

Multiple *K. pneumoniae* STs were identified in lineage with other isolates from a global strain collection, albeit different geographical sources and exchange suggest high genetic diversity and clonal expansion of this species, as reported in other studies (28, 32). The CTX-M-15-producing *K. pneumoniae* ST type 101 was first reported in Greece in an ICU infections outbreak caused by ertapenem-resistant *K. pneumoniae* (33) and then in other countries including Spain, Italy, France and Tunisia in hospital outbreaks associated with carbapenem resistance (34). In contrast, our isolates were sensitive to carbapenems as these agents have been introduced into the Ghanaian clinical practice in recent times, are comparatively more expensive than the mainstay antibiotics and used as last-resort agents in treating serious infections. Thus, there is relatively low selection pressure for the development of carbapenem resistance. Global phylogeny investigation indicated that two of the isolates (P26-75 and P26-81) were of the same sequence type (ST101) as the main cluster of carbapenemase-producing *K. pneumoniae* found in Durban, South Africa. Although, this lineage of ST101 isolates have been evolved in different local environment and resistance pattern including carbapenem resistance differed in that of our isolates. Also the P26-66 and P26-71 isolates were phylogenetically related to the South African ST323 isolate and ST14 isolate respectively (35). This could indicate regional transmission perhaps due to international travel between the two countries facilitating the dissemination of specific *K. pneumoniae* STs.

The predominance of CTX-M-15 and different TEM-types found in this study is associated with multidrug-resistance in *Enterobacteriaceae.* The CTX-M-15 and TEM-, SHV- and OXA-types of β-lactamases are plasmid encoded with the tendency to disseminate among various species to confer resistance to β-lactams and other non-β-lactam antibiotics including quinolones, chloramphenicol, tetracyclines and aminoglycosides (29, 36, 37) as reflected in this study.

The IncFIB(K) and ColRNAI plasmids was found in all the isolates being associated with CTX-M-15 and other resistance genes. This finding is consistent with studies that reported the dissemination of CTX-M-15 as mainly harboured on IncFII(K) plasmids in ESBL-producing *K. pneumoniae* isolates (38, 39). Reports from others studies have described IncFIB(K) plasmids as dynamic in nature, with the capacity for rapid multi-replicon as well as dissemination of antibiotic resistance genes among *Enterobacteriaceae* (40, 41).

Our study found *qnrB10-like, qnrB49* and *qnrB66* variants of the *qnrB* gene in the isolates (P27-02, P26-66, P26-75 and P26-81) which mediated quinolone resistance, consistent with a study that reported this gene as predominantly encoding for fluoroquinolone/quinolone resistance among *K. pneumoniae* in Africa (27). *oqxA* and *oqxB* genes were found together in all the isolates, suggesting that, the *oqxA* and *oqxB* genes cannot be a major mechanism, particularly as they were detected in isolates of both susceptible and much higher MICs or perhaps *oqxAB* in synergy with other mechanism increased fluoroquinolone resistance in the isolates (35). However, studies conducted in Africa have reported *oqxA* and *oqxB* genes encoding oqxAB protein to mediate high fluoroquinolones resistance commonly in *K. pneumonia*e (35, 42). We also identified multiple aminoglycoside (*aac(6')Ib-cr, StrA, StrB*) and quinolone (*qnrB, qnrB66*) resistance genes which have been reported in other studies to mediate resistance to gentamicin and ciprofloxacin (42, 43).

Analysis of quinolone resistance-determining regions of *gyrA*, *gyrB* and *parC* genes revealed the presence of multiple and diverse mutations in *gyrA* (S83Y, S83F, D87A, T408A, and D445E), *gyrB* (S558A, A692T) and *parC* (S440N, S673N, S80I, and N304S) in isolates that were clonally distinct. Mutations in *gyrA*, *gyrB* and *parC* genes have been reported as a major mechanism of fluoroquinolone/quinolone resistance associated with DNA gyrase and topoisomerase IV

alterations in Enterobacteriaceae (44, 45), as the plasmid-mediated resistance genes commonly mediate low-level fluoroquinolone/quinolone resistance (46, 35). However, mutations in both *gyrA* and *parC* are often common and associated with high-level quinolone resistance in Enterobacteriaceae than alterations in *gyrB* (44) as evident in this study. ST101 (P26-75, P26-81) and ST152 (P26-66) isolates had the same mutation codons 83 and 87 of the *gyrA* and at 80 in *parC* genes with no mutation in *gyrB* gene among isolates with a ciprofloxacin MIC of ≥4mg/L. Mutations at 83 and 87 in *gyrA* and 80 in *parC* genes are most common mutation points which display major alterations among clinical isolates, associated with fluoroquinolone resistance (44, 47, 48), with codon 83 commonly reported in fluoroquinolone resistant *K. pneumoniae* (45, 49). The combined effect of S83Y/F, D87A and S80I detected in *gyrA* and *parC* genes in the isolates, P26-66, P26-75 and P26-81 (MIC ≥4mg/L) in our study could be associated with increased-level of ciprofloxacin resistance as previously reported (44, 48). The complexity and diversity of resistance gene combinations detected among *K. pneumoniae* strains in this study and their potential for dissemination poses a serious threat to the management of infections by this species in Ghana.

Conclusion

This study identified genes encoding resistance for β-lactams, fluoroquinolones, aminoglycosides and other antibiotics in diverse permutations and combinations among multidrug-resistant *K. pneumoniae* bacteria in Komfo Anokye Teaching Hospital. There is thus an urgent need for epidemiological and molecular studies to understand the dynamics of antibiotic resistance transmission to inform strategies for containment.

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Authors' contribution

The study was co-conceptualized and jointly designed by NA, UG, AO, MA, DG, TP, AS and SE. NA collected the data undertook the preliminary laboratory work and present result as tables. AO supervised the sampling and preliminary laboratory work. UG supervised the isolates phenotypic screening and analysis. MA and DG contributed to the bioinformatics data analysis. TP contributed to isolation of high quality DNA and illumina bioinformatics analyses. AS contributed to data analyses and assuring the quality of the result. SE contributed to assuring the quality of the final manuscript. All the authors contributed in preparation and submission of manuscript.

Competing interests

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Supplementary Material: MICs

Isolate code	SPM	Diagnosis	Susceptibility profile - MICs (mg/L)																
			AMC	TZP	CXM	FOX	CTX	CAZ	CFP	ETP	IMP	MEM	AMK	GEN	CIP	TET	NIT	COL	SXT
P27-01 [20]	urine	UTI	≥32	≥64	≥64	4	≥64	16	8	≤0.5	≤0.25	≤0.25	≤2	≥16	≤0.2	1	64	≤0.5	≥320
P27-02 [46]	urine	UTI	≥32	16	≥64	≤4	≥64	4	2	-	≤0.25	≤0.25	≤2	≥16	2	1	64	≤0.5	≥320
P26-62 [70]	urine	UTI	≥32	16	≥64	16	≥64	16	16	≤0.5	≤0.25	≤0.25	≤2	≥16	≤0.25	2	64	≤0.5	≥320
P26-63 [76]	urine	UTI	≥32	32	≥64	≤4	32	16	2	≤0.5	≤0.25	≤0.25	≤2	≥16	≤0.25	1	128	≤0.5	≥320
P26-66 [117]	urine	UTI	16	32	≥64	≤4	≥64	16	2	≤0.5	≤0.25	≤0.25	≤2	≥16	≥4	4	≥128	≤0.5	≥320
P26-71 [155]	gastric lavage	gastritis	16	≥64	≥64	≥64	16	16	2	≤0.5	≤0.25	≤0.25	≤2	≥16	≤0.25	1	≤16	≤0.5	≥320
P26-75 [183]	urine	UTI	16	≥64	≥64	16	≥64	16	2	≤0.5	≤0.25	≤0.25	≤2	≥16	≥4	4	>256	≤0.5	≥320
P26-78 [201]	aspirate	sinusciti s	≥32	32	≥64	≤4	≥64	16	2	≤.5	≤.25	≤.25	≤2	≥16	≤.25	≤1	≤16	≤0.5	≥320
P26-79 [202]	urine	UTI	≥32	128	≥64	≤4	≥64	16	2	≤0.5	≤0.25	≤0.25	≤2	≥16	≤0.25	≤0.5	32	≤0.5	≥320
P26-81 [206]	urine	UTI	16	32	≥64	16	≥64	16	2	≤0.5	≤0.25	≤0.2	≤2	≥16	≥4	≤0.5	≥512	≤0.5	≥320
13	urine	UTI	≤2	8	≥64	≤4	≤1	≤1	2	≤1	≤0.25	≤0.25	≤2	≥16	≥4	≤0.5	≤16	≤0.5	≥320
16	blood	sepsis	≤2	8	≥64	≤4	≤4	4	2	≤0.5	≤0.25	≤0.25	≤2	≥16	≥4	≤0.5	≤16	≤0.5	≥320
27	urine	UTI	≥32	8	≥64	≥64	≤4	4	2	≤0.5	2	≤ 0.5	≤2	≤1	≤0.25	1	≥512	_	80
39	urine	UTI	≤2	≤4	≤1	≤4	≤1	≤1	≤1	≤0.5	≤0.2	≤0.25	≤2	≤1	≤0.25	≤0.5	32	≤0.5	≤20
40	urine	sepsis	≥32	≥64	≥64	≤4	8	16	8	≤ 0.5	≤0.25	≤0.25	≤2	≥16	≤0.25	1	64	≤ 0.5	≥320
53	blood	enteriti s	16	64	≥64	16	≤4	4	2	≤0.5	≤0.25	≤0.25	≤2	≥16	≥4	4	256	≤0.5	≥320

56	w/swab	W/I	16	8	≥64	≤4	16	2	2	≤0.5	≤0.25	≤0.25	≤2	≥16	≥4	≤0.5	≤16	≤0.5	≥320
61	urine	nephritis	16	8	≥64	≤4	8	8	2	≤0.5	≤0.25	≤0.25	≤2	≥16	1	1	≤16	≤0.5	≥320
64	urine	UTI	16	8	≥64	≤4	32	4	2	≤0.5	≤0.25	≤0.25	≤2	≥16	1	1	≤16	≤0.5	≥320
75	urine	UTI	8	≤4	≥64	16	8	8	8	≤0.5	≤0.25	≤0.25	≤2	≥16	≥4	≤0.5	≤16	≤0.5	≥320
83	Pleural swab	tuberculosis	16	64	≥64	16	8	2	2	≤0.5	≤0.25	≤0.25	≤2	≥16	≥4	≥4	256	≤0.5	≥320
84	w/swab	W/I	16	8	≥64	≤4	32	8	2	≤0.5	≤0.25	≤0.25	≤2	≥16	≥4	≤0.5	64	≤0.5	≥320
99	urine	UTI	≤2	16	≥64	16	≤4	16	2	≤0.5	≤0.25	≤0.25	≤2	≥16	≥4	≥4	≥128	≤0.5	≥320
104	urine	UTI	≥32	64	≥64	≤4	32	8	2	≤1	≤0.25	≤0.25	≤2	≥16	≥4	≤0.5	≤16	≤0.5	≥320
105	w/swab	W/I	16	8	≥64	≤4	32	8	2	≤0.5	≤0.25	≤0.25	≤2	≥16	≥4	≤0.5	64	≤0.5	≥320
106	urine	UTI	16	≥64	≥64	32	16	8	4	≤0.5	≤0.25	≤0.25	4	≥16	≥16	≤0.5	≤16	≤0.5	≥320
116	urine	urosepsis	≥32	8	≥64	32	4	8	≤1		≤1	≤0.25	≤2	≤1	≥8	≥4	≥512	≤0.5	≥320
120	urine	UTI	8	≤4	≥64	16	16	4	8	≤0.5	≤0.25	≤0.25	≤2	≥16	≤2	≥4	≤16	≤0.5	≥320
122	urine	nephritis	16	16	≥64	≤4	≥64	4	2	≤0.5	≤0.25	≤0.25	≤2	≥16	2	4	64	≤0.5	≥320
126	urine	UTI	8	≤4	2	16	≤4	≤1	≤1	≤0.5	≤0.25	≤0.25	≤2	≤1	≤2	≤0.5	≤16	≤0.5	≥320
127	blood	sepsis	4	16	≥64	≤4	16	16	2	≤0.5	≤0.25	≤1	≤2	≥16	≤0.25	1	64	≤0.5	≥320
132	w/swab	W/I	≥32	≥64	≥64	8	≥64	16	2	≤0.5	≤0.25	≤0.25	≤2	≥16	≥4	4	≥128	≤0.5	≥320
135	sputum	tuberculosis	16	8	≥64	≤4	32	8	2	≤0.5	≤0.25	≤0.25	≤2	≥16	1	≤0.5	≤16	≤0.5	≥320

143	urine	UTI	16	≤4	16	2	16	≤1	≤1	≤0.5	≤0.25	≤0.25	≤2	≤1	≤0.25	1	32	≤0.5	≥320
165	B/L	bronch	≤2	8	≥64	≤4	32	8	2	≤1	≤0.25	≤0.25	≤2	≥16	≥4	≤0.5	≤16	≤0.5	≥320
172	w/swab	WI	≥32	32	≥64	≤4	≥64	4	2	≤0.5	≤0.25	≤0.25	≤2	≥16	≤0.25	≤1	≤16	≤0.5	≥320
193	sputum	pneum	16	8	≥64	≤4	32	4	2	≤0.5	≤0.25	≤0.25	≤2	≥16	≥4	≤0.5	64	≤0.5	≥320
208	w/swab	WI	16	≤4	64	≤4	32	8	2	≤0.5	≤0.25	≤0.25	≤2	≤1	2	1	128	≤0.5	≥320

AMC- Amoxicillin-Clavulanate, TZP- Piperacillin Tazobactam, CXM-Cefuroxime, FOX-Cefoxitin CTX- Cefotaxime , CAZ- Ceftazidime, CFP- Cefepime, ETP- Ertapenem, IMP- Imipenem, MRP-Meropenem, AMK- Amikacin, GEN-Gentamicin, CIP-Ciprofloxacin, TET- Tetracycline, NIT- Nitrofurantoin, COL-Colistin, SXT- Trimethoprim-sulfamethoxazole, W/swab-wound swab, W/I-wound infection, pneum-pneumoniae. B/L-bronchial lavage, bronch-bronchitis, UTI-urinary tract infection.

CHAPTER FOUR

MANUSCRIPT THREE: Characterization of *bla*$_{\text{DIM-1}}$ and *bla*$_{\text{IMP-34}}$-Encoding Genetic Structures in *Pseudomonas aeruginosa* From a Tertiary Hospital in Ghana

Author's contributions

- N Agyepong, as the principal investigator, co-conceptualized the study, undertook the preliminary laboratory work, and drafting of manuscript
- A. Owusu-Ofori as co-supervisor, co-conceptualized the study, supervised the sampling and preliminary laboratory work
- T. Pedersen, contributed to data analyses and writing process, focusing on the results and discussion
- Ø. Samuelsen contributed to data analyses and writing process, focusing on the introduction and discussion
- J. Janice contributed to data analyses, figures and writing process
- A. Sundsfjord undertook critical revision of the manuscript
- S. Y. Essack, as principal supervisor, co-conceptualized the study and undertook critical revision of the manuscript

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Characterization of *bla*$_{DIM-1}$- and *bla*$_{IMP-34}$-encoding Genetic Structures in *Pseudomonas aeruginosa* From a Tertiary Hospital in Ghana

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Abstract

Metallo-β-lactamases (MBLs) are significant resistance factors in Gram-negative bacterial pathogens, which confer resistance to broad-spectrum β-lactams including carbapenems. A number of such enzymes have been described over the past decade. This study characterized isolates from a single, clinically significant MBL-producing *P. aeruginosa* clone from a tertiary hospital in Ghana whose MBL genes were identified in a class 1 integron. Isolates were identified by the Vitek-2 (Biomerieux, France) automated systems and confirmed by MALDI-TOF MS (Bruker Daltonic. Genomic DNA extraction was carried out using the NucliSens

easyMAG® (BioMérieux) kits. Extracted DNA was subjected to whole genome sequencing on the Illumina MiSeq platform and analysed using CLC Genomics Workbench version 10. The *bla*$_{\text{DIM-1}}$ was 100% identical to the *bla*$_{\text{DIM-1}}$ prototype gene, whereas *bla*$_{\text{IMP-34-like}}$ had two base pair changes in thymine (T) to cytosine (C) and guanine (G) to adenine (A) at 190 bp and 314 bp respectively compared to *bla*$_{\text{IMP-34}}$ suggesting evolution of the gene perhaps through selection pressure. Both *bla*$_{\text{DIM-1}}$ and *bla*$_{\text{IMP-34-like}}$ genes were linked to the type 1 integron structure. The four isolates belonged to the sequence type ST234. All the isolates carried other resistance genes most commonly *aph*(3')-*IIb*-like, *qnrVC*, *fosA-like*, *sul1*, *dfrB5, catB7*-like and *arr-2* that mediated resistance to aminoglycoside, fluoroquinolone, fosfomycin, sulphonamide trimethoprim, phenicol and rifampin respectively. Detection of acquired MBL in clinical *P. aeruginosa* isolates is a clear cause for concern in Ghana as they eliminate the last resort carbapenems as therapeutic options for the management of multi-resistant Gam-negative pathogens.

Keywords: *Pseudomonas aeruginosa,* IMP, DIM, carbapenemase, Ghana.

Introduction

Pseudomonas aeruginosa is a significant cause of nosocomial infections particularly in patients with compromised immunity. It is widespread in nature and readily acquires resistance to multiple classes of antibiotics. The emergence of multi-drug resistant (MDR) *P. aeruginosa* in hospitals and communities has led to a loss in the efficacy of most currently available antibiotics used for infection management (1). The mortality associated with MDR *P. aeruginosa* remains high. Its resistance to β-lactam antibiotics may be attributed to one or a combination of three mechanisms, i.e., porin alteration or loss, efflux and the expression of β-lactamases from all

four Ambler classes (2). The β-lactamase genes may be encoded chromosomally or borne by a diversity of mobile genetic elements (3, 4).

The class A ESBLs including Temoneira (TEM-24), sulfhydryl variable (SHV-12), Cefotaximase-München (CTX-M-1, CTX-M-2, CTX-M-3), Vietnam extended-spectrum β-lactamase (VEB) as well as *Klebsiella pneumoniae* carbapenemases (KPC-2) have all been isolated from *P. aeruginosa* (5, 6). The metallo-β-lactamases, comprising of Verona integron-encoded metallo-β-lactamases (VIM), impenemases (IMP), New Delhi metallo-β-lactamases (NDM) and Dutch impenemases (DIM) have also been isolated from *P. aeruginosa* in many parts of the world, as have been the cephamycinase (CMY), an AmpC-β-lactamase, and the oxicillinases (OXA) of the class D β-lactamases (7, 8). Various mobile genetic element (MGEs) that carry MBL genes have been identified, but the majority of them are found in the form of gene cassettes (*bla*$_{IMP}$-like, *bla*$_{VIM}$-like, *bla*$_{DIM}$-1, and *bla*$_{GIM}$-1) embedded into class 1 integron structures (9). Delineation of the clones and MGEs encoding the resistance genes in this pathogen is critical to contain their proliferation. We report on the complexity and diversity of MBL-mediated resistance from four isolates of *P. aeruginosa* from a teaching hospital in Ghana.

Materials and Methods

Ethical approval: Approval for this study was given by the Joint Committee on Human Research, Publications and Ethics, School of Medical Sciences, Kwame Nkrumah University of Technology, the Ethics Committee of Komfo Anokye Teaching Hospital (ref: CHRPE/AP/015/15), in Kumasi Ghana and the Biomedical Research Ethics Committee of the University of KwaZulu-Natal, South Africa with reference number BE 494/14.

Bacteria isolates: Multi-drug resistant *P. aeruginosa* were isolated from in-patients at Komfo Anokye Teaching Hospital in Ghana. The clinical isolates were identified by the Vitek-2 (Biomerieux, France) automated system and confirmed by MALDI-TOF MS (Bruker Daltonic Gmbh, Bremen, Germany). Four (4) out of thirty nine (39) isolates were selected for whole-genome sequencing analysis based on their extensively drug-resistant (XDR) (isolate resistant to at least one agent in all but two or fewer antibiotic class) profiles (supplementary material MICs). Of the four samples from the in-patients, two were obtained from wound swabs in the surgical ward and one each from urine and pleural fluid in the Obstetrics and Gynaecology and Child Health Departments of the hospital respectively. Demographic characteristics and clinical diagnosis of each patient included in the study were obtained from the clinical records.

Antimicrobial susceptibility testing: MICs of ampicillin, amoxicillin/clavulanate, piperacillin/tazobactam, cefuroxime cefotaxime, ceftazidime cefepime, gentamicin, amikacin, imipenem meropenem, ertapenem, and sulfamethoxazole/trimethoprim were ascertained by the Vitek-2 (Biomerieux, France) automated system and confirmed by broth microdilution in accordance with European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (10)

Whole Genome Sequencing: Genomic DNA extraction was carried out using the NucliSens easyMAG® (BioMérieux) kits according to the manufacturers' instructions. The genomic DNA libraries were generated using the Nextera® kit (Illumina) followed by sequencing on an Illumina MiSeq platform at the Genomics Resource Center at UiT – The Arctic University of Norway.

Bioinformatics analyses: Raw sequence reads were adaptor- and quality-trimmed using Trimmomatic (11) and subsequently assembled with Spades v.3.6.1 (12) using the careful flag.

For in-house analysis purposes, assemblies were annotated using prokka v.1.11(13) with further NCBI BLAST searches and manual annotation of mobile genetic elements and regions containing resistance genes as identified by ResFinder searches. Assemblies deposited in GenBank were annotated using the PGAP pipeline provided by NCBI (http://www.ncbi.nlm.nih.gov/genome/annotation_prok/), with additional manual curation of resistance gene and mobile genetic element annotations. Identification of resistance genes, particularly β-lactamases, was done with RAST 2.0 (14, 15). To identify the presence of plasmid DNA in isolates, raw reads were mapped to plasmid reference using bowtie2 v.2.2.6 (16) with "dovetail-no-unal"-flags enabled, and resulting bam files were passed through the BRIG coverage graph plugin to be used as input to BRIG (17) together with reference plasmids. Phylogenetic analyses were conducted by binning isolate assemblies by species and running them through parsnp v.1.2 (18) with "–c –x"-flags enabled. Phylogenetic trees were later edited with FigTree (http://tree.bio.ed.ac.uk/software/figtree/). Multi-locus sequence typing was done by feeding assemblies into the CGE MLST-server (v.1.8) (18). To gain further insight into the genetic structures one of the isolates, P26-65, was subjected to long read sequencing by Oxford Nanopore.

The raw read sequences and the assembled whole genome contigs have been deposited at the sequence read archive (SRA) and GenBank of NCBI, respectively, under bio-project number PRJNA411997.

RESULTS

Demographics, sample sources, wards, species and clones

The four extensively drug resistant (XDR) *P. aeruginosa* had similar resistance profiles (Table 1) were selected for genetic analyses by whole genome sequencing (WGS). The isolates originated

from different patients and sources, and were collected at different time points and wards at the Komfo Anokye Teaching Hospital in Ghana (**Table 1**). Bioinformatics analyses (**Table 2, Table S2**) show all four isolates had the same sequence type of ST234.

Genetic analyses of the *bla*$_{\text{DIM-1}}$ and *bla*$_{\text{IMP-34}}$-like containing regions

The strain (P26-65) chromosome was circularized (**Figure 3**) and no plasmids were present. The resistance-encoding genes are marked in the figure together with the detected integron structures, i.e., integron 1 containing *bla*$_{\text{DIM-1}}$ and integron 2 containing *bla*$_{\text{IMP-34}}$-like gene. Further insights into the *bla*$_{\text{DIM-1-}}$ and *bla*$_{\text{IMP-34}}$-like-encoding regions of the P26-85 chromosome were obtained by alignment with the corresponding regions of *P. aeruginosa* strain (SMC4389) of ST654 isolated in India by the use of Artemis Comparison Tool (ACT) (19). In **Figure 4a** (*bla*$_{\text{DIM-1}}$) and **Figure 4b** (*bla*$_{\text{IMP-34}}$-like) the putative integron structures (white arrows) are indicated. Moreover, the alignments revealed the presence of two genomic islands (green arrows) containing each of the integron structures. Both islands represent insertion of a DNA segment into the chromosome.

The detected resistance encoding genes were identical in the four ST234 isolates, with the exception of *qnrVC,* which was additionally detected in P26-65 and P26-68; all four isolates were resistant to ciprofloxacin. Of specific interest are the *bla*$_{\text{DIM-1}}$ and *bla*$_{\text{IMP-34}}$-like genes detected in these four isolates. Alignment of the *bla*$_{\text{DIM-1}}$-positive contigs from each of the four samples showed that they had identical DNA sequences and sequence sizes (3093 bp); indicating that both resistance genes are flanked by repetitive elements that impairs the DNA assembly at this site. The same was shown for the *bla*$_{\text{IMP-34-like}}$ positive contigs (2656 bp). Comparisons of the detected *bla*$_{\text{DIM-1}}$ was 100% identical to the *bla*$_{\text{DIM-1}}$ prototype gene, whereas the *bla*$_{\text{IMP-34}}$-like had two nucleotide changes in T190C and C314G respectively compared to *bla*$_{\text{IMP-34,}}$ leading to

one amino acid substitution in IMP-34-like β-lactamase. The base substitutions G:C→ A:T transitions or transversions are the most common spontaneous mutation type among bacteria (20-22). Also the variation or mismatch in GC-content is highly influenced by natural selection or biased gene conversion (23), thus, suggesting, the *bla*$_{\text{IMP-34}}$ gene may have evolved through selection pressure. BLAST analyses of the *bla*$_{\text{DIM-1}}$-positive contig as well as the *bla*$_{\text{IMP-34}}$-positive contig did not reveal identical genetic structures deposited in NCBI, neither among the nucleotide collection, completed genomes nor among the completed plasmids. Moreover both *bla*$_{\text{DIM-1}}$ and *bla*$_{\text{IMP-34}}$ genes were not found to be widespread among bacterial sequences, deposited in NCBI (assessed November 2017). A linkage of both the *bla*$_{\text{DIM-1}}$ and *bla*$_{\text{IMP-34}}$-like genes to type1 integron structures, including the *intI1* integrase encoding gene, was found by BLAST analyses of the assembled contigs.

Phylogenetic analyses

To investigate the global phylogeny and likely origins of these isolates, we added *P. aeruginosa* genome assembly datasets (*n*=417) downloaded from the PATRIC database (https://www.patricbrc.org/) as shown in **Figure 1a**. For each isolate included in the tree, country of origin, isolation source and MLST are given. The tree was coupled with the metadata using Phandango with the input files included (**Figure S1** and **Table S1**). Arrows indicate the *P. aeruginosa* isolates from this study. In **Figure 1b**, the phylogenetic branch containing the *bla*$_{\text{DIM-1}}$ positive isolates were magnified. None of the closest neighbors encodes *bla*$_{\text{DIM-1}}$ gene. They include isolates of ST111, ST381, ST252, ST654, and ST392 from different isolation sources and countries. Among the included genomes, one was positive for *bla*$_{\text{DIM-1}}$ (GCA_001548335; blue) and four for *bla*$_{\text{IMP-34}}$ (GCA_00291745; GCA_001547955; GCA_002201405;

GCA_02201455; red). These were closely related to the isolates from this study (indicated in orange) as evident in phylogenetic tree shown in **Figure 2**.

Table 1. Relevant patient data, source of specimens and antibiotic susceptibility of *P. aeruginosa* isolates (n=4) from teaching hospital in Ghana

Extensively Drug resistant (XDR) isolates

Isolate		Patient					Antibiotic Susceptibility profile - MICs (mg/L)															
Code	Collection Date	WT	Sex	Age Yrs	Source	Diagnosis	AMP	AMC	TZP	CXM	FOX	CTX	CAZ	CFP	IMP	MEM	AMK	GEN	CIP	TET	NIT	COL	SXT
P26-65 [97]	25/08/2015	OG	F	18	Urine	UTI	≥32	≥32	≥128	≥64	≥64	≥64	≥64	≥64	≥16	≥16	≥64	≥16	≥4	≥8	≥512	≤0.5	≥320
P26-67 [130]	06/08/2015	Surg	F	24	w/ swab	SSI	≥32	≥32	128	≥64	≥64	≥64	≥64	≥64	≥ 16	4	≥32	≥ 16	≥4	≥8	≥512	≤0.5	≥320
P26-68 [140]	06/09/2015	Surg	M	8	w/ swab	SSI	≥32	≥32	128	≥64	≥64	≥64	≥64	≥64	≥ 16	4	≥32	≥ 16	≥4	≥8	≥512	≤0.5	≥320
P26-69 [142]	31/07/2015	CH	F	0.5	P/ Fluid	Peritonitis	≥32	≥32	128	≥64	≥64	≥64	≥64	≥64	≥ 16	≥ 16	≥64	≥ 16	≥4	≥8	≥512	≤0.5	≥320
EUCAST clinical breakpoints																							
Resistant MICs breakpoint							_	_	>16	_	NA	_	>8	>8	>8	>8	>16	>4	>0.5	>2	_	>2	_

AMC- Amoxicillin-clavulanate, TZP- Piperacillin-Tazobactam, CXM-Cefuroxime, FOX-Cefoxitin CTX- Cefotaxime , CAZ- Ceftazidime, CFP- Cefepime, ETP- Ertapenem, IMP- Imipenem, MEM-Meropenem, AMK- Amikacin, GEN-Gentamicin, CIP-Ciprofloxacin, TET- Tetracycline, NIT- Nitrofurantoin, COL-Colistin, SXT- trimethoprim-sulfamethoxazole, CH- Child Health, O&G-Obstetrics and Gynecology, Med-Medicine, Surg- Surgery, WT-Ward type, SPM-Specimen, SSI-Surgical site infection, P/fluid-pleural fluid, w/swab-wound swab.

Table 2. Genotypic characteristics of *P. aeruginosa* isolates subjected to whole genome sequencing

Isolate P-No. [code]	Genome Size	Coverage (x)	No. of contigs	MLST	Data Files	Resistance Genes							
						β-lactams	**Aminoglycosides**	**Quinolones**	**Fosfomycin**	**Sulphonamide**	**Trimethoprim**	**Phenicols**	**Rifampin**
P26-65 [97]	6914056	134	196	234	SAMN07692776	*bla$_{DIM-1}$, bla$_{IMP-34}$-like, bla$_{OXA-10}$-like, bla$_{OXA-129}$, bla$_{OXA-50}$-like, bla$_{PAO}$-like*	*aadA1-like, aph(3')-IIb-like, aacA4*	*qnrVC*	*fosA-like*	*sul1*	*dfrB5*	*catB7-like*	*arr-2*
P26-67 [130]	6897550	98	304	234	SAMN07692777	*bla$_{DIM-1}$, bla$_{IMP-34}$-like, bla$_{OXA-10}$-like, bla$_{OXA-129}$, bla$_{OXA-50}$-like, bla$_{PAO}$-like*	*aadA1-like, aph(3')-IIb-like, aacA4*		*fosA-like*	*sul1*	*dfrB5*	*catB7-like*	*arr-2*
P26-68 [140]	6849434	111	332	234	SAMN07692778	*bla$_{DIM-1}$, bla$_{IMP-34}$-like, bla$_{OXA-10}$-like, bla$_{OXA-129}$, bla$_{OXA-50}$-like, bla$_{PAO}$-like*	*aadA1-like,aph(3')-IIb-like, aacA4*	*qnrVC*	*fosA-like*	*sul1*	*dfrB5*	*catB7-like*	*arr-2*
P26-69 [142]	6944596	54	660	234	SAMN07692779	*bla$_{DIM-1}$, bla$_{IMP-34}$-like, bla$_{OXA-10}$-like, bla$_{OXA-129}$, bla$_{OXA-50}$-like, bla$_{PAO}$-like*	*aadA1-like, aph(3')-IIb-like, aacA4*		*fosA-like*	*sul1*	*dfrB5*	*catB7-like*	*arr-2*

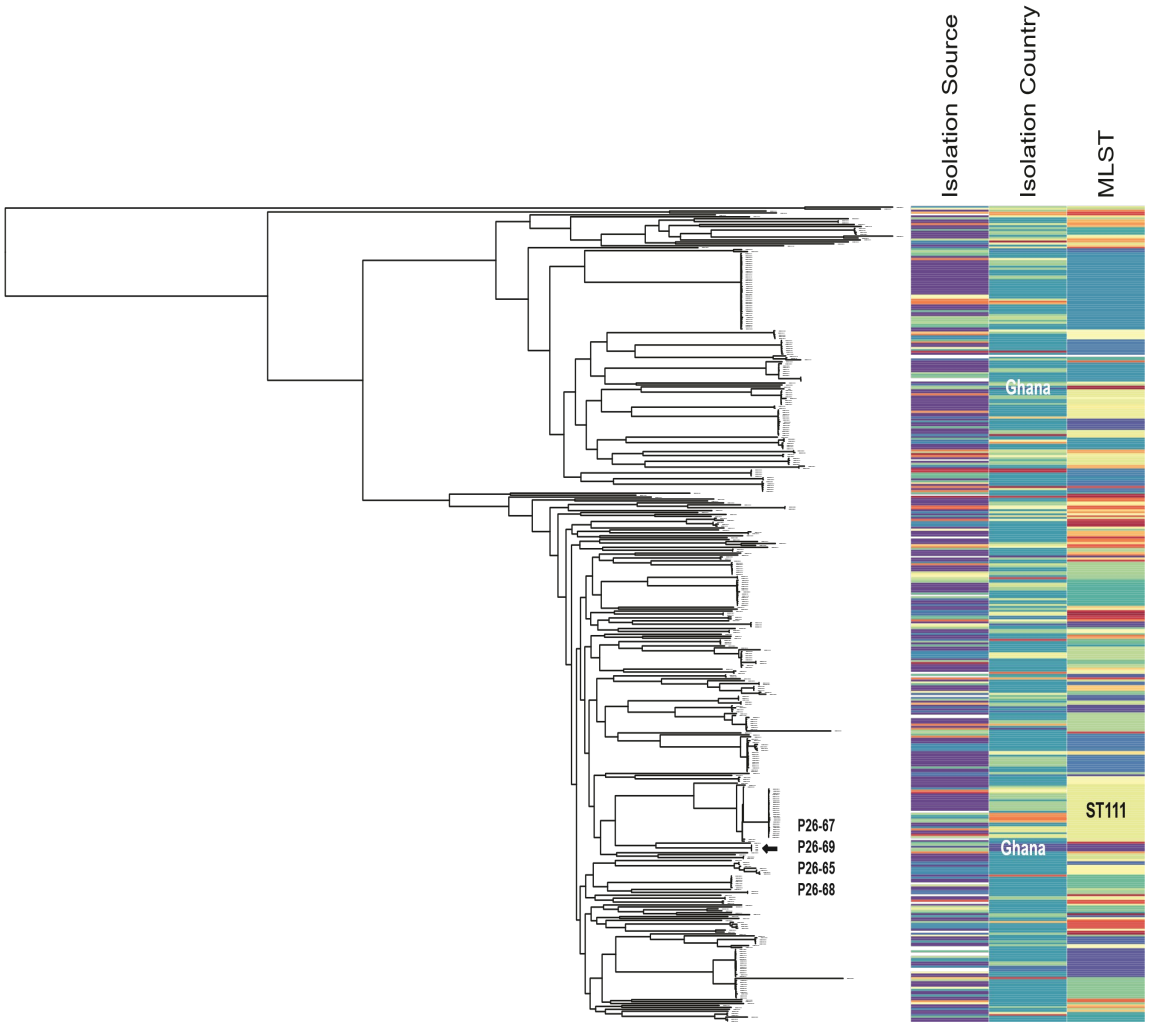

Figure 1a. Phylogenetic tree of *P. aeruginosa* genome assembly datasets (n=417) downloaded from the PATRIC database (https://www.patricbrc.org/). Clustering of the strains into clades was mainly by country of origin, isolation source and MLST. Arrows indicate the *P. aeruginosa* isolates from Ghana. MLST: multi-locus sequence typing.

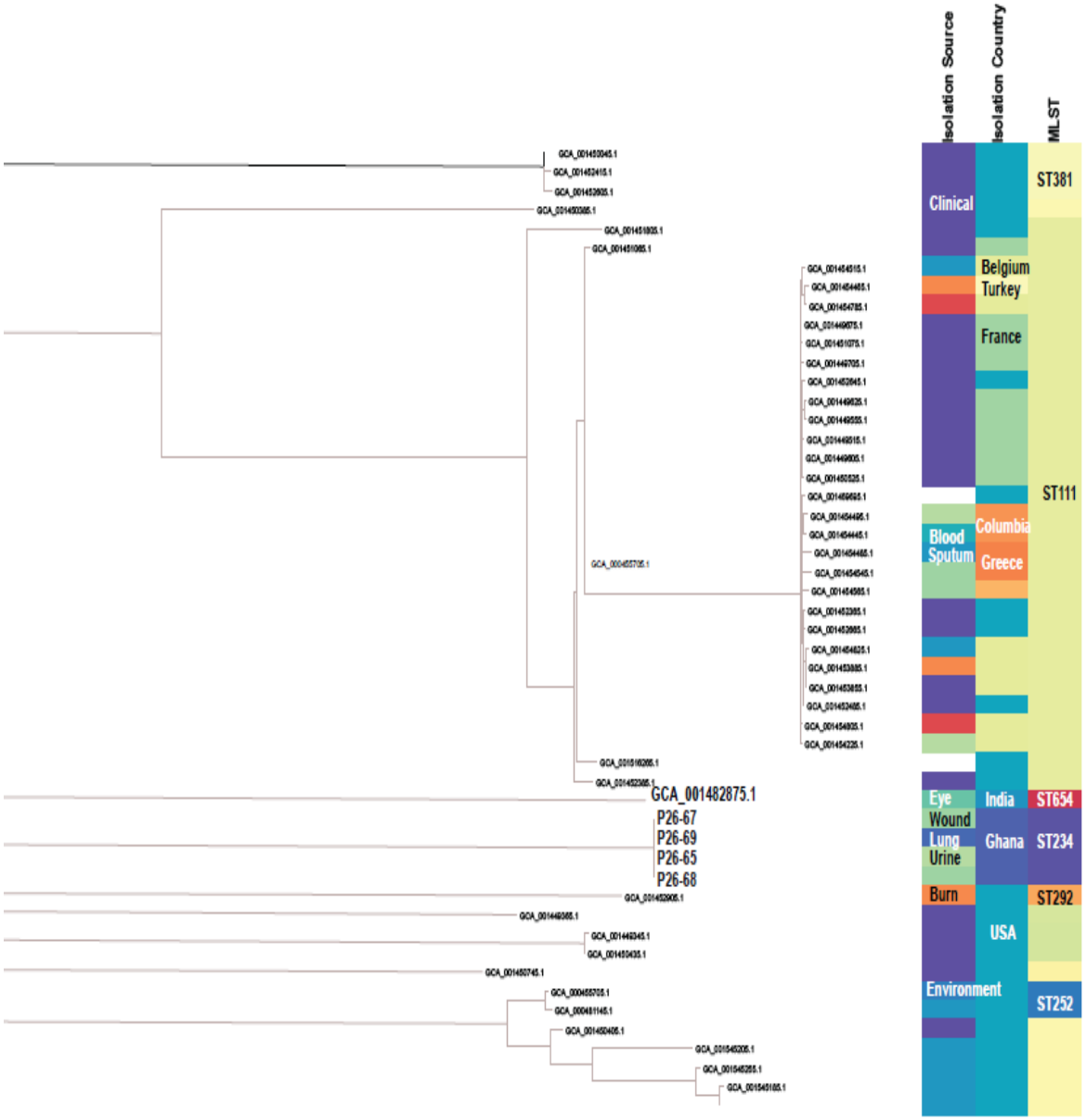

Figure 1b. **Phylogenomic tree containing encompassing *bla*$_{\text{DIM-1}}$ and *bla*$_{\text{DIM-1}}$ positive *P. aeruginosa* isolates**. Clustering of the strains into clades were isolates of ST111, ST381, ST252, ST654, and ST392 from different isolation sources (clinical and environment) and countries (Belgium, Turkey, France, Colombia, Greece, India, Ghana and USA). The genomic phylogeny shows that, none of the closest neighbors encoded *bla*$_{\text{DIM-1}}$ gene. MLST: multi-locus sequence typing.

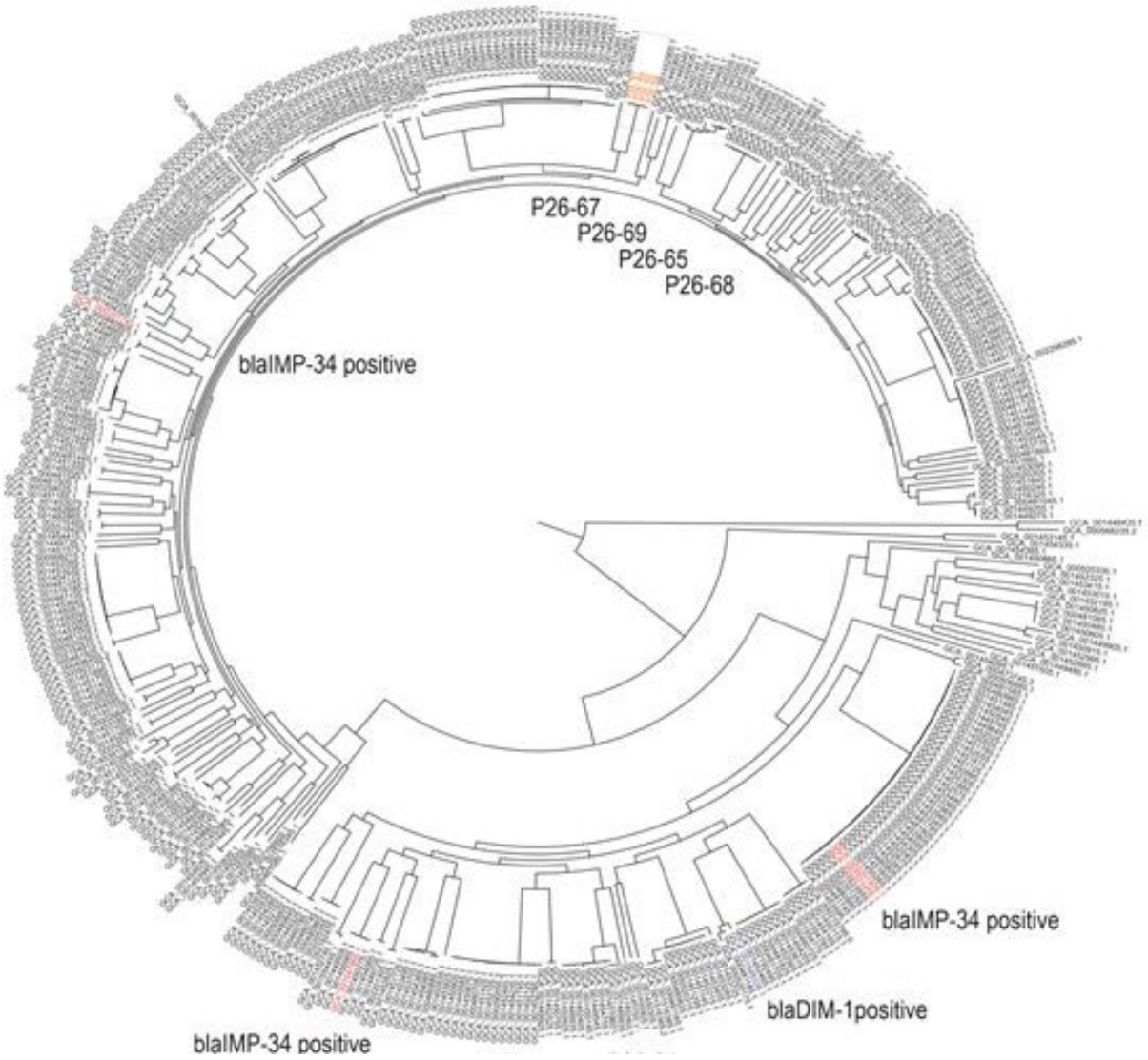

Figure 2. **Evolutionary relatedness of *P. aeruginosa* strains from Ghana.** Phylogenomic tree from metadata drawn by the use of Phandango**.** Among the genomes included, one was positive for $bla_{\text{DIM-1}}$ (GCA_001548335)[blue] and four for $bla_{\text{IMP-34}}$ (GCA_00291745; GCA_001547955; GCA_002201405; GCA_02201455) [red], which indicates that, they were closely related to the isolates from this study (orange).

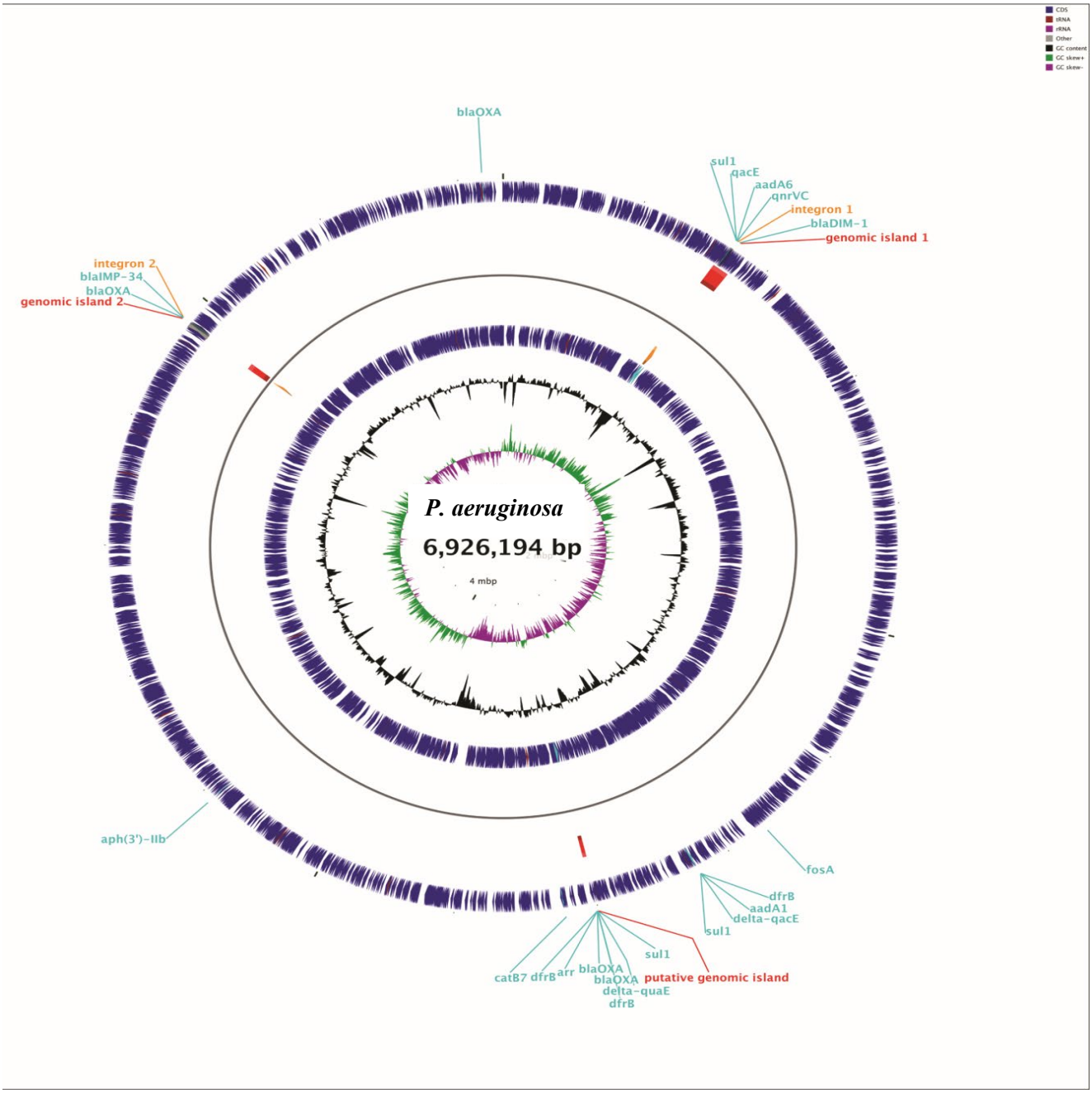

Figure 3. **Representation of chromosome structure of *P. aeruginosa* strain (P26-65) by oxford Nanopore sequence.** The resistance encoding genes are marked together with the detected integron structures, Integron 1 and Integron 2 containing $bla_{\text{DIM-1}}$ and $bla_{\text{IMP-34}}$-like respectively. A third putative class 1 integron structure was detected as indicated in the figure.

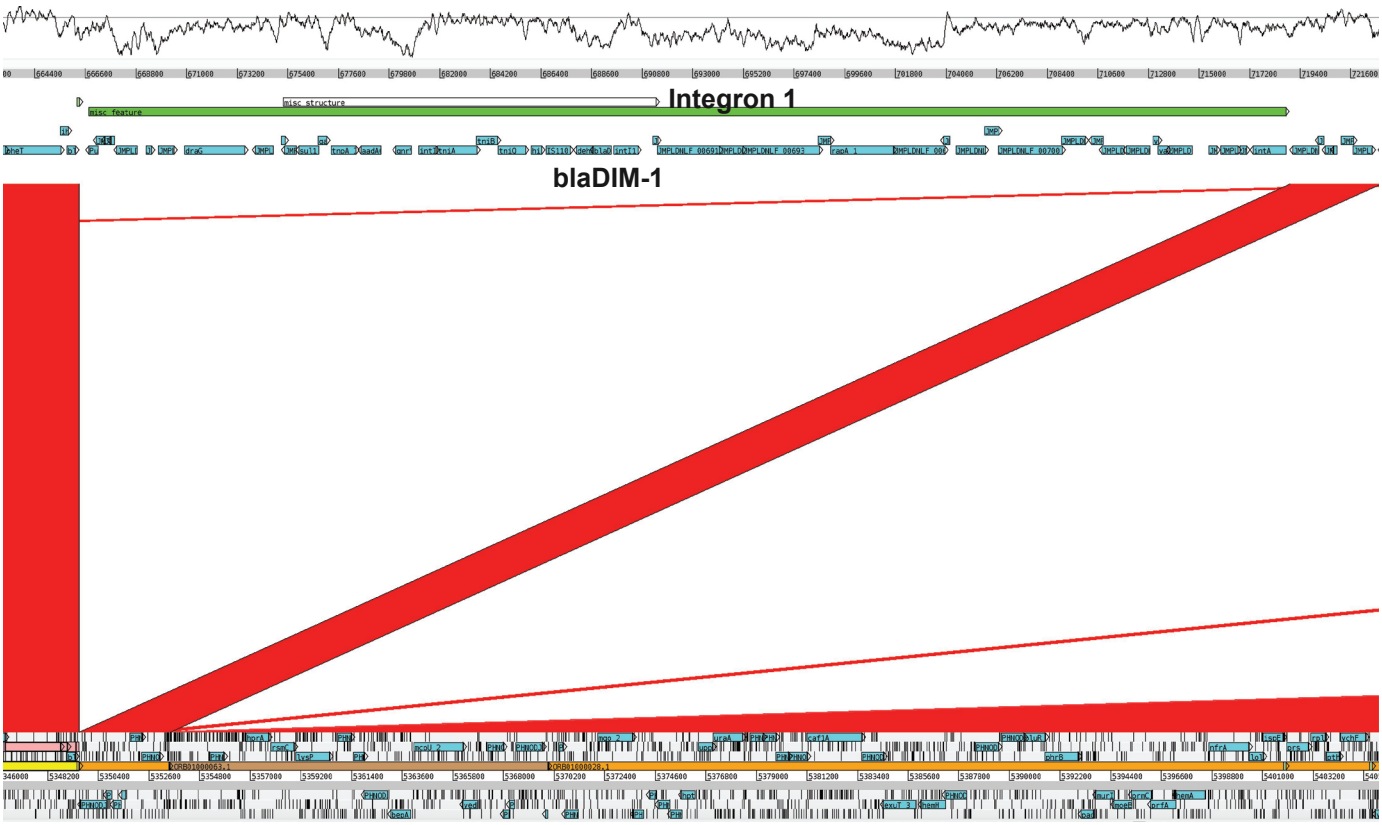

Figure 4a. Alignment of the *bla*$_{\text{DIM-1}}$ encoding regions of *P. aeruginosa* (P26-85) chromosome with the corresponding regions of SMC4389 (GCA_001482875) of ST654 isolate using Artemis Comparison Tool to graphically show the relationship between the two integron structures. The alignment revealed the presence of two genomic islands containing each of the integron structures represented in green arrows with the putative integron structures indicated in the white arrows. Both islands are representation of insertion of the DNA segment into the chromosome.

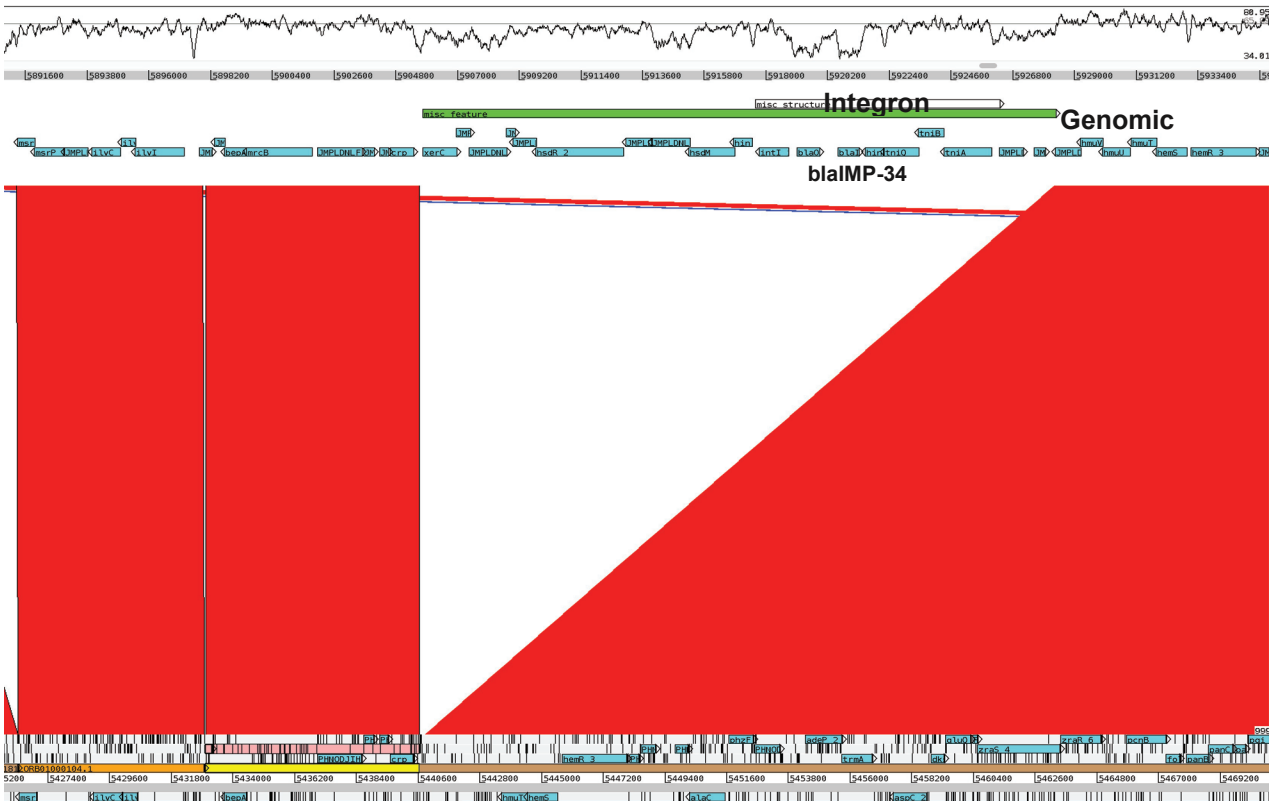

Figure 4b. Alignment of *bla*$_{IMP-34}$-like encoding regions of *P. aeruginosa* (P26-85) chromosome with the corresponding regions of SMC4389 (GCA_001482875) of ST654 using Artemis Comparison Tool **to graphically show the relationship between the two integron structures.** The putative integron structures (white arrows) with the two genomic islands containing each of the integron structures (green arrows) on top of the figure. Both islands represent insertion of the DNA segment into the chromosome.

Discussion

Metallo-β-lactamase (MBL)-producing *P. aeruginosa* is among the ESKAPE Gram-negative pathogens that have evolved to exhibit high levels of extensive drug resistance including carbapenem resistance. MBL production is a major mechanism by which *P. aeruginosa* acquires resistance to carbapenems. Despite the emergence of MBL-producing *P. aeruginosa*, no surveillance data and genomic characterization have so far been reported from Ghana. This study represents the first detailed report on molecular characterization of MBL-producing *P. aeruginosa* using whole genome sequencing. We identified four *P. aeruginosa* isolates resistant to all antibiotics tested except colistin. The four isolates harboured *bla*$_{\text{DIM-1}}$ and *bla*$_{\text{IMP-34}}$-like gene and have the same sequence type (ST234). Both *bla*$_{\text{DIM-1}}$ and *bla*$_{\text{IMP-34}}$-like positive contigs showed that the four isolates have identical DNA sequences representing one clone. Analyses of the *bla*$_{\text{DIM-1}}$ and *bla*$_{\text{IMP-34}}$ positive contigs indicated a linkage of both the *bla*$_{\text{DIM-1}}$ and *bla*$_{\text{IMP-34}}$-like to type 1 integron structure with the *intI1* integrase encoding gene.

The four XDR *P. aeruginosa* isolates were resistant to all tested β-lactams including carbapenems and the combination of β-lactam/lactamase inhibitors such as amoxicillin-clavulanate and piperacillin-tazobactam were also not active against the isolates. The resistance to carbapenems is particularly challenging to clinical practice posing a serious threat to antibiotic therapy as these agents are regarded as most effective β-lactam antibiotics against MDR Gram-negative bacteria including *P. aeruginosa* in Ghana (24). Thus, stringent antibiotic stewardship programmes should be enforced to combat the spread of MBL-producing *P. aeruginosa* in order to preserve the efficacy of carbapenems in Ghana.

The *bla*$_{\text{DIM-1}}$ and *bla*$_{\text{IMP-34}}$-like genes were identified in the XDR isolates belonging to ST234. Reports from several hospitals in Moscow have indicated the prevalence of MBL-producing *P. aeruginosa* ST234, but these were associated with VIM-2 producers (25). Genomic data analysis found one closely related isolate from India, i.e., *P. aeruginosa* strain SMC4389 (GCA_001482875) belonging to ST654. However, this isolate did not contain the *bla*$_{\text{DIM-1}}$ and *bla*$_{\text{IMP-34}}$-like genetic structure. This finding intimates that the genetic elements containing these resistance genes had been acquired, but from an unknown source, confirming reports from studies that, many African countries do not undertake epidemiological surveillance of carbapenems resistance and carbapenemase genes (26, 27). Further phylogenic investigation on our isolates from 417genome assembly datasets from different isolation sources, countries and MLST showed that none of the closest neighbors encodes *bla*$_{\text{DIM-1}}$ genes. This suggests that these carbapenem-resistant *P. aeruginosa* isolates emerged independently, possibly through selection pressure, and are disseminating locally through clonal expansion, probably due to poor adherence to infection control protocols. Our study indicated that there were no plasmids present in the circularized chromosome, implying that the IMP and DIM carbapenemases were chromosomal and corroborated the clonal dissemination of the *P. aeruginosa* strains isolated from different patients, sources and wards in the hospital.

We found a linkage of *bla*$_{\text{DIM-1}}$ and *bla*$_{\text{IMP-34}}$-like genes to type1 integron structures and *intI1* integrase encoding gene by BLAST analysis. The *bla*$_{\text{DIM-1}}$ in the form of gene cassette inserted at *intI1* is similar to other acquired MBLs in Gram-negative bacteria commonly located in class 1 integrons (28, 29). Studies have reported *bla*$_{\text{IMP}}$-types as one of the most common families of acquired MBLs in *Enterobacteriaceae* including *P. aeruginosa* (29, 30). A number of MBL types in carbapenem-resistant *P. aeruginosa*, including IMP-like and

DIM-like types, have been reported from many parts of the world (8, 31). Class 1 integrons, which carry MBL genes and genes encoding resistance determinants of other antibiotics such as aminoglycosides and trimethoprim, mobilise and transfer extensive drug resistance within and between species (32, 33). Reports from other studies have indicated that class 1 integrons are often chromosomally located and associated with genomic islands of pathogenic bacteria such as *P. aeruginosa* evident in this study (34, 35). This presents a major challenge to both clinical treatment and infection control management given the limited healthcare logistics in Ghana. Therefore, studies on dissemination of MBLs among other Gram-negative isolates, particularly the *Enterobacteriaceae* is crucial for the development of strategies to contain the spread of these resistance genes. This study should be extended to hospitals in the other regions of Ghana.

Conclusion

We report herein the first carbapenem-resistant and carbapenemase producing *P. aeruginosa* clonal outbreak in a tertiary hospital in Ghana. Chromosomally mediated *bla*$_{\text{DIM-1}}$ and *bla*$_{\text{IMP}}$-34-like carbapenemases are circulating in *P. aeruginosa* ST234 clones in Komfo Teaching Hospital, Ghana. Stricter infection control, contact precautions, antibiotic stewardship and surveillance are necessary to identify and contain the spread of this XDR pathogen.

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Supplementary Material: MICs

Isolate code	specimen		Susceptibility profile - MICs (mg/L)															
		AMP	AMC	TZP	CXM	FOX	CTX	CAZ	CFP	ETP	IMP	MEM	AMK	GEN	CIP	TET	NIT	COL	SXT
P26-65 [97]	urine	≥32	≥32	≥128	≥64	≥64	≥64	≥64	≥64	-	≥16	≥1	≥64	≥16	≥4	≥8	≥512	≤0.5	≥320
P26-67 [130]	W/swab	≥32	≥32	128	≥64	≥64	≥64	≥64	≥64	-	≥ 16	4	≥32	≥ 16	≥4	≥8	≥512	≤0.5	≥320
P26-68 [140]	W/swab	≥32	≥32	128	≥64	≥64	≥64	≥64	≥64	-	≥ 16	4	≥32	≥ 16	≥4	≥8	≥512	≤0.5	≥320
P26-69 [142]	P/fluid	≥32	≥32	128	≥64	≥64	≥64	≥64	≥64	-	≥ 16	≥ 16	≥64	≥ 16	≥4	≥8	≥512	≤0.5	≥320
P26-64 [85]	W/swab	≥32	≥32	32	≥64	≥64	8	≤1	8	-	≤1	≤0.25	≤2	4	≥4	≥8	≥512	≤0.5	160
2	W/swab	≥32	≥32	8	≥64	≥64	8	≤1	≤1	-	2	1	≤2	≤1	≤0.25	8	≥512	≤0.5	80
14	sputum	≥32	≥32	8	≥64	≥64	32	2	2		2	≤0.25	≤2	≤1	≤0.25	8	≥512	≤0.5	80
22	urine	≥32	≥32	64	≥64	≥64	≥64	4	2	-	1	1	≤2	≥16	≥4	≥8	≥512	≤0.5	≥320
25	W/swab	≥32	≥32	8	≥64	≥64	32	4	≤1	-	2	≤0.25	≤2	≤1	≤0.25	8	≥512	≤0.5	160
28	E/swab	≥32	≥32	8	≥64	≥64	32	4	2	-	2	≤0.5	≤2	≤1	≤0.25	≥8	≥512	-	80
32	urine	≥32	≥32	8	≥64	≥64	8	≤1	≤1	-	2	1	≤2	≤1	≤0.25	≥8	≥512	≤0.5	80
43	W/swab	≥32	≥32	8	≥64	≥64	16	≤1	≤1	-	2	0.5	≤2	≤1	≤0.25	≥8	≥512	≤0.5	40
44	W/swab	≥32	≥32	8	≥64	≥64	8	≤1	≤1	-	1	≤0.5	≤2	≤2	≤0.25	≥8	≥512	≤0.5	80
49	W/swab	≥32	≥32	8	≥64	≥64	16	2	≤1	-	2	≤0.25	≤2	≤1	≤0.25	≥8	≥512	≤0.5	160
51	W/swab	≥32	16	32	≥64	≥64	≥64	4	4	-	0.5	1	≤2	≥16	≥4	≥8	≥512	≤0.5	≥320
54	sputum	≥32	≥32	8	≥64	≥64	≥64	4	2	-	2	≤0.25	4	≤1	≤0.25	≥8	256	≤0.5	80

58	sputum	≥32	≥32	8	≥64	≥64	32	2	4	-	1	≤0.25	4	4	≤0.5	≥8	≥51	≤0.5	≥320
59	urine	≥32	≥32	≤4	≥64	≥64	8	≤1	≤1	-	2	0.5	≤2	≤1	≤0.25	≥8	≥512	≤0.5	≥320
62	N/swab	≥32	≥32	8	≥64	≥64	8	≤1	≤1	-	2	≤0.25	≤2	≤1	≤0.25	≥8	≥512	≤0.5	80
65	G/lavage	≥32	≥32	8	≥64	≥64	8	≤1	≤1	-	1	≤0.25	≤2	≤1	≤.25	≥8	≥512	≤0.5	80
67	urine	≥32	≥32	8	≥64	≥64	32	4	2	-	1	≤0.25	≤2	≤1	≤0.25	≥8	≥512	≤0.5	≥320
85	W/swab	≥32	≥32	32	≥64	≥64	8	≤1	8	-	≤1	≤0.25	≤2	4	≥4	≥8	≥512	≤0.5	160
86	blood	16	4	≤4	16	≥32	8	4	2	-	≤0.25	≤0.25	≤2	≤1	≤0.25	≤0.5	128	≤0.5	≤20
87	W/swab	≥32	≥32	8	≥64	≥64	32	≤4	≤2	-	≤1	≤0.25	≤2	≤1	≤0.25	8	≥512	≤0.5	160
88	W/swab	≥32	≥32	8	≥64	≥64	≥16	≤1	2	-	2	≤0.25	≤2	≤1	≤0.25	≥8	≥512	≤0.5	80
91	urine	≥32	≥32	8	≥64	≥64	32	4	2		2	≤0.25	≤2	≤1	≤0.25	≥8	≥512	≤0.5	80
119	urine	≥32	≥32	8	≥64	≥64	16	2	≤1		2	≤0.25	≤2	≤1	≤0.25	≥8	≥512	≤0.5	80
121	W/swab	≥32	≥32	8	≥64	≥64	32	4	2	-	2	≤0.25	≤2	2	≤0.25	≥8	≥512	≤0.5	160
123	W/swab	≥32	≥32	8	≥6	≥6	32	4	2		2	≤0.25	≤2	≤1	≤0.5	≥8	≥512	≤0.5	160
124	sputum	≥32	≥32	8	≥64	≥64	32	4	2		1	≤0.5	≤2	2	0.5	≥8	≥512	≤0.5	≥320
134	urine	≥32	≥32	8	≥64	≥64	32	4	2	-	2	≤0.5	≤2	≤1	≤0.25	≥8	≥512	≤0.5	80
145	urine	≥32	≥32	8	≥64	≥64	16	4	2	-	2	≤0.25	≤2	≤1	≤0.25	≥8	≥512	≤0.5	≥320
`146	urine	≥32	≥32	8	≥64	≥64	16	4	2	-	1	≤0.25	≤2	≤1	≤0.25	≥8	≥512	≤0.5	160
150	urine	≥32	≥32	8	≥64	≥64	32	4	2	-	2	≤0.25	≤2	≤1	0.5	≥8	≥512	≤0.5	160
157	W/swab	≥32	≥32	≤4	≥64	8	≤1	≤1	≤1		1	≤0.25	≤2	≤1	≤0.25	2	64	≤0.5	≥320

161	W/swab	≥32	≥32	8	≥64	≥64	1	4	≤1	-	2	≤0.25	≤2	≤1	≤0.25	≥8	≥512	≤0.5	80
176	sputum	≥32	≥32	≤4	≥64	≥64	8	≤1	≤1		1	≤0.25	≤2	≤1	≤0.25	≥8	≥512	≤0.5	≥320
210	sputum	≥32	≥32	8	≥64	≥64	32	4	2		2	≤0.25	≤2	≤2	≤0.25	8	≥512	≤0.5	≥320

AMP-Ampicillin, AMC- Amoxicillin-clavulanate, TZP- Piperacillin-Tazobactam, CXM-Cefuroxime, FOX-Cefoxitin CTX- Cefotaxime , CAZ- Ceftazidime, CFP- Cefepime, ETP- Ertapenem, IMP- Imipenem, MEM-Meropenem, AMK- Amikacin, GEN-Gentamicin, CIP-Ciprofloxacin, TET- Tetracycline, NIT- Nitrofurantoin, COL-Colistin, SXT- trimethoprim-sulfamethoxazole
P/fluid-pleural fluid, w/swab-wound swab, P/swab-pleural swab, G/lavage-gastric lavage, N/swab-nasal swab.

CHAPTER FIVE

Conclusions, Recommendation and Significance

6.1 Conclusion

Antibiotic resistance among Gram-negative ESKAPE pathogens poses a major challenge in Komfo Anokye Teaching Hospital and associated with adverse outcomes in treatment of infections in Ghana.

Key findings were as follows:

- The most predominant bacteria were *P. aeruginosa* and *K. pneumoniae* accounting for 40.2% and 39.2% respectively, followed by *E. cloacae*, 12.4% and *A. baumanii,* 8.2% as the least of the Gram-negative ESKAPE pathogens.
- The bacteria were multidrug and extensively-drug resistant, however meropenem was noted as the most effective β-lactam antibiotic in Ghana.
- The presence of β-lactamase genes in diverse combination with non-β-lactamase resistance genes was found among the pathogens.
- The ten MDR *K. pneumoniae* isolates evidenced multiple resistance genes conferring resistance to multiple antibiotic classes with β-lactams (*bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{SHV-11}}$, *bla*$_{\text{TEM-1B}}$), aminoglycosides *(aac(3)-IIa-like, aph(3')-Ia, aac(6')Ib-cr)* and fluoroquinolones (*oqxA-like, oqxB-like, qnrB10-like, qnrB2*) being the most common. Other resistance genes such as *sul2, fosA and dfrA14* encoding resistance for sulphonamide, fosfomycin and trimethoprim respectively were also identified.
- IncFIB(K) and ColRNAI were the most prevalent plasmid replicon type among *K. pneumoniae* isolates.

- Multiple and diverse mutations were detected in quinolone resistance-determining regions of *gyrA*, *gyrB* and *parC* genes among *K. pneumoniae* with S83F/Y and D87A of *gyrA* and S80I in *parC* as most common mutations observed among the fluoroquinolone-resistant isolates.
    - No mutation was detected in *gyrB* among fluoroquinolone-resistant isolates with a ciprofloxacin MIC ≥4mg/L.
- The four XDR *P. aeruginosa* isolates belonged to same sequence type ST234 had:
    - The identical resistance gene profile consisting of β-lactamases (bla_{DIM-1} bla_{IMP-34}-like, bla_{OXA-10}-like, $bla_{OXA-129}$, bla_{OXA-50}-like, bla_{PAO}-like), aminoglycosides (*aadA1-like, aph(3')-IIb-like, aacA4*), fosfomycins (*fosA*-like), sulphonamides (*sul1*), trimethoprim (*dfrB5*), phenicols (*catB7-like*) and rafampins (*arr-2*)
    - The bla_{DIM-1} and bla_{IMP-34}-like were linked to type 1 integron structures
- Although not reported in the manuscripts, we also undertook WGS of two each of *E. cloacae* and *A. baumanii* (Supplementary data).
    - WGS revealed β-lactamases (carbapenemases) encoding genes bla_{ADC-25} and bla_{OXA-91} and aminoglycosides resistance gene *(aadB)* co-expressed with the tetracycline resistance gene *tetB* was found in *A. baumannii*.
    - β-lactamases (bla_{TEM-1B}, bla_{SHV-11}, bla_{OXA-1} and $bla_{CTX-M-15}$), resistance genes to aminoglycosides (*aac(6')Ib-cr, aac(3)-IIa, strB and strA*) were expressed in *E. cloacae* with quinolones/fluoroquinolones efflux pumps (*oqxA, oqxB and qnrB*) most predominant *E. cloacae* (n=1) isolate.

6.2 Limitations

- The use of Illumina sequencing precluded the definitive association of antibiotic resistance genes with plasmids and detailed plasmid analysis.

- Financial constraints precluded the WGS of all isolates
- Isolates for whole genome sequencing were not randomly selected, presenting a potential bias. Notwithstanding, random sampling may have precluded stratified sampling precision and equal representation of isolates collected from each group of Gram-negative ESKAPE pathogens.

6.3 Recommendations

- High prevalence of multidrug resistance was observed among Gram-negative ESKAPE pathogens in KATH. Studies should be extended to other health care settings over a longer period of time with larger sample size for representivity.
- Antibiotic stewardship programs should be strictly enforced to maintain the efficacy of the carbapenems (meropenem).
- Regular education on antibiotic resistance awareness, contact precaution and infection control measures among health care professionals should be encouraged to combat the spread of resistant bacteria.
- Comprehensive molecular characterization to delineate antibiotic resistance mechanisms is necessary to inform effective control measures and containment.

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APPENDIX I

ETHICAL APPROVAL (BREC) LETTER

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

06 July 2015

Mr Nicholas Agyepong
CHS, UKZN
Private Bag X54001
Durban
4000
agyanicho33@yahoo.com

PROTOCOL: Molecular Profile of Eskape Pathogens in Ghana: Degree Purposes (PhD) - School of Health Sciences (Pharmaceutical Sciences) Student Number: 214583993. **BREC REF: BE494/14.**

EXPEDITED APPLICATION

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application received on 20 November 2014.

The study was provisionally approved pending appropriate responses to queries raised. Your responses received on 11 June 2015 to queries raised on 07 February 2015 have been noted by a sub-committee of the Biomedical Research Ethics Committee. The conditions have now been met and the study is given full ethics approval.

This approval is valid for one year from **06 July 2015.** 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 **11 August 2015.**

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

Yours sincerely

Professor J Tsoka-Gwegweni
Chair: Biomedical Research Ethics Committee

cc: Supervisor - Prof Sabiha Essack
Postgrad Office - Ms Phindile Nene

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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ETHICAL APPROVAL (CHRPE) LETTER

KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY
COLLEGE OF HEALTH SCIENCES
SCHOOL OF MEDICAL SCIENCES / KOMFO ANOKYE TEACHING HOSPITAL
COMMITTEE ON HUMAN RESEARCH, PUBLICATION AND ETHICS

Our Ref: CHRPE/AP/015/15

13th January, 2015.

Mr. Nicholas Agyepong
School of Health Sciences
University of KwaZulu-Natal
Private Bag X54001
Durban
4000
SOUTH AFRICA.

Dear Sir,

LETTER OF APPROVAL

Protocol Title ***"Molecular Profile of ESKAPE (Enterococcus Faecium, Staphylococcus Aureus, Klebsiella Pneumoniae, Acinetobacter Baumannii, Pseudomonas Aeruginosa and Enterobacter Spp) Pathogens in Ghana."***

Proposed Site: ***Komfo Anokye Teaching Hospital, Department of Microbiology, Kumasi.***

Sponsor: ***Principal Investigator.***

Your submission to the Committee on Human Research, Publications and Ethics on the above named protocol refers.

The Committee reviewed the following documents:

- A notification letter of 2nd December, 2014 from the Komfo Anokye Teaching Hospital (study site) indicating approval for the conduct of the study in the Hospital.
- A Completed CHRPE Application Form.
- Research Proposal.
- Specimen Collection Form.

The Committee has considered the ethical merit of your submission and approved the protocol. The approval is for a fixed period of one year, renewable annually thereafter. The Committee may however, suspend or withdraw ethical approval at any time if your study is found to contravene the approved protocol.

Data gathered for the study should be used for the approved purposes only. Permission should be sought from the Committee if any amendment to the protocol or use, other than submitted, is made of your research data.

The Committee should be notified of the actual start date of the project and would expect a report on your study, annually or at close of the project, whichever one comes first. It should also be informed of any publication arising from the study

Thank you Sir, for your application.

Yours faithfully,

Osomfuor Prof. Sir J. W. Acheampong MD, FWACP
Chairman

Room 7 Block J, School of Medical Sciences, KNUST, University Post Office, Kumasi, Ghana
Phone: +233 3220 63248 Mobile: +233 20 5453785 Email: chrpe.knust.kath@gmail.com / chrpe@knust.edu.gh

INFORMED CONSENT/ASSENT FORM

Committee on Human Research Publication and Ethics
School of Medical Sciences, Kwame Nkrumah University of Science and Technology
Kumasi, Ghana. Tel: 233 3220 63248 or 233 20 5453785. Email: chrpe.knust.hath@gmail.com

Participant Information Leaflet and Consent Form

This leaflet must be given to all prospective participants to enable them know enough about the research before deciding to or not to participate

Title of Research: Molecular profile of ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp) pathogens in Ghana.

Name(s) and affiliation(s) of researcher(s): This study is being conducted by Nicholas Agyepong of the Department of Pharmaceutical Sciences, University of Kwa-Zulu Natal (UKZN), Dr. Alex Owusu-Ofori of Department of Clinical Microbiology, KNUST, Prof. Essacks Sabiha and Dr. Usha Govinden both of Department of Pharmaceutical Sciences, UKZN, South Africa.

Background (Please explain simply and briefly what the study is about): There is current increasing frequency of infections caused by resistant bacteria and the decline in research and development of new antibiotics which is now threatening to take us back to the pre-antibiotic era. Curtailing the spread of resistant bacteria has met with limited success in the Sub-Saharan Africa, especially Ghana. This has consequently resulted in bacterial infections being the major cause of morbidity and mortality due to the emergence of antimicrobial resistance by clinical Gram negative ESKAPE isolates, which undermines the effective control of infectious diseases (Newman et al., 2011). Hence the study is critical to aid in health care policies, antibiotic protocols and prescription regulations in Ghana. The research can become a baseline for further research and comparative studies in Ghana as the Sub regions.

Purpose(s) of research: The purpose of this study is to ascertain the prevalence and describe the phenotypic and genotypic characteristics of resistance of clinical Gram negative ESKAPE pathogens from Komfo Anokye Teaching Hospital (KATH) in Kumasi in the Ashanti region of Ghana to beta-lactam antibiotics.

Committee on Human Research Publication and Ethics
School of Medical Sciences, Kwame Nkrumah University of Science and Technology
Kumasi, Ghana. Tel: 233 3220 63248 or 233 20 5455785. Email: chrpe.knust.kath@gmail.com

Procedure of the research, what shall be required of each participant and approximate total number of participants that would be involved in the research:

Isolates will be identified from samples sent to the microbiology lab. Persons who have their samples sent to the lab will be contacted by the principal investigator (PI). The patient participation in the study will then be explicitly explained to him/her before they voluntarily sign the consent form. One hundred and fifty samples in all will be collected for the study.

Risk(s):
There is risk associated with stigmatization and panic if patients get to know some organisms have been isolated from them. However calming and assuring them of privacy and confidentiality will be paramount in the study

Benefit(s):
There would be no direct benefit to the participants; however the aim of this study is to find solution to overwhelming increase in bacterial resistance to commonly used antibiotics (β-lactams) in Ghana will help to guide in the future infections treatment.

Confidentiality:
Patients will be assigned specific study numbers after initially using the name and personal information to trace the sample to the patient, which will be used during the study and for analysis. Therefore data collected cannot be linked to any patient in anyway, whether in publications or reports.

Voluntariness:
Patients' participation in the research will be solely voluntary. Participant's information leaflet will be given. If they are literate they can read or you may make translated versions available for them. Spending time to explain the study to them, will also allow them to ask questions and when they are satisfied and you are sure they have understood the study, they can sign the informed consent. All consents by illiterate subjects will also be signed by an independent witness.

Alternatives to participation:
Patient's surety of fair treatment will be paramount whether he/she chooses to participate or decides otherwise.

Withdrawal from the research:

The informed consent form is not legal binding document and that participant may choose to withdraw or not to answer any question he finds uncomfortable or private.

Consequence of Withdrawal: (For example: There will be no consequence, loss of benefit or care to you if you choose to withdraw from the study. Please note however, that some of the information that may have been obtained from you without identifiers (name etc), before you chose to withdraw, may have been modified or used in analysis reports and publications. These cannot be removed anymore. We do promise to make good faith effort to comply with your wishes as much as practicable.)

Costs/Compensation: (For example: For your time/inconvenience/transport to the hospital, we will compensate you with Ghȼ2.00 to show our appreciation for your participation).

Contacts:
For any question concerning this study, please do not hesitate to contact Nicholas Agyepong (0261305798) or Dr. Alex Owusu-Ofori (0209149370)

Further, if you have any concern about the conduct of this study, your welfare or your rights as a research participant, you may contact:

The Chairman
Committee on Human Research and Publication Ethics
Kumasi
Tel:0322063248/0205453785

Committee on Human Research Publication and Ethics
School of Medical Sciences, Kwame Nkrumah University of Science and Technology
Kumasi, Ghana. Tel: 233 3220 63248 or 233 20 5453785. Email: chpe.knust.kath@gmail.com

CONSENT FORM

Statement of person obtaining informed consent:
I have fully explained this research to _____ and have given sufficient information about the study, including that on procedures, risks and benefits, to enable the prospective participant make an informed decision to or not to participate.

DATE: _____ NAME: _____

Statement of person giving consent:
I have read the information on this study/research or have had it translated into a language I understand. I have also talked it over with the interviewer to my satisfaction.

I understand that my participation is voluntary (not compulsory).

I know enough about the purpose, methods, risks and benefits of the research study to decide that I want to take part in it.

I understand that I may freely stop being part of this study at any time without having to explain myself.

I have received a copy of this information leaflet and consent form to keep for myself.

NAME:_____

DATE: _____ SIGNATURE/THUMB PRINT: _____

Statement of person witnessing consent (Process for Non-Literate Participants):

I _____ (Name of Witness) certify that information given to _____ (Name of Participant), in the local language, is a true reflection of what I have read from the study Participant Information Leaflet, attached.

WITNESS' SIGNATURE (maintain if participant is non-literate): _____

MOTHER'S SIGNATURE (maintain if participant is under 18 years): _____

MOTHER'S NAME: _____

FATHER'S SIGNATURE (maintain if participant is under 18 years): _____

FATHER'S NAME: _____

Supplementary Data

Table 1: Phenotypic Profile of WGS *A. baumanii* and *E. cloacae* Isolates

Code		Susceptibility profile - MICs (ug/mL)															
	Species	**AMC**	**TZP**	**CXM**	**FOX**	**CTX**	**CAZ**	**CFP**	**ETP**	**IMP**	**MEM**	**AMK**	**GEN**	**CIP**	**TET**	**NIT**	**COL**	**SXT**
151	*A. baumannii*	≥32	≥128	≥64	≥64	≥64	≥64	≥64	-	0.5	1	≤2	≥16	≥4	4	≥512	≤0.5	≥320
198	*A. baumannii*	8	16	≥64	≥64	32	16	8	-	≤0.25	≤0.25	≤2	≤1	≤0.25	≤.5	≥512	≤0.5	≤20
170	*E. cloacae*	≥32	64	≥64	≥64	≥64	≥64	≤1	≤0.5	≤0.25	≤0.25	≤2	≤1	≤0.25	1	128	≤0.5	≤20
194	*E. cloacae*	≥32	≥128	≥64	≥64	≥64	≥64	≥64	≤1	≤0.25	≤0.25	≤2	≥16	≥4	2	256	≤0.5	≥320

Abbreviations: AMC- Amoxicillin-clavulanate, TZP- Piperacillin Tazobactam, CXM-Cefuroxime, FOX-Cefoxitin CTX- Cefotaxime , CAZ- Ceftazidime, CFP- Cefepime, ETP- Ertapenem, IMP- Imipenem, MRP-Meropenem, AMK- Amikacin, GEN-Gentamicin, CIP-Ciprofloxacin, TET- Tetracycline, NIT- Nitrofurantoin, COL-Colistin, SXT-Trimethoprim-sulfamethoxazole, Obs& Gyn. -Obstetrics and Gynaecology,

Table 2: Patient Demographic and Clinical Records

Isolate P-number	Code	Species	Collection date	Patient				
				Specimen	**Age (yr)**	**sex**	**diagnosis**	**ward type**
P26-70	151	*A. baumannii*	09/09/2015	Urine	5	F	UTI	ICU
P26-77	198	*A. baumannii*	09/03/2015	Urethral swab	30	F	UTI	ICU
P26-73	170	*E. cloacae*	11/06/2015	Aspirate	3	M	Scalp abscess	OPD
P26-76	194	*E. cloacae*	18/03/2015	Sputum	45	M	Tuberculosis	Medicine

Table 3: WGS and Bioinformatics Analyses with Resistance Genes

Isolate P-number	Assembly			Resfinder							
	Genome Size	Coverage	# Contigs	Aminoglycoside	Quinolone/fluoroquinolone	Fosfomycin	β-lactam	Macrolide	Sulphonamide	Tetracycline	Rifampicin
P26-70	4049510	172x	230	*aadB-like*			*blaADC-25-like,blaCARB-8-like,blaOXA-91*		*sul2*	*tet(39)*	
P26-77	4024532	213x	86	*aadB-like*			*blaADC-25-like,blaCARB-8-like,blaOXA-91*		*sul2*	*tet(39)*	
P26-73	5096234	175x	64			*fosA-like*					
P26-76	5460117	153x	204	*aac(3)-IIa-like,strA,strB*	*aac(6')Ib-cr qnrB1-like*	*fosA-like*	*blaCMY-4,blaCTX-M-15, blaOXA-1-like,blaTEM-1B*		*sul2*		

Table 4. WGS and Bioinformatics Analyses with Plasmid/Replicon Types

Isolate P-number	Species/ MLST Scheme	Sequence Type	PlasmidFinder			
			Plasmids	IncHI1	IncF	pMLST summary
P26-70	*A. baumannii*	ST-1418				
P26-77	*A. baumannii*	ST-1418				
P26-73	*E. cloacae*	Unknown ST	NA			
P26-76	*E. cloacae*	ST-456	IncQ1,ColRNAI			