Investigating the Mechanisms of Antimicrobial Resistance and Virulence of Bacteria Isolated from Hospital Environments

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DECLARATION OF ORIGINAL AUTHORSHIP

This thesis contains work originally done by me as guided by my supervisors: Dr. Lydia Mosi, Dr. Samuel Duodu and Prof. Miguel Valvano. It has not been previously submitted to meet requirements for an award at any higher education institution. It contains no previously written or published material by another person except where due reference is made.

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DEDICATION

This thesis, by heart and acts, is dedicated to the memory of my late father, Lawrence Isawumi (*Senior Advocate of the Masses*), who initiated this dream with the soundest footing when he called me a Doctor at age seven. To my mum, Cecilia Isawumi, it is a fulfilled dream to see you witness this. And to the precious gifts called people, without which this dream would not have been a reality, I dedicate this.

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LIST OF ABBREVIATIONS

ABA	–	Acrylonitrate-butadiene acrylate
ABC	–	AbiMosi Bacterial Culture
AMR	–	Antimicrobial Resistance
AMPs	–	Antimicrobial Peptides
APS	–	Ammonium Persulfate
ARLN	–	Antibiotic Resistance Lab Network
ATCC	–	American Typed Culture Collection
BLAST	–	Basic Local Alignment Sequencing Tool
BSA	–	Bovine Serum Albumin
CDC	–	Centre for Disease Control and Prevention
CFU	–	Colony Forming Unit
CLSI	–	Clinical Laboratory Standards Institute
CRE	–	Carbapenem-Resistant Enterobacteriaceae
CV	–	Crystal Violet
DNA	–	Deoxyribonucleic Acid
EMB	–	Eiosin Methylene Blue Agar
EUCAST	–	European Committee on Antimicrobial Susceptibility Testing
E-Test	–	Epsilometer Test
GAA	–	Glacial Acetic Acid
GMI	–	*Galleria mellonella* infection
HAIs	–	Hospital Acquired Infections
ICU	–	Intensive Care Units
KPC	–	Klebsiella pneumoniae carbapenemase
LB	–	Luria Bertani
LPS	–	Lipopolysaccharides
MDR	–	Multidrug Resistance
MHB	–	Mueller Hinton Broth
MRSA	–	Methicillin Resistant Staphylococcus aureus
MIC	–	Minimum Inhibitory Concentration
MOI	–	Multiplicity of Infection
MS	–	Mass Spectrometry
NICU	–	Neonatal Intensive Care Units
OD	–	Optical Density
PAGE	–	Poly Acrylamdide Gel
PAP	–	Population Analysis Profile
PBS	–	Phosphate Buffered Saline
PCR	–	Polymerase Chain Reaction
PmB	–	Polymyxin B
QC	–	Query Cover

QL	–	Query Length
QS	–	Quorum Sensing
RND	–	Resistance Nodulation Division
SDS	–	Sodium Dodecyl Sulfate
SNP	–	Single Nucleotide Polymorphism
TEMED	–	Tetra-methyl-ethylene-diamine
TCS	–	Two Component System
VRSA	–	Vancomycin Resistant Staphylococcus aureus
VRE	–	Vancomycin Resistant Enterococcus faecalis
WGS	–	Whole Genome Sequencing

ABSTRACT

Hospitals are hubs for transmission of different pathogens. In Ghana, little is known about the diversity and antimicrobial resistance (AMR) profiles of bacterial pathogens from hospital environments. Essential is also the rising challenges of antimicrobial heteroresistance, which has contributed to the increase in bacterial AMR. In this study, bacterial strains were isolated from air and fomites of the Intensive Care Unit (ICU) and characterized with phenotypic and molecular techniques. Bacterial AMR profiles to conventional and last resort antibiotics were determined with agar disc diffusion and micro-broth dilution assays. Antimicrobial heteroresistance to polymyxins was determined using E-test and population analysis profiling assays. *Galleria mellonella* infection (GMI) model was employed to evaluate the virulence and pathogenicity of the identified bacterial strains. Lipopolysaccharides (LPS) was analyzed and profiled using Sodium Dodecyl Sulfate (SDS-PAGE) and matrix assisted laser desorption ionization-time of flight (MALDI-TOF) Mass Spectrometry analysis. AMR markers and LPS-modifying genes were characterized using specific primers targeted Polymerase Chain Reaction (PCR) and sequencing. Our findings confirmed that there is diversity of Gram-Positive and Gram-negative bacteria with high (> 80%) level of resistance to conventional and last resort antibiotics in sampled Ghanaian hospital environments. Majority of these strains are hereroresistant phenotypes and are highly virulent and pathogenic in GMI. The mechanisms of AMR to conventional antibiotics were associated with the presence of resistance markers (such as β-lactamases, quinolone resistant, gyrase, *KPC* carbapenemase, methicillin, aminoglycosides and macrolide efflux genes). The resistance of the strains to polymyxins (B and E) are on account of LPS modifications, especially with the identifications of the LPS modifying markers, *PhoP, PhoQ, PmrA and PrmB*. The structural differences in the lipid A moieties reported in this study are possible pointers to mechanisms of resistance exhibited by these strains. Overall, this study provides evidence on the potential high risk associated with bacteria in Ghanaian hospital environments.

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CHAPTER 1

Introduction and Study Rationale

1.0 Introduction

Bacteria are essential components of all ecosystems. They inhabit soil, water, air and even travel in space (Afshinnekoo *et al.,* 2015; Osman *et al.,* 2008). Bacteria also survive on inanimate objects such as fomites either transiently or for long periods (Boone and Gerba, 2007; Miller and Diep, 2008). Some bacteria that live in contact with or infect animals, have been implicated in zoonotic infections, which can be a health risk for humans (Cantas and Suer, 2014) and can modulate diverse activities in relation to human health (Allegranzi *et al.,* 2011). Acting as symbiotic agents, bacteria undertake various metabolic and immune-boosting activities that promote human health (Huttunen and Aittoniemi, 2011), and as pathogens, they can cause mild to severe disease in humans (Ashgar and El-said, 2012). Over the years, various forms of bacterial life have evolved harrowing human healthy living (Brown *et al.,* 2014). Thus bacteria are ubiquitous indoor, in working environments and outdoors and are mostly classified as opportunists (Price *et al.,* 2017).

The hospital environment harbors potential pathogens posing serious health risks (Scheckler *et al.,* 2014). Evidence on the public health-risks of bacteria residing in hospital environments are becoming increasingly important (Poza *et al.,* 2012). Studies have indicated that the hospital environments, especially indoors of Intensive Care Units (ICU) and Neonatal Intensive Care Units (NICU) harbor pathogenic bacteria (Lee *et al.,* 2009; Patel *et al.,* 2015). Factors facilitating their occurrence are diverse and have been established (Bereket *et al.,* 2012; Yallew *et al.,* 2017). Major among others involve interactions of different hospital users with facilities in hospital environment (McOrist *et al.,* 2002; Peters *et al.,* 2012; Gupte *et al.,* 2015). This has contributed to the prevalence of bacteria in healthcare settings, ultimately encouraging the emergence of new bacteria with health threatening potential.

In developing countries, where healthcare delivery systems are poor and where there is improper monitoring of the hospital environment, the prevalence of pathogenic bacteria is higher (European Commission, 2008; Liu *et al.,* 2011; Maina *et al.,* 2012; Hibberd *et al.,* 2016). These pathogens can become opportunistic agents (Price *et al.,* 2017), and they are responsible for deadly opportunistic infections in hospitalized patients (Brown *et al.,* 2014). Globally, opportunistic infections represent a challenge as they are difficult to treat, since the opportunistic bacteria are generally highly antibiotic resistant.

The World Health Organization has listed a series of opportunistic bacteria as Global Priority Pathogens (WHO, 2004; Breu *et al.,* 2013). These bacteria are designated the ESKAPE group, which include ***E****nterococcus faecium*, ***S****taphylococcus aureus*, ***K****lebsiella pneumoniae*, ***A****cinetobacter baumannii*, ***P****seudomonas aeruginosa* and ***E****nterobacter* spp., and all of them are linked with most of the known Hospital Acquired Infections (HAIs) (Pendleton *et al.,* 2013; Khan *et al.,* 2017). HAIs is a global health crisis with an average of 1 in 10 cases of people on hospital admission, and they are linked with significant mortality rate (Frca and Frca, 2005). The prevalence is higher in the ICU and the risk increases especially with immunocompromised patients (Frca and Frca, 2005). The risk of contracting HAIs in the NICU is between 6-30% and unclean environments increases the risk by 10% in Sub-Saharan Africa (Sydnor and Perl, 2011). An average of 10% of all hospitalized patients in North America and Europe has been reported to develop HAIs (Mehta *et al.,* 2014; Sydnor and Perl, 2011), while the risk of HAIs is more than 40% in Asia and Sub-Saharan Africa (Mehta *et al.,* 2014; Wondimagegn, 2012). In Ghana, the burden of HAIs is still not well defined as with other West African countries, the prevalence of HAIs is under-reported (Reed and Kemmerly, 2009). However, nationwide laboratory-based surveillance of AMR in clinical samples from Ghana indicated the presence of resistant bacteria

including *Salmonella* spp., *S. aureus*, *Streptococcus* spp., *Pseudomonas* spp. and *E. coli* (Opintan *et al.,* 2015).

High risk of infections have been associated with indoor bacteria, and hospitals are such a unique environment that have been reported as drivers of emergence of new bacteria (Adams *et al.,* 2015). The probability of contracting infectious agents is higher in hospitals (Smith *et al.,* 2013; Lax *et al.,* 2017). Not only admitted infected patients, but also non-infected patients with restricted mobility become vulnerable to infectious agents, particularly as a result of proximity to each other (Beggs, 2003; Gizaw *et al.,* 2016). Therefore, the microbiome of ICU is largely influenced by the presence of infected patients and this significantly determines the diversity of bacteria that are prevalent and transmitted in comparison to other environments (Smith *et al.,* 2013; Adams *et al.,* 2013).

The influences of outdoor diverse bacteria on indoor bacteria distribution, more specifically in hospitals with open ward systems have been shown (Adams *et al.,* 2013, 2015; Leung & Lee, 2016). Information on the diversity of bacteria from Ghanaian hospital environments is limited. However, the worldwide emergence of bacteria responsible for uncontrollable infections has been associated with hospital environments (David and Gill, 2008; Vouga and Greub, 2016; Almagor *et al.,* 2018). Different transmission routes in hospital indoors, such as air and fomites may play a role in the spread of majority of these infectious agents (Tagoe *et al.,* 2011; Suleyman *et al.,* 2018).

Transmission of infectious diseases is either direct or indirect (Eames *et al.,* 2009). By direct, there is a contact with infectious agents, majority of communicable HAIs engages this route (Vanhems *et al.,* 2013). Most of the reported infections in the hospital environments are via indirect means, such as vector-borne, airborne and vehicle-borne (Kleef *et al.,* 2013; Graber, 2017; Kov, 2017;

Jiang *et al.,* 2018). Airborne and fomite-mediated transmission of infections has contributed to causing most of the known HAIs (Qudiesat *et al.,* 2009; Kanamori *et al.,* 2017). While there is a need for contact with fomites directly with hands or other means, airborne-fomites interplay has been reported (Miller and Diep, 2008; Li *et al.,* 2016). Pathogens utilize more than one route of infection transmission to the susceptible hosts. Different bacterial infectious agents can utilize different or same infectious routes to cause the same disease (Vanhems *et al.,* 2013). Contacts with fomites and airborne pathogens (bio-aerosol) are unarguably two most potent means of contracting infections in the hospital environments (King *et al.,* 2013; Tagoe *et al.,* 2011; Fletcher *et al.,* 2002).

Aerosolized pathogens can move over a long distance from an infected person to the susceptible host to cause infection (Labler and Trentz, 2007; Eames *et al.,* 2009). It becomes worse due to formation of bacterial droplet nuclei, which can remain suspended for long periods in the air and can easily access this host via the nose (Lowen *et al.,* 2007; Bertolini *et al.,* 2012; Li *et al.,* 2016). This is a challenge in the ICU, which are sometimes overpopulated with patients in close proximity. Also airborne transmission facilitates fomite contamination, as aerosolized bacteria are deposited on various surfaces (Barker and Jones, 2005; Nicas and Sun, 2006). The first step towards fomite-mediated transmission is contamination. There is a follow-up requirement of survival of the infectious agents on the fomite for sufficient time to establish contact with susceptible individual (Kramer and Assadian, 2006). Further, the persisting agents on fomites must be transferrable to a point of entrance on a susceptible host to cause infection (Toussaint and Merlin, 2002; Tagoe *et al.,* 2011; Kleef *et al.,* 2013; Almagor *et al.,* 2018). Bacteria can survive on fomites for a longer period forming biofilm in some cases (Vickery *et al.,* 2012; Pirrone *et al.,* 2016). Biofilm forming bacteria can better resist chemicals, especially disinfectants (Vickery *et al.,* 2012) and antibiotics (Hu *et al.,* 2015; Vickery *et al.,* 2012).

In addition to biofilms, other factors including misuse, abuse and overuse of antibiotics are associated to bacterial resistance in hospital environments (Levy and Marshall, 2004; Ventola, 2015). Most ICU worldwide use lots of antibiotics, and also contributes to selection of resistant bacteria, which in turn leads to therapeutic failure (Prashanth and Badrinath, 2006; Lee *et al.*, 2009; WHO, 2012). Luyt *et al.* (2014) indicated that 30-60% of antibiotics prescribed in the ICU are inappropriate or unnecessary and this is regarded as a key factor for the emergence of resistant pathogens. Also, hospital environments played an important role in the dissemination of resistance (Wright, 2010; Ashbolt *et al.*, 2013; Martínez, 2008). Acquisition of resistant genes by mobilization or through transfer is a potent factor for spread of resistance in hospital environments (Finley *et al.*, 2013; Pruden, 2014). This is common with opportunistic pathogens, as they take advantage of hospital environmental facilities to develop resistance.

Antimicrobial resistance is a global health crisis (WHO, 2012; Report, 2014), as superbugs with the potential to resist virtually all available antibiotics are increasingly emerging (Contie *et al.*, 2014; Naylor *et al.*, 2018). Several studies illustrate the burden of AMR as a threat to public health, especially in complex environments like the hospital (Michael *et al.*, 2014; Mehrad *et al.*, 2015). High mortality rates of patients with AMR pathogens is common (Sengupta *et al.*, 2013; Vadivoo and Usha, 2018). In developing countries, particularly in Sub-Saharan Africa, this scenario is aggravated as the use of antibiotics is non-regulated and pathogens display high level of resistance to multiple antibiotics (Ventola, 2015; Yevutsey *et al.*, 2017). Bacteria usually respond to antibiotics by expressing either inherent or acquired resistant genes (Levy & Marshall, 2004; Bengtsson-palme *et al.*, 2018). The ability of bacteria to develop genetic mutations has also been linked with resistance especially with resistant gene sorting and possible gene rearrangements (Stokes & Gillings, 2011; Bengtsson-palme *et al.*, 2018).

1.1 Rationale

In Ghana, data on the diversity of bacteria in relation to hospital air and fomites are limited. While air and fomites are globally recognized as potential factors for bacteria distribution and transmission of infection, the spectrum of bacterial species engaging this medium in Ghanaian hospitals is not well defined. Also, the pathogenicity and virulence profiles will determine the potential health risks posed by these strains to all hospital users. As different bacteria are evolving and other new strains emerging, the risk associated with AMR in Ghanaian hospitals demand attention. The lack of the knowledge of how resistance to antibiotics are mediated and developed makes mitigation of spread and resistance a challenge. Overcoming the challenge of antibiotic resistance in the hospital appeals to a deeper understanding of the current reservoirs of resistance and the principle guiding the resistance. Exposure to antibiotics may favor selection of resistant bacteria. Besides, opportunistic pathogens resident in the environment already harbor resistant genes, or acquire resistant genes from related bacteria resident in human body, which facilitates resistant infections (**Figure 1.1**). Resistant genes are sometimes acquired from the environmental resistome (this comprises of all antibiotic resistant genes in the genome of both pathogenic and non-pathogenic bacteria). However, infections that might be very difficult to treat might results from such acquisitions, as new genes are now present in their reservoir of resistance.

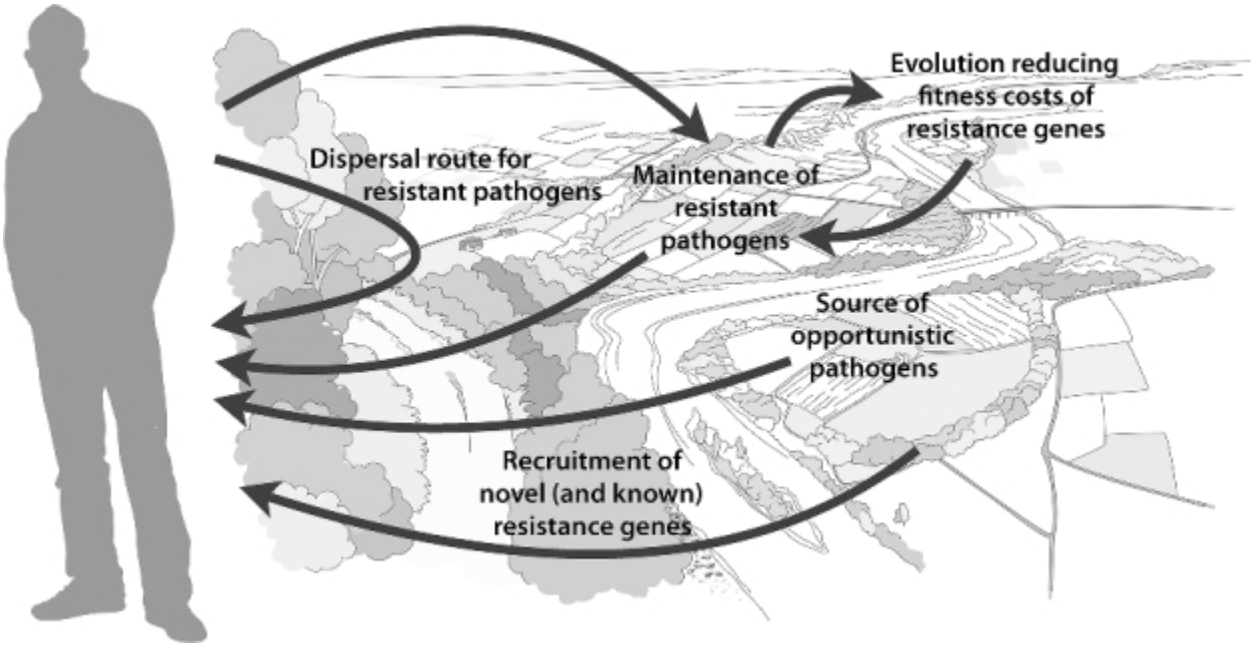


Figure 1.1: **Antibiotic resistance development and spread in the environment (**Bengtsson-palme *et al.*, 2018**)**

The rationale of this study is embodied under 5 main concepts (**Figure 1.2**):

1.) Ghana is a population of **> 28 million people** with over **300 hospitals (**WHO, 2012; Impacts, 2012**)**. Salient factors unique to this region are exposure of the population to **parasitic diseases** (notably malaria) and high prevalence of **systemic infections, sepsis** and others, these conditions influence the immune system.

2.) This observation leads to increase in hospitalization of patients with overpopulation especially within the ICU (**> 80% patients on admission**), a situation leading to overuse of antimicrobial agents.

3.) This possibly facilitates the emergence of **ESKAPE** pathogens, with **air-fomites** routes of transmission to initiate **opportunistic infections** that might be difficult to treat.

4.) These strains have potentials to resist all available antibiotics (conventional and last resort) resulting in the emergence of **superbugs** with no option for treatments (mortality rate: 1 million, 2017; 10 million, 2050; (WHO, 2012; Essack *et al.*, 2016; Prestinaci *et al.*, 2016).

5.) Essential to managing AMR (and **heteroresistance**) infections is a detailed understanding of their mechanisms, especially of the frequency and type of AMR bacteria in hospital.

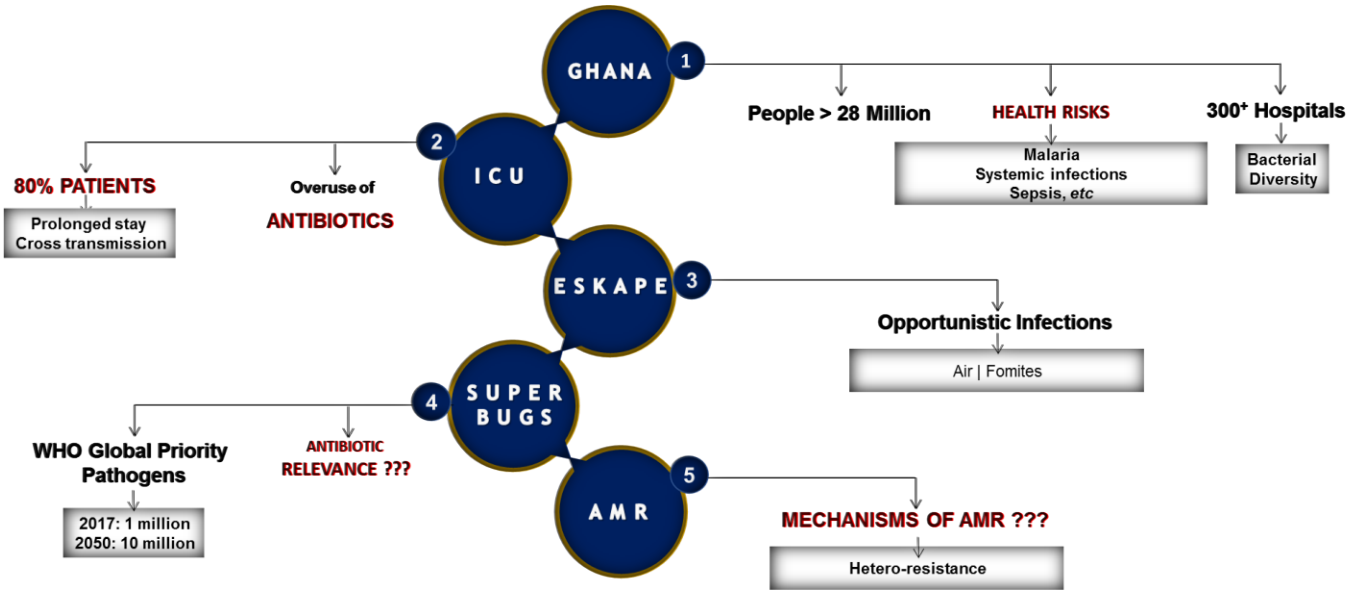


Figure 1.2: Summary of Study Rationale (Abiola Isawumi and Lydia Mosi, 2019)

1.2 Hypotheses and aims

Overall, the rate of hospital-based infection with antibiotic-resistant bacteria is on the rise. Investigating the antibiotic resistance patterns and determining resistance markers encoding virulence will inform on choices of antibiotics and serve as pointer for possible therapeutic interventions. Therefore, the study explored if **1.)** Ghanaian hospitals are dominated by **'diverse pathogenic and virulent bacterial strains' 2.)** These bacterial strains are ***'extensively resistant'***

to common and last resort antibiotics; and **3.)** Unique ***'resistant signatures'*** are drivers of their resistance mechanisms.

Specific objectives are to:

1. Phenotypically identified and molecularly characterized isolated bacteria from some Ghanaian hospital environments,

2. Determine the antimicrobial resistance profiles of the identified bacteria,

3. Determine the level of virulence and pathogenicity of the multiple antibiotic resistant bacteria in *Galleria mellonella* infection model,

4. Investigate the mechanisms of virulence and antimicrobial resistance of the highly resistant bacteria.

1.3 Organization of thesis

This thesis consists of six chapters. **Chapter 1** is the introductory section, and it provides a general overview and background to the study. The original research is presented in the four middle chapters (**Chapters 2-5**) with each chapter prepared as stand-alone manuscript. **Chapter 2** is devoted to isolation, identification and characterization of hospital air and fomite-borne bacterial strains. **Chapter 3** was designed to establish the antimicrobial profiles of the identified strains. **Chapter 4** describes the virulence and pathogenicity of these strains. **Chapter 5** investigated resistance mechanisms of the multidrug-resistant strains identified in this study. Each chapter has its own abstract, specific aims, introduction, methods, results and discussion. **Chapter 6** provides overall conclusions, future directions, and public health engagements suggestions. The last section is the **General Appendix** and **Information**; it contains relevant information and other materials for the study.

CHAPTER 2

Phenotypic Identification and Molecular Characterization of Isolated Bacteria

2.0 Abstract

A collection of bacterial strains obtained from some hospital environments in Ghana were phenotypically and molecularly characterized. The findings showed that hospital environments, especially the ICU and NICU are dominated by diverse bacteria with 80% Gram-negative resident on fomites and 53% Gram-positive circulating in the air. The study demonstrates that contamination rates of the sampled sites are above the recommended threshold (30-300 CFU/ml) for bacterial loads in hospital environments. Indoor air bacterial loads ranged from $1.80x10^3$ to $4.2x10^3$ CFU/ml, while those resident on fomites ranged from $0.7x10^3$ to $5.8x10^3$ (CFU/ml). Therefore, fomites and air are sources of diverse bacteria of pathogenic potential. Bacterial strains that are not commonly reported in association with hospital air and fomites were also discovered; an indication of a possible emergence of new bacteria in Ghanaian hospitals.

2.1 Specific Aims:

1. Isolation of bacterial strains from fomites and air samples;
2. Identification and speciation of the isolated strains using phenotypic and molecular techniques;
3. Determination of key pathogenic properties; and
4. Building a library of specific identified strains for future reference.

2.2 Introduction

Bacterial infections are a leading cause of death, especially in the hospital environment. The mortality associated with severe bacterial infections is increasing at an alarming rate in developing countries (Hibberd *et al.,* 2016). Recently, the World Health Organization and Centre for Disease Control released a list of different Gram-positive and Gram-negative bacteria with dangerous potential to cause infections because of their inherent pathogenic traits (WHO, 2017; Tacconelli, 2017). This, coupled with other forms of healthcare related problems caused by bacteria has underlined the importance of identifying and characterizing possible agents of disease transmission in both the community and other defined environments such as the hospital (Tagoe *et al.,* 2011; Fang *et al.,* 2014; Yadav *et al.,* 2015).

Hospitals are hubs for different pathogens (Ekrami *et al.,* 2011) and play special roles in dissemination of bacteria across distinct areas, thereby leading to progressive contamination that endangers the lives of both the in-patients and other hospital users (Bakkali *et al.,* 2015; Da Fonseca *et al.,* 2016; Khan *et al.,* 2017). In Ghana, as in other developing countries, little is known about the diversity and prevalence of these pathogens. Data on the identity of bacteria in sensitive hospital environments such as ICU, NICU, Maternity Unit (MU) and Surgical Wards (SGW) are limited and Hospital Acquired bacterial infection are often under-reported. However, Potential pathogenic bacteria are emerging, which demands proper identification and characterization (Armelagos et al., 1991; Vouga & Greub, 2016).

Environmental bacteria, especially those thriving in clinical settings have the potential to survive harsh conditions (Best *et al.,* 2018). Research to understand the ubiquity of these bacteria in Ghanaian hospitals is increasing, but the clinical effects are still not well defined. Understanding the roles of different infectious disease transmission agents, such as hands, surfaces, droplets,

aerosols, water, invasive devices and fomites, would help in possible bacterial infection preventive measures (Kanamori *et al.,* 2017). Studies have established that most of the Hospital Acquired bacterial infections arise from aerosols (air) and spread by fomites (**Figure 2.1**) (Fletcher *et al.,* 2002; Zemouri *et al.,* 2017). Also, most of the blood-related infections such as bacteremia and sepsis, and other immune-deficient associated bacterial infections are as a result of direct or indirect contacts with aerosol-droplets and fomites (Fletcher *et al.,* 2002; Zemouri *et al.,* 2017). Prolonged hospital stays as a result of HAIs, especially in the ICU/NICU, increases contact with fomites. Further, human-to-human transmission via infected patients occurs (Fernstrom and Goldblatt, 2013; Karo *et al.,* 2017).

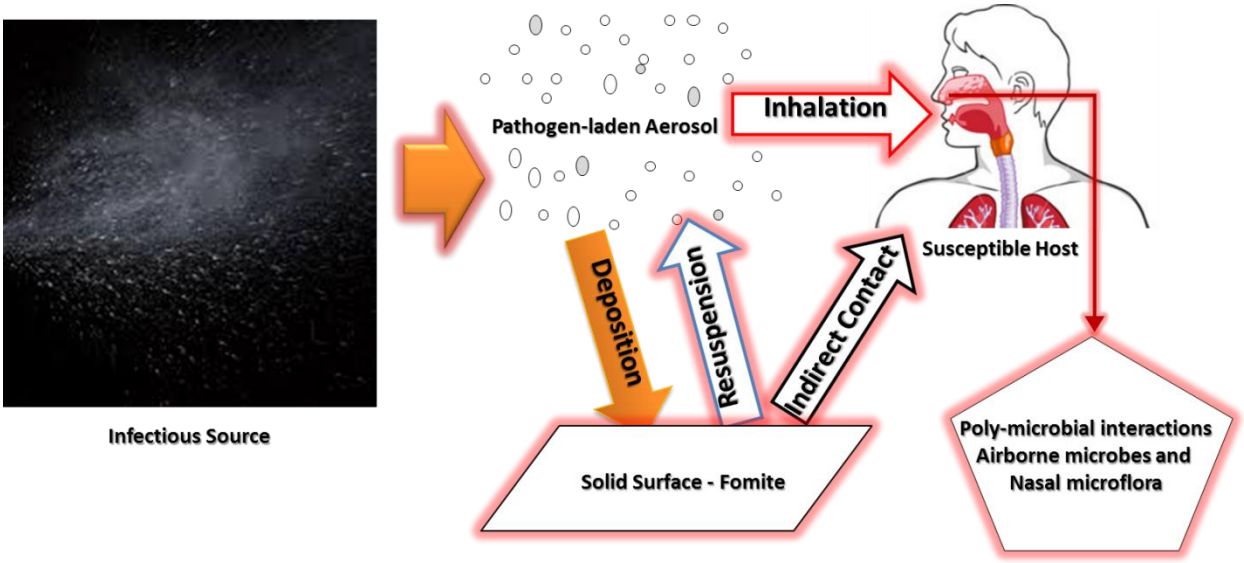


Figure 2.1: Hypothetical representation of bacterial airborne-fomites transmission in hospital environment (Abiola Isawumi and Lydia Mosi, 2019)

Environmental surfaces in the hospitals are potential reservoirs for propagation of bacteria (Fernstrom & Goldblatt, 2013; Karo *et al.,* 2017). Tables, chairs, desk surfaces, hospital door and toilet handles, taps, toilet sinks and water tub, toilet seats, flat-stairs, vents, trash-cans, waste bins, used tissue-rolls/papers and left-over food are possible potential bacterial reservoirs (Fernstrom & Goldblatt, 2013; Karo *et al.,* 2017). The contribution of fomites to the spread of infection in

Ghanaian hospitals is not well described, although some evidences indicate that hand touched fomites mediate the transmission of infections (Eze, 2012; Odigie *et al.*, 2017). The situation is worse with children; frequently touched fomites by children contribute to the increased mortality associated with diarrheal, common cold, skin rash, hand-foot-and-mouth diseases (Boone and Gerba, 2007; Miller and Diep, 2008).

Human hands harbor lots of bacteria (Zapka *et al.*, 2011), ranging from the normal residential hand microflora to transient-bacteria picked from fomites (Edmonds-wilson *et al.*, 2015). Some of these pathogens include species *Salmonella* and *Shigella*, *Clostridium*, *Escherichia coli*, and a few viruses, especially Hepatitis A virus (Boone and Gerba, 2007). Through hand contact these bacteria can be transferred from one patient to another (Kramer *et al.*, 2006; Olise *et al.*, 2018). *Staphylococcus aureus* can thrive successfully on patients' gowns and dry indoor exposed surfaces for up to six months (Neely and Maley, 2000). *Pseudomonas aeruginosa*, *E. coli* and some carbapenem-resistant Enterobacteriaceae (CRE) can survive longer on indoor wet and dry surfaces for a longer period (1-2 years) (Neely and Maley, 2000). Further, these bacteria can survive in the presence of some disinfectants (Nuñez and Moretton, 2007; Bridier *et al.*, 2011).

Areas within the hospital described as 'frequently touched surfaces', both in indoor and outdoor environments have been linked with the deposition of different pathogens (King *et al.*, 2013). Air plays special roles in transfer of pathogens from one unit of the hospital to the other. Given the potential risks of hospital contaminated air, the WHO has suggested a consistent monitoring of air quality of hospital environments (Yassin and Almouqatea, 2010). Besides, the contamination of fomites by aerosolized bacteria, fungi and viruses has been reported (Rusin *et al.*, 2002; Xiao *et al.*, 2017). Studies have reported bacterial growth on surgical table tops, bed linens and gowns of both patients and health workers (Miller and Diep, 2008; Xiao *et al.*, 2017).

Airborne bacteria can cohabit with skin microflora as a result of deposition by aerosols (Ii and Marr, 2015). Though, the skin serves as the major component of first line of natural defenses (Abdallah *et al.*, 2017); however, the interactions of airborne bacteria with bacteria resident on the skin may contribute to an increase in the bacterial opportunistic infections (Price *et al.*, 2017). Species of *Staphylococcus* and *Streptococcus*, which are normal skin flora are also the common bacteria causing skin infections (Sergent *et al.*, 2012). Mycobacteria, especially the resistant *Mycobacterium tuberculosis* has also been associated with HAI-respiratory infections (Arjomandzadegan *et al.*, 2016). Recently, airborne Gram-negative bacteria (members of Enterobacteriaceae family) have been reported to be associated with 'colonized skin and wound' infections (Sergent *et al.*, 2012).

In Ghana, bacterial infections, especially of those associated with hospital environment, play significant role in disease burden. Gram-negative enteric bacterial species of *Klebsiella, Enterobacter, Proteus, Serratia, and Citrobacter* arise from hospital environments in Ghana, posing a risk. With this view, this study was designed to phenotypically and molecularly identify and characterize bacterial isolates from air and fomites in selected hospitals of Ghana.

2.3 Method

2.3.1 Study design and Sample Collection

Ethical clearance was obtained from the Ghana Health Service and Hospital managements (GHS-ERC01/02/17) to carry out a bacteriological survey of three different selected hospital environments from Greater Accra, Eastern and Central Regions of Ghana. For ethical reasons and sensitivity of this study, the identity of the selected hospitals is described as anonymous. The hospitals were randomly selected based on the severity of health cases handled by each of the hospitals. Fomites and air samples were collected in duplicate from ICU, NICU and other locations within these selected hospitals. Over two hundred fomites (156 samples) and air (71 samples) were obtained from the selected hospitals. Fomites were obtained with sterile swabbing of different surfaces before and after cleaning with disinfectants. They include tables, chairs, desk surfaces, hospital door and toilet handles, faucets, toilet sinks and seats, flat-surfaces, waste-bins, bed-lines, used tissue-rolls/papers. Air samples were collected using passive open plate techniques as described previously (Abiola *et al.*, 2018). Briefly, plates were exposed for 60 minutes during daily active hospital working hours at different sites and temperature of the collection sites were determined.

2.3.2 Bacterial growth and culture conditions

The fomites swabbed samples were first enriched in LB broth before they were cultured on selective, differential and general-purpose agar media. Nutrient agar (Oxoid, England, CM0003), MacConkey agar (Oxoid, England, CM0007B), Blood agar (Oxoid, England, CM0055), Chocolate agar, Eiosin Methylene Blue (EMB) agar, Sorbitol agar and Mannitol salt agar (Oxoid, England, CM0085) plates were used to process the samples under aerobic conditions for 24–48 hours at 37^{o}C. The air samples collected using passive open plate agar (same media as for fomites) were

also processed using standard microbiological methods as described earlier (Abiola *et al.,* 2018; Napoli *et al.,* 2012). Non-sampled closed plates were included as controls. The plates and non-sampled plates controls were incubated at 37^{o}C under aerobic conditions for 24–48 hours. Anaerobic bacteria were isolated in a closed bacteriological Jar in the absence of oxygen (Stieglmeier *et al.,* 2009).

2.3.3 Isolation and Identification of bacterial strains

2.3.3.1 Phenotypic microbiological Identifications

Isolates were identified using phenotypic microbiological methods as described by Alonso *et al.* (2015). Microscopy (Gram's staining) and biochemical reactions were performed (Napoli *et al.,* 2012). Standard plate count was performed to determine the bacterial loads of fomites and air across the sampled sites (Napoli *et al.,* 2012). Quantitation in colony forming unit per ml (CFU/ml) was determined using an equation adapted from Samuel (2015). The sampling and overall experimental approach are described in **Figure 2.2**.

2.3.3.2 Mass Spectrometry Analysis (MALDI-TOF)

To further Identify and characterize the isolated bacterial strains, matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) was carried out as described (Ge *et al.,* 2017). Briefly, pure colonies of the strains were harvested in 20 µl of sterile deionized water. One microliter of the mixture was smeared on a target plate (Bruker Daltonics, Bremen, Germany) in replicates (to avoid or minimize random effects) and allowed to dry at room temperature. After which 1 µl of absolute ethanol was added to each well and the mixture was allowed to dry. Then, 1 µl of matrix solution (2, 5-dihydroxybenzoic acid, 50 mg/ml; 30% acetonitrile and 0.1%

trifluoroacetic acid) was added and allowed to stand for some time for co-crystallization with the sample.

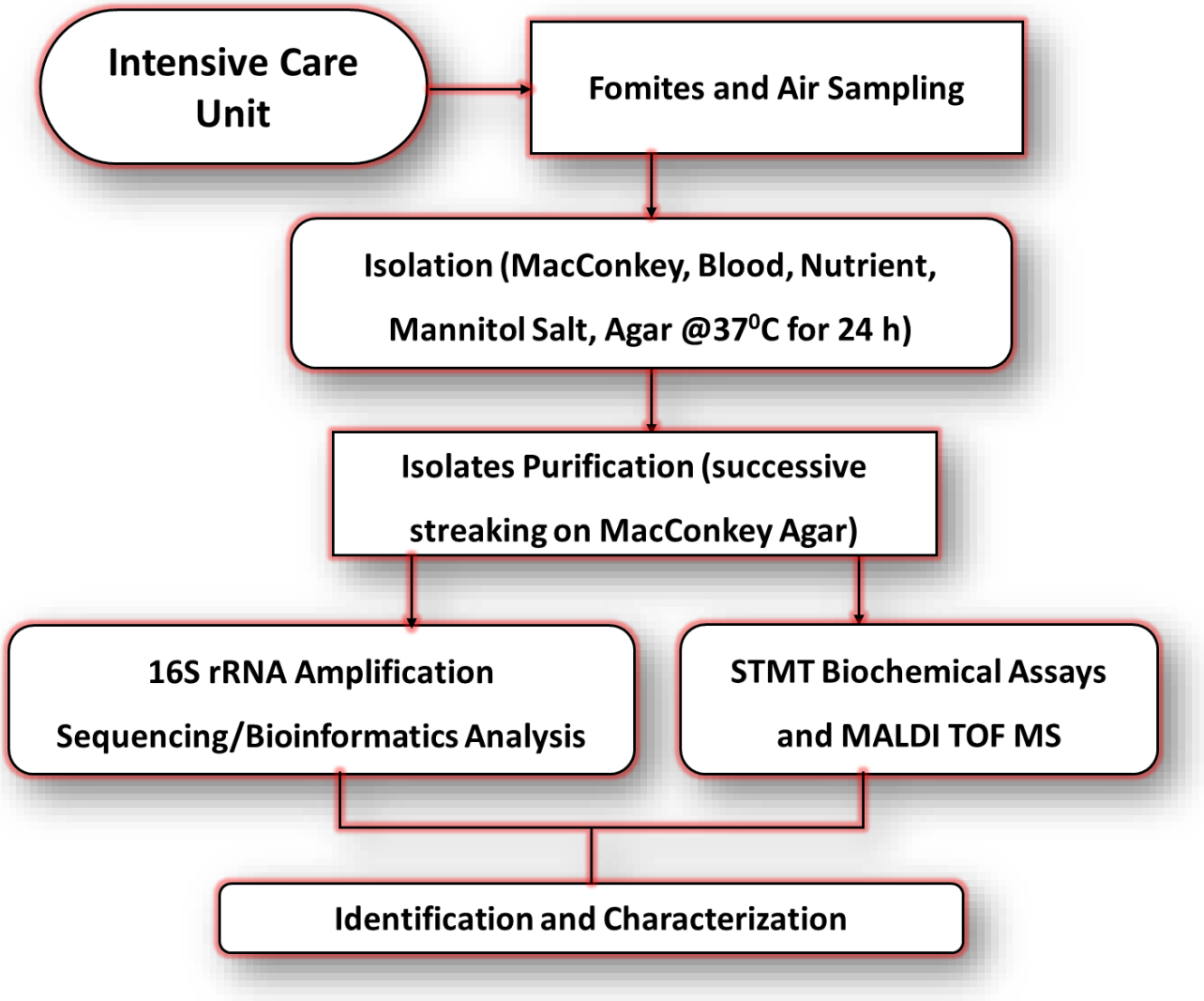


Figure 2.2: Summary of experimental approach

Next, the samples were processed with the MALDI-TOF spectrometer (MALDI-Biotyper) and the spectra data was analyzed using a flex analysis software v3.0. The peaks were compared with referenced bacterial strains in the database. Probable species identification was ranked using log-score value reflecting the peak matching the standard. Scores between 0 and 3 indicating 0 to 100%

peak-matches were used for identification. Correct and secure species identification was ranked as ≥2.0 whiles values less than 2 and ≥1.7 for genus identification (Ge *et al.*, 2017).

2.3.3.3 Polymyxin Biofilm and Motility Assays

Biofilm assay was performed using crystal violet dye as previously described (Toole, 2011). Briefly, bacterial cultures were prepared in 96-well microtiter plates containing minimal media supplemented with glucose. The plates were incubated for 48-72 hours at 37^{o}C; the planktonic cells (non-adherent cells) were removed with 0.9% normal saline (2-3 times) and washed gently with sterile ultrapure water. Two hundred µl of 0.1% crystal violet was added to each well and incubated at room temperature for 30 minutes. The crystal violet (solubilized with 96% ethanol or glacial acetic acid) was transferred into another fresh microtiter plate and the Optical Density (OD) was measured at wavelength of 590 nm and the experiment was done in triplicate. Motility swarming agar assay was prepared using 0.3% Eiken minimal media supplemented with 0.8% glucose in nutrient broth (Morales-soto *et al.*, 2015). Two to five microliters of bacterial cultures were spotted on the agar and incubated at 37^{o}C for 24 hours and the diameters of the swarm were measured.

2.3.3.4 *Preparation of Genomic DNA*

Two methods were used for DNA extraction. First was based on guanidine hydrochloride (GHCl), where a single pure colony was homogenized with 450 µl of the lysis buffer in 2 ml microcentrifuge tube. The contents were beads-beaten for 15 minutes (DNA disruptor) incubated for 20 minutes in water bath at 65^{o}C and centrifuged at 5600 g for 2 min. Potassium acetate was added to 400 µl of the supernatant and 600 µl of GHCl was pipetted into fresh tubes. Seven hundred microliter of the mixture was transferred to the spin filter and centrifuged for 2 min at 5600 g. Five hundred microliters of wash solution was added, centrifuged for 2 min at 5600 g. Five hundred

microliters of absolute ethanol was added, spun and flow through discarded. Finally, elution buffer was added, incubated for 10 min and spun at the same speed and time to elute the DNA. The second method was based on QIAGEN DNA extraction (column-based technique) kit (QIAGEN, Hilden, Germany) as an alternative DNA extraction method. Single pure colony of the bacterial strain was suspended in 150-180 µl of ATL (tissue lysis buffer) and 20 µl of proteinase K. Manufacturer's instructions were followed for the continuation and completion of DNA extraction.

2.3.3.5 *Amplification of 16S rRNA gene of bacterial strains and sequencing*

Primer sequences for the amplification of 16S rRNA genes were obtained from GenBank and designed with Primer3 design program (http://bioinfo.ut.ee/primer3-0.4.0/). Four sets of primers were used, they include; 5'-AGGAGGTAGATCCAACCGCA and 5-AACTGGAGGAAGGTGGGAT-3' as forward and reverse primers respectively. Also 5′-AGAGTTTGATCCTGGCTCAG-3′ and 5′-GGTTACCTTGTTACGACTT-3′ were used as complementary primer sequences for the 16S rRNA genes. The Basic Local Alignment Software Tool (BLAST) was used to determine the primer specificity of binding to the DNA of interest (http://bioinfo.ut.ee/primer3-0.4.0/). The Polymerase Chain Reactions (PCR) conditions were as described by the manufacturer. In general, the PCR reactions contained 10 X PCR buffer without $MgCl_2$, 1.5 mM of $MgCl_2$, 10 mM dNTPs, 10 µM of forward primer and reversed primers, 0.13 µl of Taq polymerase. Each reaction contains 2.5 µl template DNA, and was made up to a final volume of 25 µl with sterile PCR water. The amplification was carried out using a thermocycler (Biometra-T professional TRIO Thermocycler, Sheffield, UK). PCR products (5-10 µl with 0.5X DNA SYBR® dye) were resolved with 1-2% Midouri Green and Gel Red stained agarose gel (100 V, 100 mA for two hours; Consort Ev243, Antwerp, Belgium) and were viewed on Ultra Violet (UV) transilluminator and Gel Doc™ imager (Amershan™ Imager 600, Tokyo, Japan). The size

and size of amplicons were determined using Gene Ruler and DNA Molecular Weight Marker (100bp, 1Kbp, Roche).

The PCR amplicons were taken through purification process using QIAGEN PCR purification kit following the manufacturer's instructions. The PCR sample was mixed with 5-volumes of the PB buffer, applied to spin column (QIAGEN QUICK) and centrifuged to facilitate the binding of the sample to the column. The column was washed with PE Buffer; the amplicon was eluted with BE Buffer and quantified using the Nanodrop (Thermo Scientific, Washington, USA) before Standard-Seq. (Macrogen Inc., Netherlands). BLAST algorithm and SNAPGENE software (version 4.1.7) was used to determine the identity of the strains. Strain identifications were determined based on percentage similarity of bacteria in NCBI database. Percentage similarity above 95% was considered the real identity of the bacterial strain (Schlaberg *et al.,* 2012). Descriptive statistics were used in this study (with SPSS 16.0 and GraphPad 6.0) and the data presented in tables and graphs.

2.4 Results

2.4.1 Identification of isolated bacteria

2.4.1.1 General characteristics of isolated bacteria

Gram-positive and negative bacteria were isolated and identified from fomites and air samples collected from the three hospital environments sampled using both biochemical and molecular techniques. More than 400 bacterial strains were recovered and identified from over 200 samples collected aseptically. An average of two strains was isolated from each sample. The higher percentage of bacteria identified was Gram-negative and originated from fomites, especially from door handles of rooms and toilets, sink handles, flat surfaces (tablets), faucet, toilet sinks/seats and beddings. Both indoor and outdoor air samples cultured bacteria, also with higher prevalence of

Gram-negative isolates. There was similarity (>95%) observed among the isolated strains from fomites and air. Overall, fomites and air are possible sources of pathogens in the hospital environments.

2.4.1.2 Morphological and Gram's staining profiles of isolated bacterial strains

Morphological characteristics of the strains were analyzed using different agar media as earlier mentioned. Colony size, colour, margin, opacity, elevation; cell shape, sporulation and lactose-metabolic activities were used for strain identification. Strains positive with specific morphological traits were hypothetically assigned to different genera and probable species (**Table 1: Appendix IA**). Crystal violet retaining nature was used to identify the strains as Gram positive or negative bacteria. Purple or violet coloration under oil immersion light microscope indicated the Gram-positive and red or pink as negative. These strains were subjected to different biochemical tests for further confirmation. Strains were grouped as Gram-positive strains include *Staphylococcus, Streptococcus, Enterococcus, Bacillus* and *Clostridium*. The negative strains were grouped as *Acinetobacter, Acetobacter, Campylobacter, Citrobacter, Enterobacter, Klebsiella, Proteus, Salmonella* and *Serratia*.

2.4.1.3 Biochemical identification and carbohydrate fermentation of the isolated strains

Biochemical profiles of the strains were established using different tests including catalase, urease, coagulase, motility, oxidase, citrate utilization, starch hydrolysis, nitrate reduction, Indole and many others. Various reactions resulting from these tests were indicated as either positive or negative. These responsive data were further used to determine the possible genera and presumptive species of the isolated strains (**Table 2: Appendix IB**). Also, nine different sugars were used for the evaluation of the ability of the strains to metabolize various carbohydrates. Strains were identified on the basis of acid production through color change from pink (orange) to

yellow which indicated positive carbohydrate fermentation (**Table 3: Appendix IC**). In general, *Staphylococcus, Streptococcus, Enterococcus, Bacillus* and *Clostridium* were identified as common Gram-positive strains, while *Acinetobacter, Acetobacter, Campylobacter, Citrobacter, Enterobacter, Klebsiella, Proteus, Salmonella, Serratia* and *Escherichia coli* as the Gram negative.

2.4.1.4 Molecular identification of strains by Amplification of 16s rRNA genes and MALDI-TOF scoring

Genomic DNA extracted from the isolated strains was amplified using 16S rRNA sets of primers as earlier stated. The bacterial specific primers yielded amplification products ranging from 350-600 base pairs. The amplicons were sequenced using standard sequencing method at Macrogen (Netherland). The sequences were cleaned and followed by BLAST analysis. BLAST analysis revealed different species with 95% minimum similar identity to the reference strains in the databases (**Table 2.1**). There was significant correlation between the query length (QL) and the percentage query cover (QC) with minimum of 86%. This indicated that the percentage of the bacterial sequenced data successfully aligned with the sequences of the standard. Strains were scored using a very robust and diverse bacterial library MALDI Biotyper (version). Strains with scores $\geq$ 1.7 but with Score < 2 were accurately assigned to specific genus, while scores $\geq$ 2.0 qualifies strains as specific species.

Table 2.1: MALDI-TOF and 16S rRNA Identification of Isolated Strains

16S rRNA nucleotides BLAST			MALDI-TOF Biotyper	
Query Length (nucleotides)	**Query Cover (%)**	**% Similarity**	**Assigned Genus (≥ 1.7 Score < 2)**	**Species Identity (Score ≥ 2.0)**
400	95	99	Staphylococcus	*Staphylococcus aureus*
400-600	86	95	Streptococcus	*Streptococcus pneumoniae, S. pyogenes, S. durans, S. entericus*
350-650	90	99	Bacillus	*Bacillus cereus, B. subtilis, B. atrophaeus, B. manliponensis B. thuringiensis, B. abyssalis*
400	92	96	Enterococcus	*Enterococcus faecalis, E. faecium*
450	99	95	Clostridium	*Clostridium perferingens*
500	95	90	Acinetobacter	*Acinetobacter baumannii*
460	90	94	Acetobacter	*Acetobacter aceti*
520	93	90	Campylobacter	*Campylobacter enteritis*
500	99	99	Citrobacter	*Citrobacter freundii*
450-500	100	99	Enterobacter	Enterobacter cloacae, *Enterobacter cloacae* complex
400	100	100	*Escherichia coli*	*Escherichia coli*
500-600	100	100	Klebsiella	*Klebsiella pneumoniae, K. oxytoca*
500	99	97	Proteus	*Proteus mirabili, P. vulgariss*
500	95	98	Pseudomonas	*Pseudomonas aeruginosa*
450	99	95	Salmonella	*Salmonella enterica*
500	99	90	Serratia	*Serratia marcescens*

2.4.1.5 Prevalence of bacteria in hospital air

Air samples from three different hospitals in Ghana (Locations: A, B and C) were collected using passive open plate techniques at temperature ranges of 25-41^{0}C (ICU/NICU), 18-20^{0}C (Surgical ward), 25-27^{0}C (Waiting Room) and 25-28^{0}C (Maternity Department). One hundred and forty-four bacterial strains were recovered and identified from air samples. Out of the 53 indoor air samples collected, eighty-five 85 bacterial strains were identified, 32 from NICU, 53 from ICU, 9 from surgical ward, 10 from Waiting room and 11 from Maternity unit respectively (**Table 4: Appendix ID**). Twenty-nine bacterial strains from 18 outdoor samples obtained were identified with 13 from NICU and 16 from ICU.

Diverse bacterial strains were identified (using phenotypic and molecular methods as previously described) from both indoor and outdoor air samples of which 53% were Gram-positive and 47% were Gram-negative (**Figure 2.3a**).

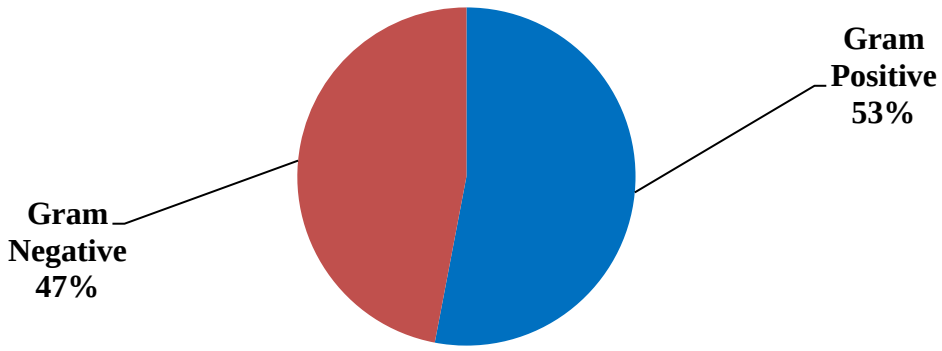


Figure 2.3a: Prevalence of bacteria from hospital air

This Gram-positive species included *Bacillus cereus, B. subtilis, B. thuringiensis, B. atrophaeus, B. manliponensis, B. abyssalis, Streptococcus pneumoniae, S. durans S. pyogenes, S. entericus, Enterococcus faecalis, Staphylococcus aureus* and *Clostridium perfringens.* The Gram-negative bacteria included *Enterobacter aerogenes, E. cloacea* complex, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Pseudomonas* spp., *Citrobacter freundii*, *Serratia marcescens*, *Escherichia coli* and *Proteus mirabilis* (**Table 5: Appendix IE**). *Pseudomonas aeruginosa*, *Bacillus cereus* and *subtilis, E. feacalis, S. aureus and E. coli* were also isolated from surgical room, waiting room and maternity room respectively. Overall, there is similarity in the diverse species of bacteria distributed across the indoor and outdoor hospital air with a few that are commonly reported, and some others that are less common, especially the Gram negative *C. freundii*, *Serratia marcescens*, *E. cloacae* complex, as well as *C. perferingens* and other species of *Bacillus* aside *B. cereus* and *B. subtilis*.

2.4.1.6 Rate of air contamination in sampled hospital environments

Bacterial load in the samples was also determined as colony forming units per cubic meter (CFU/ml) (**Figure 2.3b**). The concentration of bacteria in indoor air ranged from $1.80x10^3$ to $4.2x10^3$ CFU/ml, with the ICU sites having the highest contamination rate and the surgical rooms the lowest. The highest rate of contamination of outdoor air was observed in the ICU with $2.90x10^3$ CFU/ml as compared to the NICU. The waiting room and maternity rooms showed high rate of contamination with $2.80x10^3$ and $2.50x10^3$ CFU/ml respectively. Therefore, the air of the sampled hospital environments is significantly contaminated and also dominated by diverse bacteria with pathogenic potential.

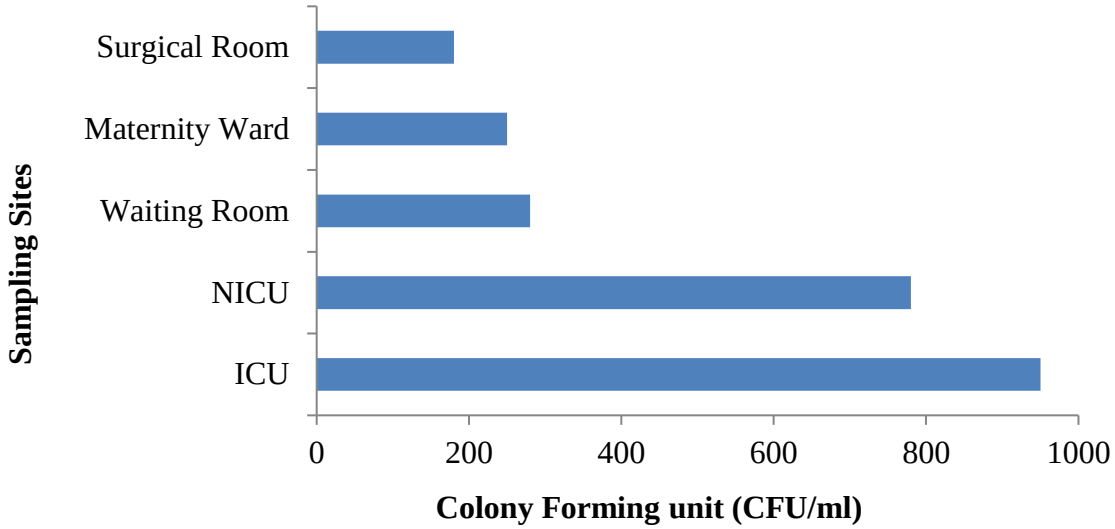


Figure 2.3b: Rate of contamination of hospital air

2.4.1.7 Prevalence of bacteria in hospital fomites

The diversity of bacterial strains on fomites in three hospital environments sampled was also assessed. One hundred and fifty-six fomites samples were collected from NICU, ICU, waiting room and maternity departments of the three hospitals. Two hundred and ninety-six bacterial strains were recovered from sampled fomites, with 54.36% (137) from ICU, 36.90% (93) from NICU, 5.5% (14) from maternity unit and 3.1% (8) from the waiting room (**Figure 2.4**). All the surfaces sampled, which include faucet, tablets, room handles, toilet (seats, sinks and handles), sinks, beddings and waste-bins from the major locations within the hospitals, harbor diverse bacterial strains. The highest number of strains was isolated from handles, toilets and faucets across the three sampled hospitals. There is variation in the number of strains resident on beddings, sinks, waste-bins and tablets across the sampled sites, however the bacterial loads relative to the sample sites are considered significant with Wilcoxon Signed Rank test ($p < 0.05$; **Page 195**).

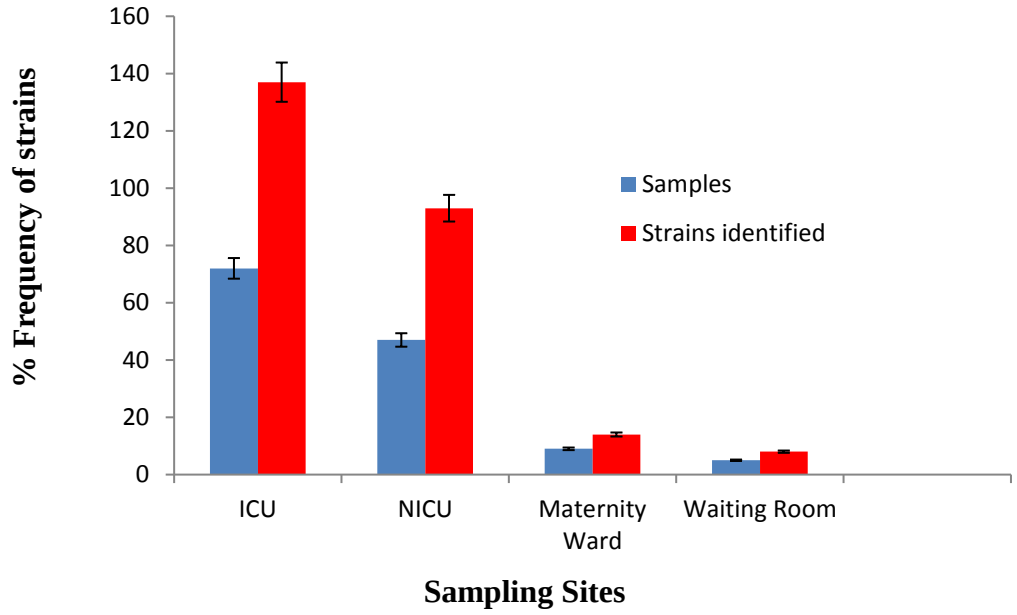


Figure 2.4: Diversity of bacteria in hospital fomites

About 80% of the identified strains were Gram-negative with an approximate amount of 20% being Gram-positive. The Gram-negative bacteria recovered and identified include those that have been implicated in HAIs and are commonly reported to reside on hospital fomites. These include *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa and Acinetobacter baumannii*. Other pathogens that are less commonly reported in hospital fomites include *Enterobacter cloacae, E. aerogenes, E. cloacae complex, E. cowanii, K. oxytoca, Citrobacter freundii, Serratia marcescens, Proteus mirabilis, P. vulgaris, Campylobacter entiritis, Salmonella enterica* and *Acetobacter aceti.* Others are *Bacillus cereus, B. subtilis, E. faecalis, Streptococcus pyogenes, S. entericus* and *S. aureus*. Faucets, handles, toilet (handles, sinks, seats) and beddings harbor more than 65% of these isolated bacterial strains. The ratio of samples obtained to the specific strains per site is 1:3 which was statistically confirmed significant with t-test ($p < 0.05$; **Page 195**).

2.4.1.8 Fomites contamination load in sampled hospital environments

The bacterial loads in the sampled fomites across the three hospitals were determined in colony forming unit (CFU/ml). The concentration of bacteria ranged from $0.7x10^3$ to $5.8x10^3$ (CFU/ml) (**Figure 2.5**). Room handles, faucets, toilets (sinks, handles, seats) and beddings of the ICU and NICU had the highest rate of contamination, followed by room handles and faucets of maternity and waiting rooms. Generally, table tops, waste-bin has the lowest rate of contaminations across the sampled sites. Room handles, faucets, toilet (sinks, handles, seats) and beddings of the ICU and NICU harbored most of the rare Gram-negative bacteria. Most of the Gram-positive strains are resident on table tops, waste-bins and chairs. An alarming rate of contamination was observed in waiting room of one of the hospitals sampled, as CFU of $2.7x10^3$ was recorded with *Citrobacter freundii, P. aeruginosa, E. faecalis and S. pneumoniae* among other isolated potential pathogens.

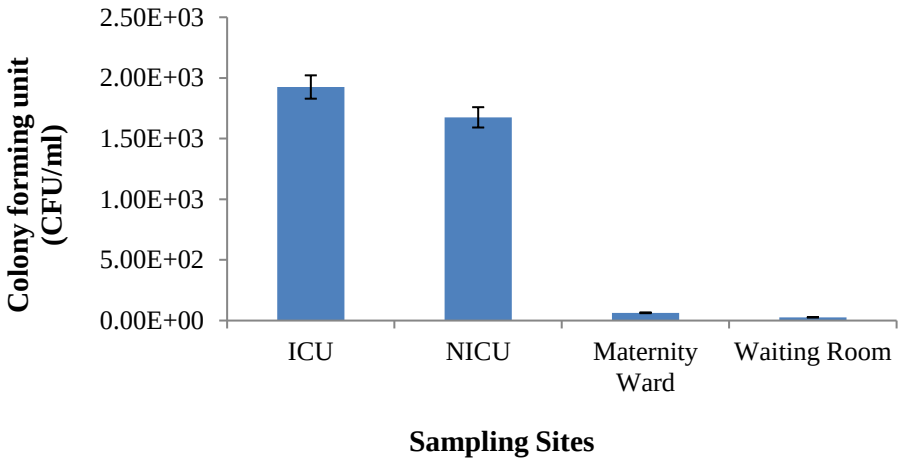


Figure 2.5: Rate of fomites contamination in sampled hospital environments

There was significant correlation between the number of samples obtained from different sites and the rate of contamination as determined by CFU/ml. There was variation in some of the strains resident on the fomites, but the similarity of the strains across the sampling sites was statistically significantly higher. Specific strains matched to specific sampled fomites are as represented on

Table 2.2. As with the air sampling, contaminated fomites in hospital environments are also dominated by diverse bacteria with a potential for pathogenicity.

Table 2.2: Summary of the Isolated and Identified Strains from Air and Fomites

Isolated Strains		
	Fomites	**Air**
Gram Positive	***Bacillus*** *cereus, B. subtilis;* ***Streptococcus*** *pyogenes, S. entericus;* ***Staphylococcus*** *aureus;* ***Enterococcus*** *faecalis*	***Bacillus*** *cereus, B. subtilis, B. thuringiensis, B. atrophaeus, B. manliponensis, B. abyssalis;* ***Streptococcus*** *pneumoniae, S. durans, S. pyogenes, S. entericus;* ***Enterococcus*** *faecalis, E. durans, E. avium;* ***Staphylococcus*** *aureus,* ***Clostridium*** *perferingens*
Gram Negative	***Enterobacter*** *cloacae, E. aerogenes, E. cowanii, E. cloacae complex;* ***Klebsiella*** *pneumoniae, K. oxytoca,* ***Escherichia coli****;* ***Pseudomonas*** *aeruginosa, P. alcaligens;* ***Citrobacter*** *freundii, Citrobacter* spp.*;* ***Serratia*** *marcescens, S. marcescens subsp.,* ***Proteus*** *mirabilis, P. vulgaris, Proteus* spp.*;* ***Acinetobacter*** *baumanni, Acinetobacter* spp.; ***Campylobacter*** *enteritis;* ***Salmonella*** *enterica;* ***Acetobacter*** *aceti*	***Enterobacter*** *aerogenes, E. cloacae* complex, ***Klebsiella*** *pneumoniae*; ***Pseudomonas*** *aeruginosa, Pseudomonas* spp.; ***Citrobacter*** *freundii*; ***Serratia*** *marcescens*; ***Escherichia coli****;* ***Proteus*** *mirabilis*

2.4.2 Characterization of representative strains

Selection of specific strains for further characterization was based on the Global Priority Pathogen list developed by World Health Organization in 2016/2017 (Tacconelli *et al.,* 2017). In general, they include the potential of the pathogens to cause outbreaks or infections that might be difficult to treat, such as ESKAPE bacteria (***E****nterococcus faecium,* ***S****taphylococcus aureus,* ***K****lebsiella pneumoniae,* ***A****cinetobacter baumannii,* ***P****seudomonas aerugionosa and* ***E****nterobacter* spp.). Some

of the strains that are less commonly reported from air and fomites samples in hospital environments as indicated in **Table 2.3** are also included.

Table 2.3: Selected strains from fomites and air samples for characterization

Test Strains	Code
Enterobacter cloacea complex	ENAB1, ENAB2
Pseudomonas aeruginosa	PGAB1, PGAB2
Citrobacter freundii	CTAB1, CTAB2
Klebsiella pneumoniae	KBAB1
Klebsiella oxytoca	KBAB2
Serratia marcescens	SRAB1, SRAB2
Acinetobcter baumanii	ACNAB1, ACNAB2
Escherichia coli	ECAB01, ECAB02
Proteus mirabilis	PRMAB1, PRMAB2
Enterococcus faecalis	ETAB1, ETAB2
Staphylococcus aureus	STAB1, STAB2

2.4.2.1 Bacteria survival at different temperatures

Growth of the selected bacterial strains was determined at different temperatures, 20^{o}C, 25^{o}C, 30^{o}C and 37^{o}C, consistent with the temperature variations at the different environments from where they were sampled. Most of the selected strains (**Table 2.4**) grew very well at 25, 30 and 37^{o}C, and also some including *Proteus mirabilis*, *Acinetobacter baumanii* and *Klebsiella oxytoca* survived at 20^{o}C. *Staphylococcus aureus* and *Enterococcus faecalis* did not survive well at temperature lower than 25^{o}C.

Table 2.4: Bacteria growth at different temperatures

Strains	Growth at different temperatures (^{o}C)			
	20	25	30	37
ENAB1	+[a]	+++[d]	+++	+++
ENAB2	+	+++	+++	+++
PGAB1	-[c]	+++	+++	+++
PGAB2	+	+++	+++	+++
CTAB1	++[c]	+++	+++	+++
CTAB2	-	+++	+++	+++
KBAB1	+	+++	+++	+++
KBAB2	+++	+++	+++	+++
SRAB1	-	++	+++	+++
SRAB2	+	+++	+++	+++
ACNAB1	++	+++	+++	+++
ACNAB2, PRMAB1	+++	+++	+++	+++
ECAB01, PRMAB2	+	+++	+++	+++
ECAB02	-	+++	+++	+++
ENTAB1	-	+	+++	+++
ENTAB2, STAB2	-	-	+++	+++
STAB1	-	+	+++	+++

[a]- = no growth, [b]+ = slight growth, [c]++ = appreciable growth, [d]+++ = strains grew well

2.4.2.2 Standard growth curve of strains at optimum temperature

Standard growth curve of the strains was determined at optimum growth temperature of 37^{o}C for 24 hours using Bioscreen C analyzer incubator. It was observed that the strains grew very well relative to the control *E. coli* ATCC 3047 over the time course with minimum optical density at (600 nm) of 0.2 at 5 hours to a maximum of 1.4 in less than 24 hours (**Figure 2.6a & b**).

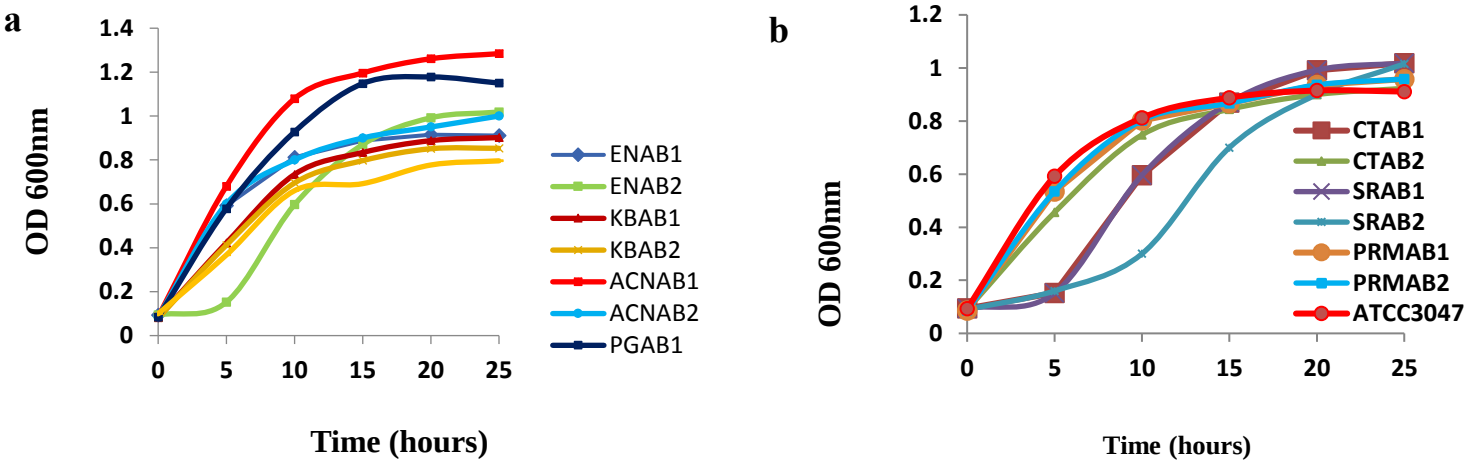


Figure 2.6: Standard Growth Curve of strains at 37ºC for 24 hours

2.4.2.3 Effects of salinity on bacteria strains

Conditional growth stresses including salt concentrations have been reported to affect the growth of different bacteria resident in the environment (El-Halfawy and Valvano, 2013). To determine if the model strains isolated from hospital environment in this study are salt tolerant, salinity test was conducted using a standard temperature gradient Bioscreen C analyzer incubator with maximum shaking. The strains were exposed to different concentrations of salt, 25%, 30%, 35% and 40% and analyzed at 37ºC for 24 hours. It was observed that the strains survived different concentrations of salt as tested in three different experiments done in triplicates with optical density (600 nm) of an average of 0.2-1.2 OD (**Table 6: Appendix IF**). The maximum growth of the strains was observed at 25% concentration with slight decrease in growth rate with increased concentrations. Bacterial growth was not inhibited at increasing concentrations of salt. This further confirms the uniqueness of these strains since most of them are not commonly isolated from air and fomites as sampled.

2.4.2.4 Swarming motility profiles of strains

Motility is a property used by enteric bacteria during colonization and biofilm formation (Kearns, 2011). Motility was assessed using standard swarm motility agar with a few additional supplements. The selected tested strains were highly motile thereby relative to the controls,

spreading at a minimum of 1 mm in ≤ 24 hours to 6.5 mm in 96 hours (**Figure 2.7** and **Figure 1**: **Appendix IG**).

2.4.2.5 Biofilm profiles of strains

The ability of the selected strains to form biofilms was tested using the crystal violet (CV) assay with and without Polymyxin B (8-32 µg/ml). As observed, 95% of the tested strains are strong biofilm producers with and without antibiotic relative to the ATCC 3047 control used. ATCC 3047 is a known biofilm producer but very sensitive to Polymyxin B. The control strain had > 1 CV absorbance (590 nm) in the presence of the antibiotic, as compared to the tested strains with minimum of 3 nm with/without antibiotic and maximum of 6.5 nm (**Figure 2.8**). This suggested that the strains were able to resist PmB. Species of *Enterobacter* (ENAB1/2), *Klebsiella (*KBAB1/2), *Acinetobacter* (ACNAB1/2), *Serratia* (SRAB1/2), *Proteus* (PRMAB1/2), *Citrobacter* (CTAB2), *and Pseudomonas* (PGAB2) are strong biofilm producers (> 3.5 strong ≥ 4), while CTAB1 and PGAB1 were classified as moderate biofilm producers (>1 moderate ≤ 3) (**Figure 2: Appendix IIH**).

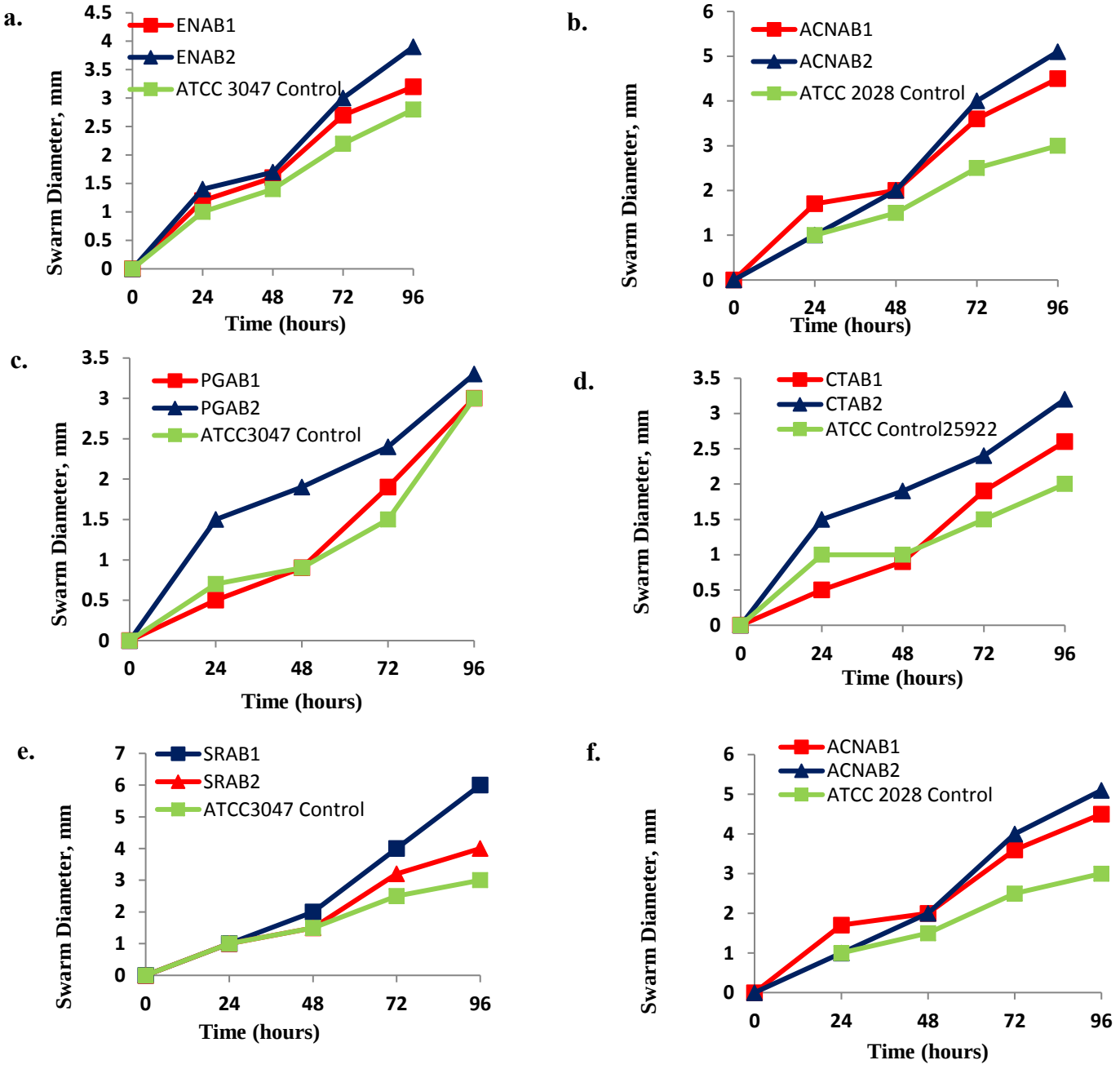


Figure 2.7: Advanced swarm motility Relative to ATCC Controls: Very Motile Strains

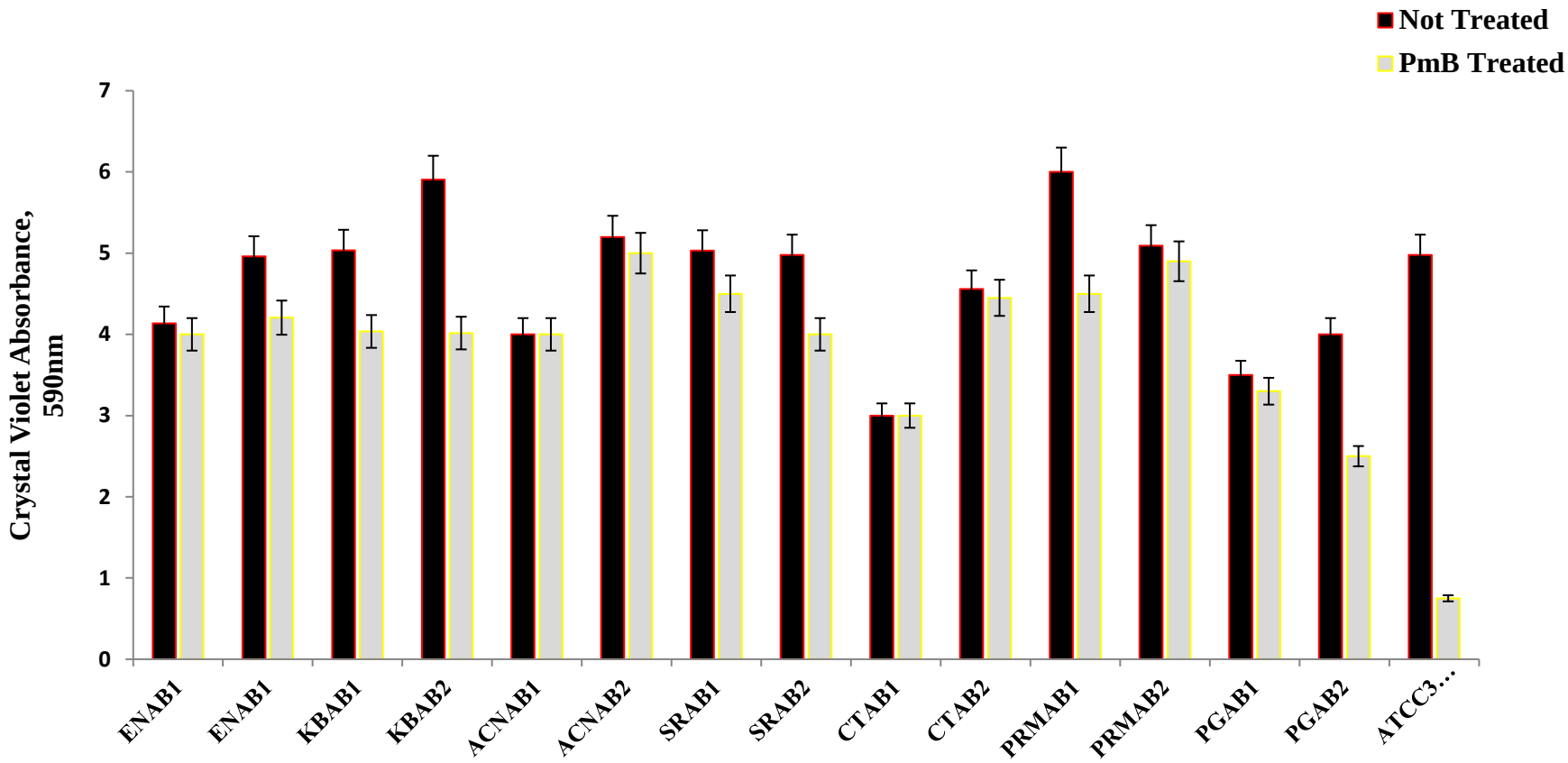


Figure 2.8: Relative biofilm quantification of strains with (Polymyxin B: 32 µg/ml) and without antibiotic; Higher absorbance indicated more biofilm production

2.5 Discussion

Hospitals accommodate patients at various stages of disease, healthcare workers, care-givers and family visitors on daily basis. Therefore, the hospital environments may also be a source for contracting infections. In Ghana as in other Sub-Sahara African developing countries, infection control measures and consistent surveillance practices to uncover possible pathogenic bacteria that are diversely distributed across most hospitals are poor (Opintan et al., 2015; Newman, 2017; Agyepong *et al.,* 2018; Labi *et al.*, 2019). The present study was conducted to consider the extent of bacterial contamination in air and fomites in three selected hospital environments in Ghana to provide data on the hygienic state of our hospitals and also uncover the bacterial strains that can be implicated in HAIs. This is important since most of the HAIs are directly linked to airborne fomite resident bacteria (Nunes *et al.*, 2005; Eze, 2012; Russotto *et al.,* 2015; Odigie *et al.*, 2017; Ngonda, 2017).

In this study, it was observed that the air and fomites across the hospitals sampled are contaminated with diverse strains, most of which were not previously reported in Ghana. Some of the identified strains were similar to those reported in studies conducted elsewhere in Africa, Europe and parts of Asia as established nosocomial pathogens (Duncan *et al.*, 2009; Gibbons *et al.,* 2011; Jung *et al.,* 2011; Celandroni *et al.,* 2016; Bazinet, 2017). However, the prevalence of airborne bacterial strains in the ICU and NICU of the Ghanaian hospitals was alarming and considered unsafe for patients, especially for those who are immunosuppressed as well as children.

This study revealed that more than 50% of the Gram-positive bacteria isolated are not commonly reported from hospital indoor and outdoor environmental air. Species of *Bacillus atrophaeus, B. manliponensis, and B. abyssalis* have not been reported from hospital environments in Ghana

before. They are opportunistic pathogens that could cause mild to severe respiratory infections (Duncan *et al.,* 2009; Jung *et al.,* 2011). Even some of them are known as surrogates *Bacillus anthracis* strains that have been linked with severe global health cases. Others like *Streptococcus durans* and Streptococcus *entericus*, also isolated across the sampled sites are linked with zoonotic infections (Warwick and Corning, 2013; Lawson *et al.,* 2018) and their involvement in nosocomial zoonoses is becoming increasingly important (Morse, 2001; Weber and Rutala, 2001). Members of the genus *Enterococcus* and *Clostridium*, especially *E. feacalis* and *C. perferinges* are notorious pathogens associated with diverse infections from hospital environments (Yang *et al.,* 2013; Lawson *et al.,* 2018). Most of these strains are global human pathogens that cause a wide variety of acute infections including pharyngitis, sore throat, common cold, skin infections, septicemia and sepsis (Prieto *et al.,* 2016; Yang *et al.,* 2013). Studies have reported the presence of species of *Staphylococcus aureus*, *Streptococcus*, *Enterococcus*, *Clostridium* and *Staphylococcus* in the environmental air of a comprehensive general hospital in Asia with prevalence rate similar to this present study (Ekrami *et al.,* 2011). Although the majority of these strains are from the air in NICU and ICU, a significant few was also reported from surgical rooms, waiting rooms and maternity departments. This is a more worrying incidence since sensitive health cases are handled in these parts of the hospital environments. Besides, more sensitive methods and robust study approach used in this study may have account for detection of these bacterial strains.

Nearly half of the strains isolated from the outdoor and indoor hospital air are Gram-negative bacteria. This suggests that the air can be a source for Gram-negative bacteria contrary to what has been reported (Park *et al.,* 2013; Getachew *et al.,* 2018; Saadoun *et al.,* 2018). Factors such as over-population of the ICU and prolonged hospital stay could provide thriving conditions for the dispersion of diverse Gram-negative pathogens (Schwab *et al.,* 2014; Saadoun *et al.,* 2018).

Acinetobacter baumannii, *Pseudomonas aeruginosa Enterobacter aerogenes*, *E. cloacae* complex, *Klebsiella pneumoniae*, *Citrobacter freundii*, *Serratia marcescens*, *Escherichia coli* and *Proteus mirabilis* were identified from the hospital environments sampled.

Most of the Gram-negative bacteria are globally prevalent in hospital and community environments, and there is likelihood for hospital-community transmission and vice-versa (Park *et al.,* 2013; Saadoun *et al.,* 2018). *Acinetobacter baumannii*, *P. aeruginosa, K. pneumoniae, Enterobacter* spp., *Escherichia coli* (Pendleton *et al.,* 2013) are opportunistic pathogens that are most often encountered in the ICU and surgical rooms (Khan *et al.,* 2017; Price *et al.,* 2017). They have been associated with bloodstream and invasive HAIs, respiratory and cystic fibrosis, especially in children, adults and immunecompromised (Pendleton *et al.,* 2013). *Citrobacter freundii*, *Serratia marcescens*, and *Proteus mirabilis* were also isolated from surgical rooms, waiting rooms and maternity department. Collectively, these strains are responsible for causing severe infections beyond their natural habitat. This observation was similar to a study conducted in Nigeria, when some of the Gram-negative bacteria were reported to be associated with HAIs in the maternity (Nwankwo, 2012). The presence of some of these bacteria in indoor hospital environments poses a serious concern (Getachew *et al.,* 2018; Suleyman *et al.,* 2018). Also, in Northwest Ethiopia, a study conducted highlighted the serious contamination of indoor air of hospital wards (Gizaw *et al.,* 2016). This study suggested that unhygienic attached toilets, poor waste management and health delivery system are possible factors responsible for the high prevalence of these bacteria in those indoor environments (Wojgani *et al.,* 2012; Umar *et al.,* 2015; Odigie *et al.,* 2017; Bhatta *et al.,* 2018).

Data from this study indicated that fomites from Ghanaian hospital environments are dominated with diverse bacteria that could be potentially associated with HAIs. This study demonstrated that

the bacterial load from frequently touched fomites such as the faucets and door handles is much higher than that of surfaces such as chairs and table tops. This is in line with studies conducted on characterization of bacterial strains from hospital fomites in other parts of Africa by Bakkali *et al.* (2015) Morocco, Ngonda (2017) Malawi, Baker *et al.* (2018) Kenya, Odigie *et al.* (2017) and Olise *et al.* (2018) Nigeria and Sserwadda *et al.* (2018) in Uganda. These studies also revealed that frequent movements of visitors, patients and health care-givers in-and-out of different sections of the hospital environments are contributing factors (Yagoub *et al.,* 2010; Ekrami *et al.,* 2011; Umar *et al.,* 2015). The door handles are engaged with no proper hand hygiene thus spread their flora to the door handles (Odigie *et al.,* 2017). As observed, this is not too different from experiences in the three hospitals sampled in this study. Also, in an attempt for health-workers to carry out their daily routines at different departments of the hospital, there was probability for cross-contamination especially when appropriate disinfection practices are compromised (Bakkali *et al.,* 2015; Odigie *et al.,* 2017; Anderson & Elmi, 2017; Yallew *et al.,* 2017).

Generally, fomites samples from NICU and ICU especially from the door handles, faucets and toilets have the largest percentage of both Gram-negative and Gram-positive bacteria. About 78% of the Gram-negative bacteria identified with fewer Gram-positive bacteria (20%) are similar to the strains recovered from the indoor air as earlier stated. They include species of *Enterobacter, Klebsiella, Pseudomonas, Cirtobacter, Acinetobacter, Salmonella, E. coli, Campylobacter, Proteus, Serratia, Bacillus, Enterococcus, Staphylococcus and Streptococcus*. Since most of these strains were isolated from ward/toilet door handles and the toilets (sinks and seats), it was observed that door locations played significant role in the distribution and transmission of pathogens. This is also supported by previous studies that reported a higher prevalence of bacterial strains in toilets as compared to other indoor environments ((Eze, 2012; Abiola *et al.,* 2018).

The lowest number of bacterial strains was recovered from the waiting room. They include *Citrobacter freundii, E. coli, S. aureus, P. aeruginosa, B. subtilis, E. faecalis*, *S. aureus*, and *S. pneumonia*. Common fomites such as the waste-bins and beddings were also found to harbor some known nosocomial pathogens, although at a very minimal proportion. It was observed that *Acinetobacter baumanni, Enterobacter cloacae, B. subtilis, E. faecalis, S. aureus, S. pneumoniae, Citrobacter freundii, E. coli, S. aureus, P. aeruginosa* are also resident on these fomites. Contamination of beddings with some of these strains might be as a result of possible secretions from patients (Olise *et al.,* 2018). This is somehow worrisome since it suggest horizontal dissemination of these strains to visitors, patients and healthcare workers as they come in contact with them, thereby increasing the chances of possible transmission of HAIs (Wojgani *et al.,* 2012; Bhatta *et al.,* 2018).

Overall, the rates of contaminations recorded in this study are above those reported in previous studies conducted in hospital environments elsewhere in Africa (Gizaw *et al.,* 2016; Sserwadda *et al.,* 2018). Reasons for this are not too clear, but geographical locations, infection control practices and ethics of health conducts are likely factors. The international standard of contamination available as provided by WHO on acceptable bacterial loads was recommended not to exceeds 1000 CFU/ml (Gizaw *et al.,* 2016). Also, Nunes *et al.* (2005) and Cappitelli *et al.* (2009) in their studies considered 750 CFU/ml as the limit for bacterial loads. Our studies recorded higher CFU counts than the recommended, an indication of extreme contaminations of the air and fomites from sampled hospitals. Sinks and waste-bins from maternity, ICU and NICU showed contaminations rate of $0.7x10^3$ to $1.4x10^3$ CFU/ml, but this is still not safe for the users.

Growth of bacteria at different temperatures has been directly linked to their ability to cause infection (Rampelotto, 2010; Schwab *et al.,* 2014; Russotto *et al.,* 2015). Our data show that

bacteria from hospital environments can survive at different temperatures, thereby suggesting that they can be transmitted and cause infections without limitation of typical ambient temperatures. This study also demonstrated that the strains are tolerant to salt, an indication of unusual response to stress. It was suspected that these strains might also survive different conditional stresses including disinfectants and antibiotics. This is also compounded by an observation that these strains are highly motile and can form biofilm. In this work, it was shown that hospital environments, especially the ICU and NICU are dominated with diverse bacteria, most of which have been implicated as global nosocomial pathogens. Also, our data demonstrate that the rates of contaminations are much higher than what is recommended for bacterial loads in hospital environments. Potentially pathogenic bacteria in hospital environments facilitate the transmission of bacteria that can cause HAIs. Majority of the identified bacteria, especially *A. baumanni, K. pneumoniae, P. aeruginosa, S. pneumoniae, S. aureus, E. faecalis* have serious clinical implications for example in blood stream infections (septicemia), pneumonia, wound/surgical infections and sepsis (Newman *et al*., 2011; Pendleton *et al.*, 2013; Khan *et al.*, 2017). Further, some bacterial strains that have not been reported in previous studies were isolated, indicating the probable emergence of new bacteria in Ghanaian hospitals. This study suggest that strong decontamination and health education measures should be in place to curtail the magnitude of the bacterial contamination of hospital environments, which in turn may contribute to the incidence of HAIs and the spread of potentially dangerous bacteria to the community.

CHAPTER 3

Antimicrobial Resistance Profiles of the Identified Strains

3.0 Abstract

Antimicrobial resistance profiles of the strains isolated from fomites and air from hospital environments, particularly the ICU and NICU were determined and established. All the tested strains displayed >80% level of resistance to conventional and last resort antibiotics. The strains displayed multiple antibiotic resistant (with 10 diverse resistance patterns) and majority are heteroresistant phenotypes with difference between highest inhibitory and highest non-inhibitory concentrations ≥8-fold. They are therefore considered potential high-risk pathogens with ≥0.2 MAR Indices standard cut-off. bb

3.1 Specific Aims:

1. Determine antibiotic susceptibility level and patterns of resistance,
2. Multiple antibiotic resistant phenotypic determination,
3. Determination of antibiotic heteroresistant phenotypes,
4. Determination of Multiple antibiotic resistance indices,
5. Selection of resistant variants and to ascertain the levels of resistance,
6. Development of micro broth dilution assays for population analysis profiling (PAP) of strains.

3.2 Introduction

Bacteria can readily adapt to changing environments as exemplified by switching from planktonic to biofilm lifestyle, changes in gene expression, and horizontal transfer of antimicrobial resistance genes and virulence factors (Ventola, 2015). Antimicrobial resistance in particular, has become a major threat to mankind due to multiple factors including excessive use of antibiotics in agriculture, breakdown in health surveillance, overuse of antibiotics in the clinic, and lack of new antibiotics in commercial pipelines (Rossolini *et al.*, 2014; Spellberg and Gilbert, 2014; Mehrad *et al.*, 2015).

Antimicrobial resistance to nearly all clinically available antibiotics reduces treatment options and can resist multiple classes of antibiotics (MDR), especially for highly resistant bacteria that thrive in the hospital environment. Gram negative bacteria are major causes of infections acquired from the hospitals, particularly in the ICU and NICU where overuse of antibiotics is a major challenge (Levy and Marshall, 2004; Mehrad *et al.*, 2015). The prevalence rates of these MDR are higher at these two locations in developing countries (Patel *et al.*, 2015; Tran *et al.*, 2017). In Ghana, the ICU is most times overpopulated and there is progressive prolonged stays of patients. Since most of these patients are immunocompromised, they become highly susceptible to bacteria that can cause HAIs. Resistant rates of bacteria to antibiotics are highest in the ICU and also associated to improper patient isolation practices, use of invasive/indwelling devices that supports biofilms growth, comorbidities and cross-spread of resistant bacteria (Agyepong *et al.*, 2018). This is compounded by the lack of new antibiotics for the pharmaceutical industry (Pendleton *et al.*, 2013).

MDR bacteria are a significant health challenge in the hospital setting (Solomon *et al.*, 2017). The ability of bacteria to display MDR phenotypes was first detected in lactose fermenting enteric

bacteria, such as *E. coli*, *Shigella sonei* and *Salmonella typhi* (Bhatta *et al.*, 2018; Eze, 2012). Most of these bacteria were implicated in untreatable nosocomial infections causing deaths in hospitalized patients, particularly in developing countries (Gaynes and Edwards, 2005). Other Enterobacteriaceae such as *Enterobacter cloacae* and *Klebsiella pneumoniae* are known pathogens with inherent antimicrobial resistance against cephalosporins and penicillins classes of antibiotics (Pendleton *et al.*, 2013). Further, Species of *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Citrobacter freundii* and other less commonly reported Gram-negative bacteria, (*e.g.*, *Serratia* and *Campylobacter*) are becoming increasingly resistant to multiple antibiotics (Champs *et al.*, 2000; Tumbarello *et al.*, 2012; Moradigaravand *et al.*, 2016; Narvaez-bravo *et al.*, 2017) (**Figure 3.1: AMR threat assessment by CDC**).

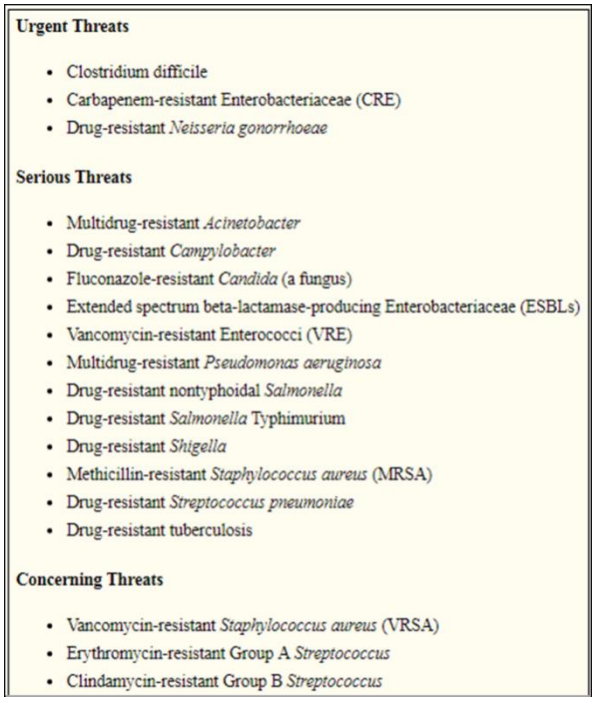


Figure 3.1: Assessment of AMR threats by CDC and WHO (CDC, 2013; WHO, 2017); http://www.cdc.gov/drugresistance/threat-report-2013

Bacteria have various ways to avoid antibiotics (Rossolini *et al.*, 2014). They can inactivate or modify antibiotics using specific enzymes such as carbapenemases that degrade β-lactam antibiotics such as penicillin and its derivatives (Levy and Marshall, 2004). Globally, carbapenem-resistant bacterial infections are difficult to treat, as the β-lactamase they produce has a broad spectrum of activity against available β-lactam antibiotics (Fair and Tor, 2014; Olaitan *et al.*, 2015). Bacteria can also alter the antimicrobial target; this prevents antibiotic action as it cannot interact with the bacterial target. This mechanism is common in quinolone resistant bacteria, as they alter the binding site for DNA gyrase A and B (Kavanagh *et al.*, 2017; Sit *et al.*, 2017). Another mechanism involves changes in metabolic pathways such with sulfonamides (Cattoir, 2016). Further, bacteria can reduce drug accumulation as a means to resistance. This is achieved by a decrease in permeability of their membrane to antibiotics or by the use of multidrug efflux genes such as *mefA* and *AcrAB* (Blanco *et al.*, 2016).

An additional problem that increases antibiotic resistance, but has not been well characterized, is the emergence of heteroresistant bacteria. "Heteroresistance" describes a phenomenon where subpopulations of seemingly isogenic bacteria exhibit a range of susceptibilities to a particular antibiotic (El-Halfawy and Valvano, 2015). Heteroresistance is potentially important in opportunistic Gram-negative and positive bacteria, which display high level, intrinsic resistance to different classes of antibiotics. These bacteria are prevalent in hospital acquired infections (El-Halfawy and Valvano, 2015), and resistance has been reported for majority of the antimicrobial classes which inclue aminoglycosides, glycopeptides, β-lactam, fluoroquinolone, and antimicrobial peptides (Rinder, 2001; Hermes *et al.*, 2013). The clinical relevance of heteroresistance is unclear, but the probability that it contributes to antimicrobial resistance is higher as selected resistant subpopulation may dominate the overall bacterial population to result

in extreme resistance to antibiotics (El-Halfawy and Valvano, 2015). Heteroresistant population contains subpopulations of bacteria with varying resistance levels to antibiotic, which makes it different from homogenous antibiotic resistance (El-Halfawy and Valvano, 2015). While information on the standard quantitative determination of heteroresistance phenomenon is limited, bacterial colonies within the zone of inhibition with E-testing or disc-diffusion are indicative of probable heteroresistant bacterial phenotype (Levin and Rozen, 2006). The gold standard method for determining heteroresitance is the population analysis profiling (PAP). This involves quantifying bacterial growth at different antibiotic concentrations to determine the difference between highest non-inhibitory and highest inhibitory concentrations (El-Halfawy and Valvano, 2013).

The MDR challenges has resulted in cases where only few antibiotics remain available, which are called last-resort antibiotics (Bartlett *et al.,* 2013). Unfortunately, resistance to these antibiotics is also appearing in different parts of the world (Arredondo-garcía *et al.,* 2007; Koch *et al.,* 2015). For example, carbapenem-resistant Enterobactericeae (CRE) have been isolated from many hospital environments around the world (Chopra *et al.,* 2012; Lodise *et al.,* 2017). There are indications for possible higher prevalence of these strains in developing countries (Agyepong *et al.,* 2018). Polymyxin B and Colistin E are very toxic antimicrobial agents that kill bacteria strains in the first 5-10 seconds of their contact (Gupta *et al.,* 2009; Cai *et al.,* 2015). Resistance to these antibiotics has also been reported especially in species of *Pseudomonas, Proteus, Serratia, Klebsiella and Enterobacter* which are mostly isolated from hospital environments in Asia, UK, US and a part of Europe (Levy and Marshall, 2004; Pendleton *et al.,* 2013).

At present, resistance to last-resort antibiotics is still not common in Africa. Studies have proposed the possible emergence of resistant bacteria to last-resort antibiotics from hospital environments

in Sub-Saharan Africa (Rossolini *et al.*, 2014; Agyepong *et al.*, 2018). Hospital acquired Infections caused by antimicrobial resistant bacteria are becoming increasingly important in Africa, also in Ghana with increasing health risks. This study sought to establish antimicrobial resistance profiles of bacteria isolated from hospital environments in Ghana and also explore these bacteria for antimicrobial heteroresistance.

3.3 Methods

3.3.1 Study design and strain collection

Strains used in this study were collected from AbiMosi Bacterial Culture (ABC©) Library, Microbiology and Molecular Biology Lab, Department of Biochemistry, Cell and Molecular Biology, University of Ghana. These strains were isolated from fomites and air from selected hospitals in Ghana. Majority of the strains selected were suspected ESKAPE and a few reported by CDC as strains of Global priority indicated in **Figure 3.1** above. Strains selected for AMR profiles are listed in **Table 3.1**.

Table 3.1: Selected strains from fomites and air samples for AMR profiles

Test Strains	Code
Enterobacter cloacae complex	ENAB1 and ENAB2
Pseudomonas aeruginosa	PGAB1 and PGAB2
Citrobacter freundii	CTAB1 and CTAB2
Klebsiella pneumoniae	KBAB1
Klebsiella oxytoca	KBAB2
Serratia marcescens	SRAB1 and SRAB2
Acinetobacter baumannii	ACNAB1 and ACNAB2
Escherichia coli	ECAB01 and ECAB02
Proteus mirabilis	PRMAB1 and PRMAB2
Enterococcus faecalis	ETAB1 and ETAB2
Staphylococcus aureus	STAB1 and STAB2

3.3.2 Strains and Reagents

Bacteria were grown in Luria-Bertani Broth at 37ºC for 24 h. Antibiotics powder used (Sigma, St Louis, MO, USA) were diluted in milli-Q water except for polymyxin, which was diluted in 0.2% bovine serum albumin/0.01% glacial acetic acid buffer. For strain recovery growth analyses, overnight cultures were diluted to an optical density of 0.005-0.05 (OD_{600}) (unless otherwise stated, and more specifically because the strains are fast grower) and incubated at 37ºC with medium continuous shaking in a Bioscreen C automated growth curve analyzer (MTX Lab Systems, Vienna, VA, USA).

3.3.3 Disc diffusion assay to determine the antibiotic susceptibility of the strains

Disc diffusion assay was employed to determine the antimicrobial susceptibility of the bacterial strains to different antibiotics following the guidelines of Clinical Laboratory Standards Institute (CLSI 12th Edition). Briefly, strains were streaked on LB-Agar (Oxoid, Hampshire, England) and incubated at 37ºC overnight to obtain pure colonies. Pure colonies were suspended in Mueller-Hinton Broth (MHB) (Oxoid, Hampshire, England) and grow until logarithmic-phase. The logarithmic-phase cells were spread on Mueller-Hinton Agar under aseptic conditions. Thereafter, antibiotic discs were applied on the plates. Antibiotics selected were those commonly prescribed by clinicians based on their general known effectiveness against bacterial infections in Ghana.

The discs used for screening Gram-positive and Gram-negative bacteria contained the following antibiotics with the respective concentrations: ampicillin (10µg), cefotaxime (30µg), chloramphenicol (30µg), ciprofloxacin (5µg), gentamicin (10µg), nalidixic acid (30µg), nitrofurantoin (200µg), methicillin (30µg), tetracycline (30µg), penicillin (15µg), flucloxacillin (5µg), cloxacillin (10µg), vancomycin (30µg), erythromycin (5µg), ceftriaxone (30µg), methicillin (250µg), ceftazidime (30µg) and cotrimoxazole (25µg) (Mast Diagnostics, Mast Group Ltd.,

Merseyside, U.K.). The reference strains of *E. coli* ATCC 3047 and *S. aureus* 25923 were used as controls.

3.3.4 E-Test antibiotic susceptibility testing assay

Polymyxin B and Colistin E (0.064-1024 µg/ml) E-test strips (AB bioMérieux, Solna, Sweden), were applied to agar plates inoculated with test bacteria by spreading overnight culture suspension in 0.85-0.9% saline diluted to OD_{600} of 0.001; plates were then incubated at 37^{o}C for 24 hours. The minimum inhibitory concentrations (MIC, µg/ml) of the strains were established at the meeting point of the bacterial halo and the test strip (Kulengowski *et al.*, 2018). Secondary or unclear halo or colonies (squatter colonies) within halo or zone of inhibition, an indication of heteroresistance were also examined as described by El-halfawy and Valvano, 2015.

3.3.5 Polymyxin µ dilution antibiotic bioassay

Micro-broth dilution antibiotic bioassay was developed to determine the MIC of bacterial strains to Polymyxin B (PmB) (**Figure 3.2**). The experimental PmB assay was made by dissolving 12,000 µg/ml Polymyxin B sulfate salt (Sigma) in a 10 ml buffer solution (0.2% bovine serum albumin/0.01% glacial acetic acid) and was filter sterilized. Overnight test bacterial culture was diluted into fresh media and grown to logarithmic-phase in double strength MHB (OD_{600} nm, 0.2 to 0.4). In a Bioscreen special well plates, 100 ul of the culture was challenged with 100 ul of double concentrations of PmB (8.0-2048 µg/ml) and incubated at 37^{o}C with maximum shaking continuously for 24 hours in a Bioscreen C automated growth curve analyzer (MTX Lab Systems, Vienna, VA, USA). Minimum inhibitory concentration was determined using the percentage of the OD of the test strain to the OD of ATCC3047 control. The percentage OD < 10 was the assigned MIC of the strains at the specific concentration (Loutet *et al.*, 2011; El-Halfawy and Valvano, 2013).

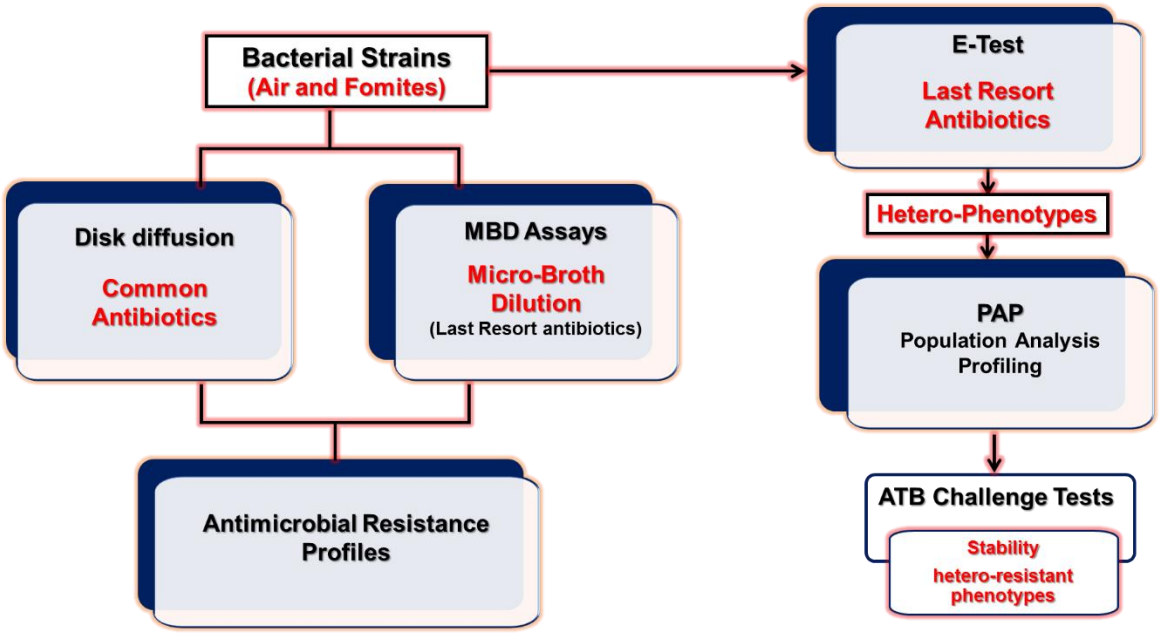


Figure 3.2: Summary of experimental approach

3.3.6 Population Analysis Profiling for Heteroresistance determination

Bacterial cultures were treated with doubling increments of antibiotic concentrations. Growth at each concentration was determined by turbidimetry in MHB (PAP by broth dilution) in Bioscreen C automated gradient incubator (MTX Lab Systems, Vienna, VA, USA), at 37^{o}C for 24 hours. Also, PAP was also determined by agar dilution with CFU count on MH/LB agar plates (PAP by agar dilution). Heteroresistance was considered when the difference between antibiotic concentrations exhibiting the highest inhibitory effects was 8-fold or higher than the highest non-inhibitory concentration (**Figure 3.3a**).

3.3.7 Polymyxin B sensitivity assay

Polymyxin B sensitive challenge assay was developed to determine the stability of the heteroresistant phenotypes and recover the PmB resistant variants (**Figure 3.3b**). Bacterial cells at the logarithmic-phase were diluted to a concentration of 2 x 10^5 CFU/ml (Loutet *et al.,* 2006; Loutet and Valvano, 2011). Hundred microliters of each of the bacterial culture was challenged

with 100 µg/ml of PmB in 0.2% BSA and 0.01% acetic acid buffer, with buffer, and buffer + strain as controls. Flash incubation of the cultures at 37°C for 2-3 hours in a rotary incubator. Viable cells were enumerated by CFU count on LB agar with 10µl at each time course (Loutet *et al.*, 2011; Loutet and Valvano, 2011). Data on the resistance profiles were summarized using descriptive statistics and resistance levels were presented as percentage errors.

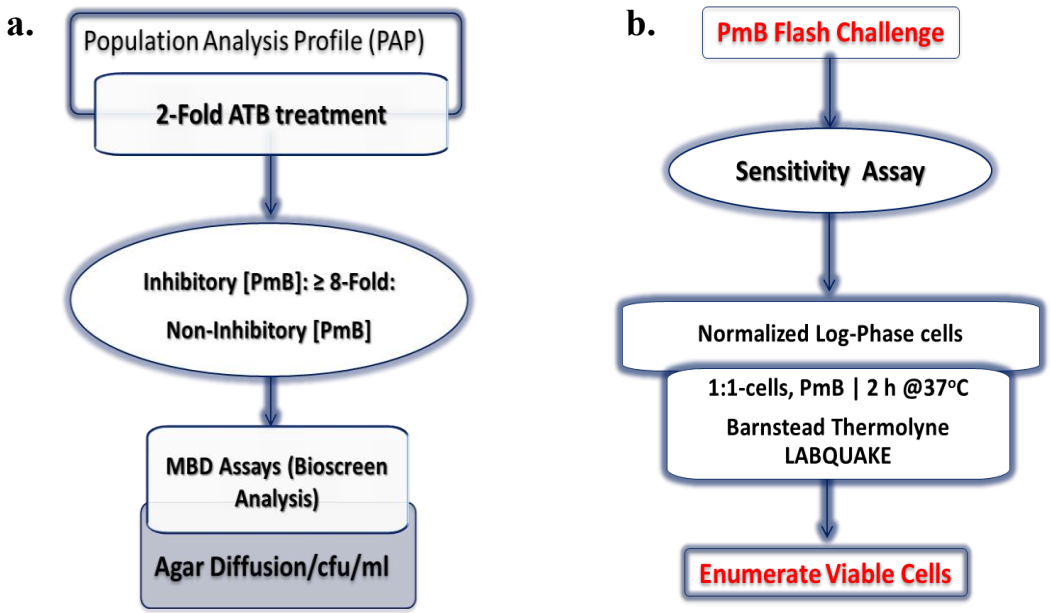


Figure 3.3: Experimental flowchart; a) PAP to determine heteroresistant phenotypes b.) Flash challenge assay to enumerate viable cells

3.4 Results

3.4.1 High level of resistance to conventional antibiotics

Eighteen antibiotics belonging to nine different classes were selected and tested to determine the antimicrobial susceptibility and resistance profiles of bacterial strains isolated. Sixteen of the antibiotics belonging to nine classes were used to challenge the Gram-positive bacteria, while all the other antibiotics without vancomycin and methicillin were tested on Gram-negative bacteria. Extreme resistance to all these antibiotics was observed relative to the controls *E. coli* ATCC 3047 and *S. aureus* ATCC25923 with established antimicrobial profiles. The AMR profiles showed that more than 90% of the Gram-positive bacteria (STAB1/2 and ETAB1/2) were 100% resistant to all the classes of antibiotics tested (**Table 3.2**). It was also noticed that the resistance rate to vancomycin and methicillin by Gram-positive bacteria was extremely high (> 97%). For the Gram-negative bacteria, more than 80% resistance rate was displayed to all the classes of antibiotic tested (**Figure 3.4**). A few secondary or unclear zone of inhibitions were observed in species of *Enterobacter* (ENAB2) to ampicillin; *Pseudomonas* (PGAB2) to chloramphenicol and gentamicin; *Citrobacter* (CTAB1) to flucloxacillin and gentamicin; *Serratia* (SRAB1) to cefuroxime; *Proteus* (PRMAB1) to nitrofurantoin; *Acinetobacter* (ACNAB1/2) to ceftazidime and flucloxacillin (**Table 1, Appendix IIA**). The *E. coli* strains showed 100% resistance to all the fifteen antibiotics tested. Overall, 100% and 97.75% resistance rates were observed in Gram-positive and negative-bacteria tested respectively, 1.25% rate of susceptibility of KAB1 (ampicillin), KBAB2 (gentamicin) and SRAB2 (ampicillin).

Table 3.2: Antimicrobial profiles of GPB strains indicating the zone of inhibitions

S[No]	Antibiotics (ug)	Resistance Profiles (Zone of inhibition, mm)				
		STAB1	STAB2	ETAB1	ETAB2	*S. aureus*
1	Methicillin (10)	[a]H[R]	[b]+	+	+	+
2	Flucloxacillin (5)	+	+	+	+	+
3	Vancomycin (30)	+	+	+	+	+
4	Erythromycin (5)	+	+	+	+	+
5	Cloxacillin (5)	+	+	+	+	+
6	Cotrimoxazole (25)	+	+	+	+	+
7	Nitrofurantoin (200)	+	+	+	+	+
8	Chloramphenicol (30)	+	+	+	+	+
9	Tetracycline (10)	+	+	+	+	+
10	Cefotaxime (10)	+	+	+	+	+
11	Cefuroxime (30)	+	+	+	+	+
12	Penicillin (15)	+	+	+	+	+
13	Ampicillin (10)	+	+	-	+	-
14	Nalidixic Acid (30)	+	+	+	+	+
15	Gentamicin (10)	+	+	+	[c]-	+
16	Ceftazidime (30)	+	+	+	+	+

[a]H[R] – unclear zone of inhibition, + = Resistance (zone of inhibition ≤5 mm), [c]- = Susceptible (zone of inhibition 10-25 mm, *S. aureus* 25923 (control)

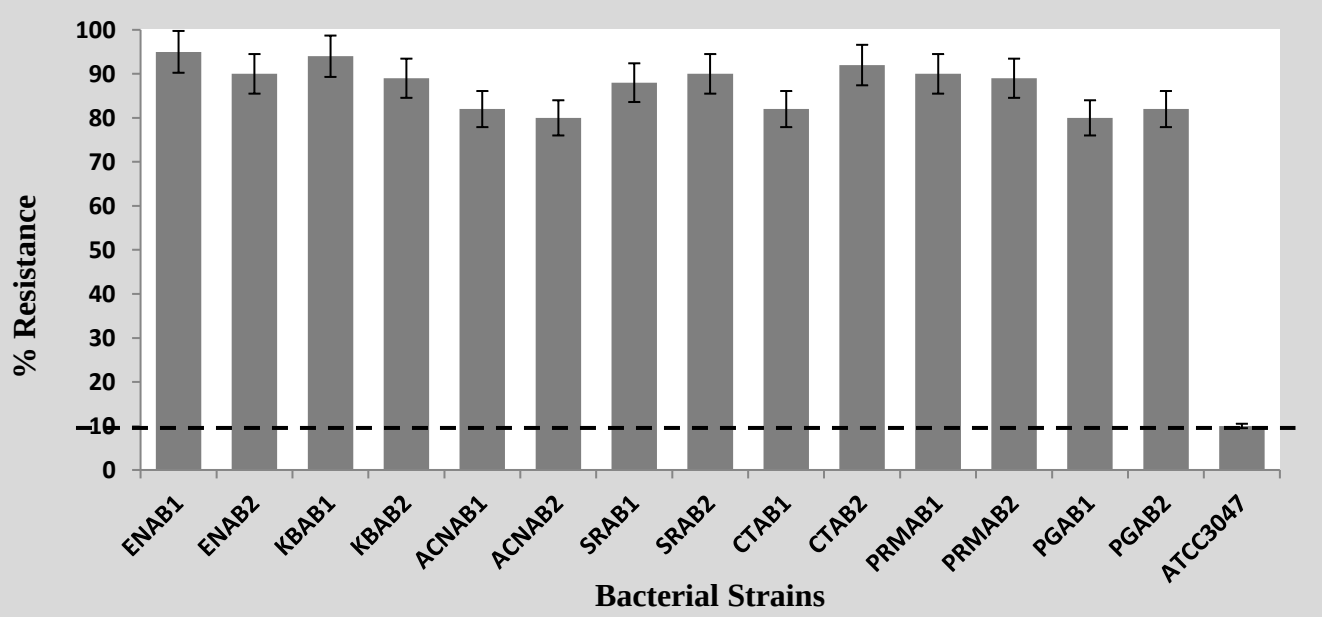


Figure 3.4: Antimicrobial Resistance Profiles of the selected Gram-negative bacteria to conventional antibiotics (CLSI and EUCAST standards); The dotted line indicates the susceptibility of the ATCC3047 control relative to the tested bacteria resistance level. The error bars indicate percentage resistance of the bacteria (x-axis) to the selected antibiotics

3.4.2 Multiple Antibiotic Resistance (MAR) Index

Multiple Antibiotic Resistance (MAR) Index of the tested strains was determined. The MAR Index was determined as the ratio of antibiotic tested to antibiotic resisted by the strains (Mandal *et al.*, 2018; Davis and Brown, 2019). The MAR Index was determined with respect to eighteen different antibiotics tested. For the sixteen antibiotics tested for Gram-positive bacteria, resistance was observed to all, indicating that the MAR Index is 1.0. Gram-negative bacteria also displayed resistance to all the fifteen antibiotics tested with MAR Index of 1.0 (**Table 3.3**). Bacterial strain is considered a high-risk pathogen when the MAR Index cut-off is **≥ 0.2** (. MAR Index of 1.0 as observed in the tested strains indicated that they are high risk pathogens.

Table 3.3: Multiple Antibiotic Resistance Index

Strains	Antibiotic Tested (a)	Antibiotics Resisted (b)	[a]MAR Index: a/b
ENAB1/2	15	15	1.0
KAAB1/2	15	15	1.0
SRAB1/2	15	15	1.0
CTAB1/2	15	15	1.0
ACNAB1/2	15	15	1.0
PRMAB1/2	15	15	1.0
PGAB1/2	15	15	1.0
ECAB01/02	15	15	1.0
STAB1/2	16	16	1.0
ETAB1/2	16	16	1.0

[a]**MAR index ≥ 0.2**: High risk Pathogen

3.4.3 Strains resisted more than two classes of antibiotics (MAR)

Multiple antibiotic resistance (MAR) was defined in this study as the resistance of the strains to at least two classes of the antibiotics tested (Magiorakos *et al.*, 2011; Abiola *et al.*, 2018). All the strains showed resistance to at least 2-classes of the nine different classes of antibiotics tested. Twenty different anti-biotypes (multiple antibiotic resistance patterns) were observed across the nine different classes of antibiotics tested for the strains. Gram-negative from **Figure 3.3** and positive bacteria on **Table 3.2** showed sixteen and four different MAR patterns respectively. Also, the nine different classes have unique patterns across strains of the same species and those belonging to the same genus (**Figure 3.5**).

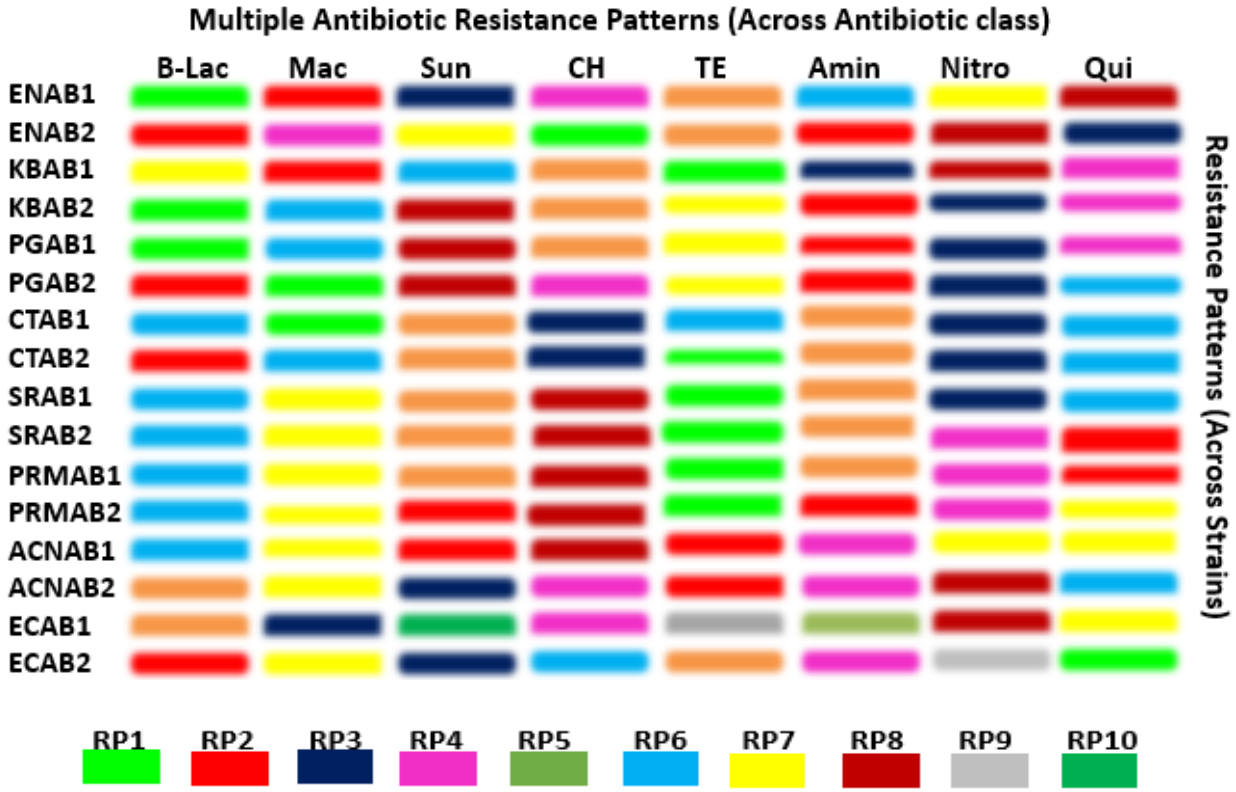


Figure 3.5: Multiple Antimicrobial Resistance (Resistance to at least 2-classes of Antibiotics) and Patterns of AMR with each color code representing a unique resistance pattern. Overall, there are 10 different antibiotic resistance patterns observed in this study indicating that the bacterial strains diverse resistance mechanisms to the selected classes of antibiotics

3.4.4 Extreme resistance to last resort antibiotics

Carbapenems, colistin E and polymyxin B, are last resort antibiotics with established effectiveness against different MAR pathogens, especially bugs that have defiled the treatment option of conventional antibiotics (CLSI, 2009; Koch *et al.*, 2015). Selected strains were challenged with the three antibiotics since they have displayed high level of resistance to other common antibiotics. All the strains tested showed extreme resistance to the last resort antibiotics. More than 80% resistance rates were observed in all the tested strains to carbapenem, polymyxin B and colistin E relative to the ATCC 3047 control which was very sensitive to the antibiotics (**Figure 3.6**). This high level of resistance observed further confirms that they are high risk potential pathogens and are superbugs that might cause serious infections in the hospital environments.

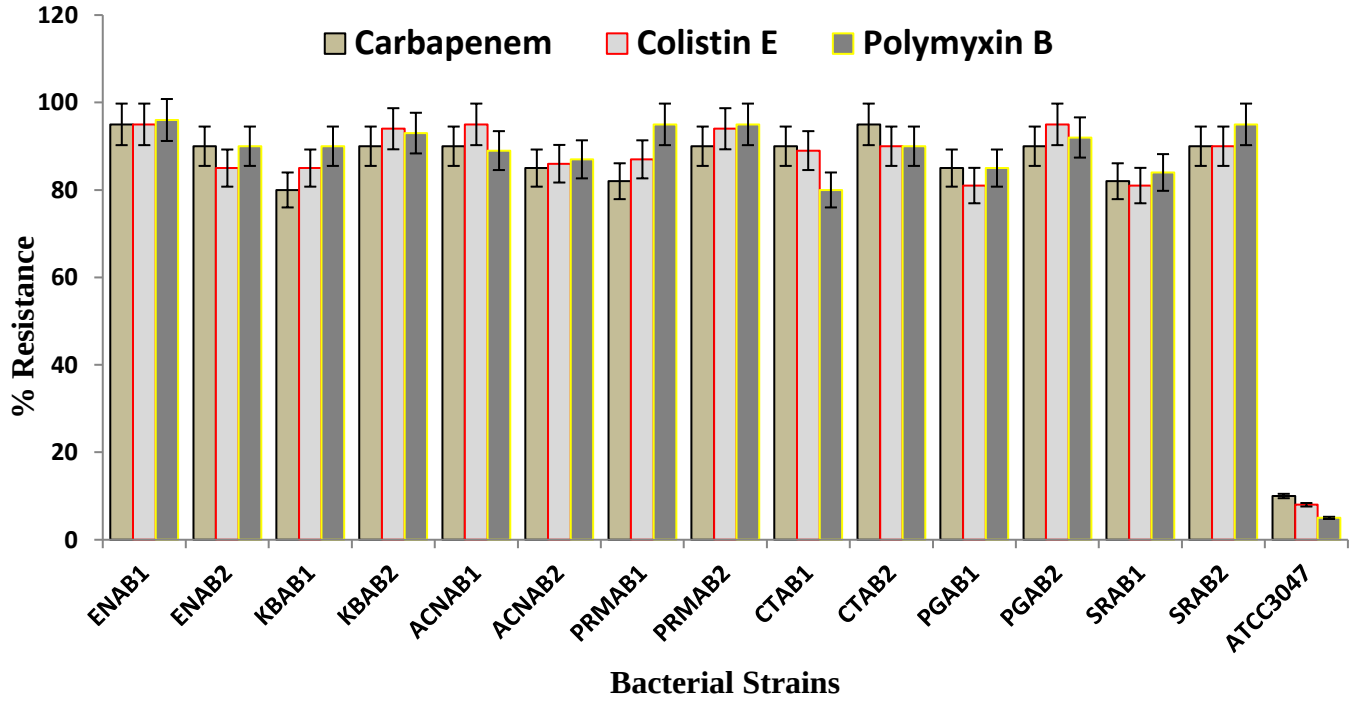


Figure 3.6: Resistance to last resort antibiotics relative to susceptible ATCC3047 control. The error bars showed the percentage resistance of the bacteria (indicated on x-axis) with more than 80% resistance level to the selected antibiotics

To further confirm how resistant these bacterial strains are, PmB micro-broth dilution sensitive assay was engaged to determine their MICs. The MIC ranges from 512 to ≥ 1024 µg/ml (**Table 3.4)**, which is far above the cut-off point for PmB and colistin resistance (Susceptible ≤ 2 µg/ml, Resistance ≥ 4 µg/ml) (Falagas and Kasiakou, 2005; Zavascki *et al.*, 2007; Srinivas and Rivard, 2017).

Table 3.4: Minimum inhibitory concentrations of strains (Micro-broth dilution)

S^{No}	Strains	[a]MIC of Polymyxin B (µg/ml)
1	ENAB1	1024
2	ENAB2	>1024
3	PGAB1	512
4	PGAB2	>1024
5	CTAB1	1024
6	CTAB2	1024
7	KBAB1	1024
8	KBAB2	1024
9	SRAB1	1024
10	SRAB2	>1024
11	ACNAB1	512
12	ACNAB2	1024
13	ECAB01	>1024
14	ECAB02	>1024
15	PRMAB1	1024
16	PRMAB2	1024

[a]CLSI standard: Susceptible ≤ 2 µg/ml, Resistance ≥ 4 µg/ml

3.4.5 Strains with heteroresistant phenotypes

Unclear or distinct colonies within the zone of inhibition indicated heterogenous response to polymyxin B and colistin E (**Figure 3.7**).

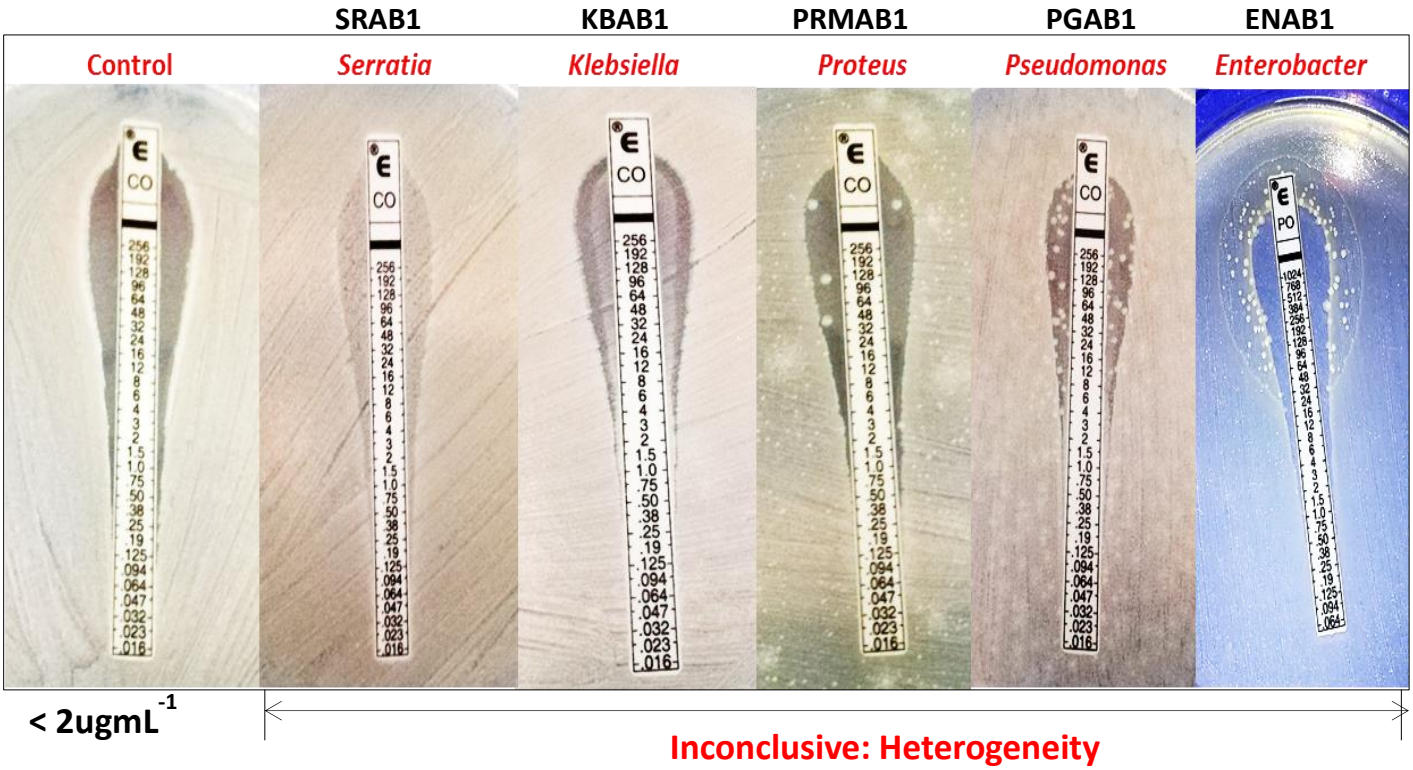


Figure 3.7: Distinct colonies within inhibition zone an indication of heterogenous response to Polymyxin B (E-Test Assay: 0.064-1024 µg/mL); The pictures are not drawn to any standard scale; they are snippets from the representative E-Test plates

Since this was revealed by E-test assay, it was further confirmed with population analysis profiling. The strains were treated with double increments of PmB concentration in a micro-broth dilution assay. The difference between the highest minimum inhibitory and non-inhibitory concentrations as 8-fold and greater was determined. It was observed that majority of the tested strains were heteroresistant positive by PAP (**Figure 1, Appendix IIB**). For instance, the highest non-inhibitory concentrations range was 512-1024 µg/ml, while the highest inhibitory concentration was 8192

µg/ml. This indicated a >8-fold difference to indicate that the strains are heteroresistant (**Table 3.5**). The stability of the heteroresistant variants was ascertained using a PmB challenge test. A frequency of 10-40% recovery rates indicated the stability of these resistant variants (**Figure 3.8**).

Table 3.5: PAP profiles of the strains

S^{No}	Strains	Highest non-inhibitory conc. (µg/ml)	Highest non-inhibitory conc. (µg/ml)	[a]PAP ≥ 8
1	ENAB1	1024	8192	+
2	ENAB2	1024	8192	+
3	PGAB1	512	8192	+
4	PGAB2	1024	8192	+
5	CTAB1	1024	8192	+
6	CTAB2	1024	8192	+
7	KBAB1	1024	8192	+
8	KBAB2	1024	8192	+
9	SRAB1	1024	8192	+
10	SRAB2	1024	8192	+
11	ACNAB1	512	8192	+
12	ACNAB2	1024	8192	+
13	ECAB01	1024	8192	+
14	ECAB02	1024	8192	+
15	PRMAB1	1024	8192	+
16	PRMAB2	1024	8192	+

[a]Strains positive for PAP: difference between highest inhibitory and non-inhibitory concentrations ≥ 8

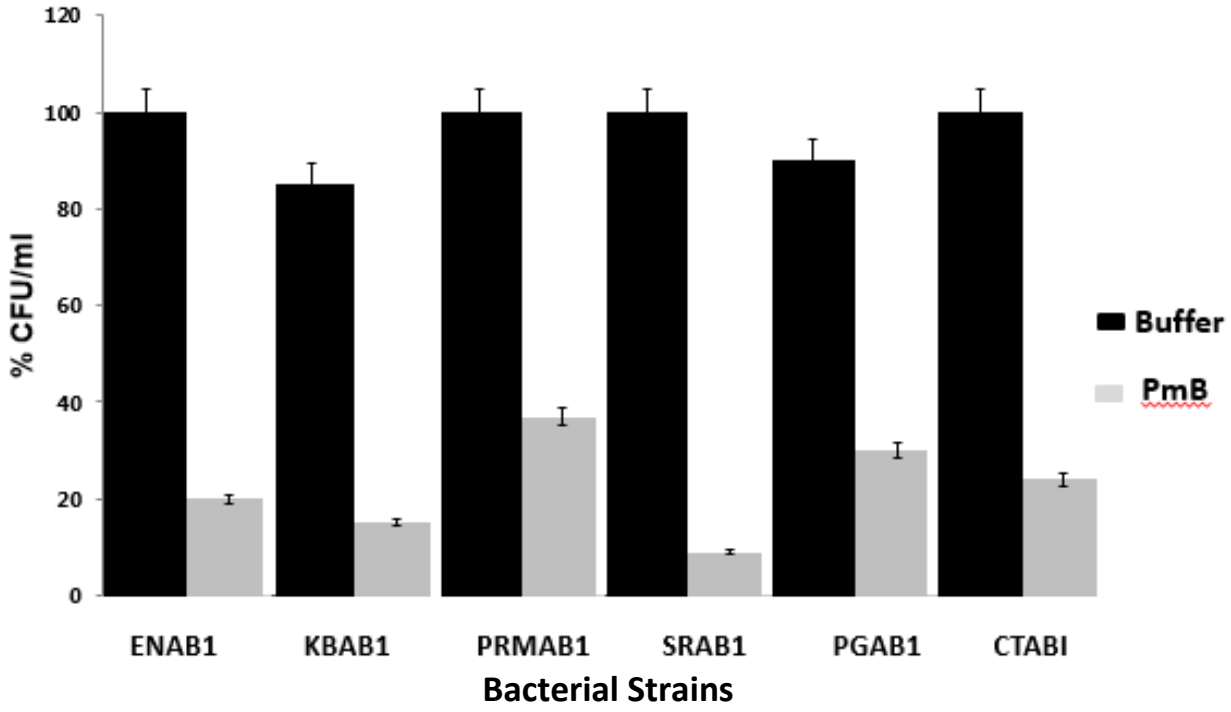


Figure 3.8: The suspected heteroresistant bacterial strains (from Table 3.5) **on x-axis were challenged with 2-fold of PmB MIC in a Flash-challenge test; this was to establish that heteroresistance was not a random occurrence. The least resistant non-heteroresistant population could not survive in the PmB challenge test, allowing the heteroresistantly stable strains to survive the 2-fold MIC. Error bars indicate percentage survival and stability of the heteroresistant strains.**

3.5 Discussion

Bacteria continuously evolve different mechanisms to display resistance to antibiotics globally (Eggleston *et al.,* 2010). Most of the HAIs caused by resistant bacteria lead to prolonged duration of ailments and have limited therapeutic treatment options (Levy and Marshall, 2004). While surveillance efforts by the developed countries have uncovered some global resistant pathogens, the challenges keep increasing in Africa where the tracking of these pathogens are not clearly defined and do not have a broad coverage (Bloom *et al.,* 2017). In Ghana, resistance of bacteria to various antibiotics in the hospital setting is now a great threat to all hospital users (Agyepong *et al.,* 2018). Previous studies demonstrated high prevalence of bacterial resistance to conventional antibiotics in the in-patients of ICU (Yevutsey *et al.,* 2017; Agyepong *et al.,* 2018). Information on the resistance profiles of bacteria in hospital environments in Ghana is limited; therefore, this study which was to establish the antimicrobial resistance profiles of bacteria from hospital environments is of highest priority.

The present study demonstrated high prevalence of resistant pathogens from air and fomites from three different hospitals in Ghana. Of the eighteen commonly used antibiotics tested, species of Gram-positive and Gram-negative bacteria showed between 80-100% levels of resistance. *Staphylococcus aureus* showed close to 100% resistance to methicillin and vancomycin. Methicillin/Vancomycin-Resistant *S. aureus* (MRSA/VRSA) is a global nosocomial pathogen in the ICUs (Miller and Diep, 2008). Studies have linked MRSA to severe infections in hospital environments (French *et al.,* 2004; Kurashige *et al.,* 2016; Dickmann, 2017). MRSA are linked with outbreaks in UK (Coll *et al.,* 2018; Ma & Bchir, 2004; S. Report, 2004), Asia (Apisarnthanarak *et al.,* 2017; Sit *et al.,* 2017), USA (Kavanagh *et al.,* 2017) and some parts of Europe (Dulon *et al.,* 2011; Johnson, 2011). Its clinical impacts in Sub-Saharan Africa have also

been reported (Falagas *et al.,* 2013; Shittu *et al.,* 2018). Studies conducted in Kenya and South Africa reported MRSA from in-patients suffering from bloodstream infections (BSI) (Aiken *et al.,* 2014; Shittu *et al.,* 2018). MRSA can survive for a longer period on frequently touched fomites (Neely and Maley, 2000; Plipat *et al.,* 2013). Fomites from ICU and NICU of Ghanaian hospitals sampled harbors MRSA.

Enterococcus faecalis was first reported in the US (Sievert *et al.,* 2013). These bacteria cause life-threatening infections in hospitalized individuals with general contact with medical equipment and invasive devices (Crank, 2015). *E. faecalis* showed almost 100% level of resistance to methicillin and vancomycin in this study. Vancomycin-Resistant *E. faecalis* (VRE) are dangerous as they cause infections more challenging to treat (Prieto *et al.,* 2016). VRE spreads faster in hospitals where more antibiotics are being used, hence the ICU (Mcdermott *et al.,* 2017). As at the time of this study, there is insufficient data on prevalence on VRE in Ghana in relation to hospital fomites or air. Most especially, VRE, as opportunistic pathogens, also survive on door handles, faucet and toilet sinks (Crank, 2015; Duckro *et al.,* 2015; Karki, 2015). Studies also indicated that *E. faecalis* showed resistance to other antibiotic aside vancomycin and methicillin, this is similar to our observation (Pendleton *et al.,* 2013; Martín et al., 2013).

Resistant Enterobacteriaceae that are well established global threats showed similar level of resistance in this study. *E. coli, Salmonella, Klebsiella and Enterobacter* showed more than 80% resistance to conventional antibiotics. Studies have also reported strains with similar high level of resistance as nosocomial agents (Eze, 2012; Bhatta *et al.,* 2018). Prevalence of bacteria with extreme resistance to antibiotics are less common in the air and on fomites, but regardless of sources they posed similar health risks (Ventola, 2015; Karo *et al.,* 2017). *Pseudomonas, Citrobacter, Serratia, Proteus* and *Acinetobacter* showed extreme resistance to more than 90% of

all the antibiotics tested. These bacteria are opportunistic resistant pathogens, most often encountered in ICU and surgical wards (Pendleton *et al.,* 2013). These strains are linked with infections that might be difficult to control (Kramer *et al.,* 2006).

Multiple antibiotic resistance indices indicated that majority of these resistant bacteria are potential high-risk pathogens. Besides, they are not only extensively resistant, but are also multiple antibiotic resistant pathogens. By this, even combination therapeutic approaches may not be effective to control them. Therefore, we may be left with no options for treatment and it calls for quick interventions to reduce their hospital-community transmission are critical. MIC and antimicrobial resistance profiles of the strains to Carbapenem, polymyxin B and colistin E were also established. The findings revealed that the Gram-negative bacteria were extensively resistant to these antibiotics. More than 80% levels of resistance were observed across the strains, thereby qualifying most of the strains as ESKAPE and Global Priority pathogens (Pendleton *et al.,* 2013; Singh, 2018). This prioritized pathogens identified in our study have also been categorized by CDC ARLN Surveillance as the possible life-threatening Gram-negative pathogens as they are highly resistant to conventional and last resort antibiotics (Davies and Davies, 2010; G. Report, 2014). Carbapenem-resistant Enterobacteriaceae (CRE) are described as threats to hospital patients with consequent high rates of mortality (Lerner *et al.,* 2013). Previous clinical study conducted on the prevalence of CRE in some Ghanaian hospitals indicated that nonfermenters such as *P. aeruginosa* and *A. baumanni* were highly resistant to carbapenems (Codjoe *et al.,* 2019). Similar study on the emergence of CRE among the clinical isolates of ESBL producers in Ghana indicates *E. coli* and *K. pneumoniae* as the dominant bacterial strains (Hackman *et al.,* 2017). The present study further confirms and complements previous studies conducted on CRE with focus on hospital environments.

Majority of the Gram-negative bacteria tested with polymyxin and colistin exhibited the phenomenon of heteroresistance. This suggested that there is more resistant subpopulation within the largest population of resistant strains. Antibiotic selection of this extremely resistant subpopulation may possibly account for the overall level of resistance observed in this study. Heteroresistance phenomenon leads to therapeutic failures (Falagas *et al.*, 2007; El-Halfawy and Valvano, 2015). While the clinical relevance of heteroresistance is still been understudied, its contribution to recurrent and chronic infections have been highlighted (Morand and Mu, 2007; Deresinski, 2009; Orton, 2011; Sola *et al.*, 2011; Campanile *et al.*, 2012). Though the prevalence of these heteroresistant phenotypic strains may not be high in Ghanaian hospital environments, however the current study indicates their occurrence. The findings, however, provide new information on the antimicrobial resistance profiles of bacterial strains in Ghanaian hospitals. This study has shown that resistant pathogens are prevalent in hospital environments, unfortunately in the air and fomites of the ICU and other sensitive sites within the Ghanaian hospitals.

CHAPTER 4

Virulence and pathogenicity of multiple antibiotic resistant bacterial strains in *Galleria mellonella* infection model

4.0 Abstract

The multiple antibiotic resistance levels of the selected set of strains indicate they are potential high-risk pathogens. *Galleria mellonella* infection model was engaged, as a surrogate virulence model to evaluate the ability of these strains to overcome mechanisms of innate immune defenses. *G. mellonella* provides an innate immune model that resembles that of mammalian hosts including human. Therefore, this study provides additional evidence on the potential high risk associated with infection by these bacteria in the hospital environment. Our results show that these strains can overcome the innate immune defenses of *G. mellonella*.

4.1 Specific Aims:

1. Determine antibiotic virulence and pathogenicity of strains *in vivo* (*Galleria mellonella* Infection model, GMI),
2. Cationic Antimicrobial peptide (AMP) resistance *in vivo* (Purified LPS in GMI)
3. Resistance markers encoding virulence,

4.2 Introduction

Many bacteria have the ability to overcome host defenses and breach through the skin and mucosal surfaces (Beceiro *et al.,* 2013). Although not all bacterial breaches are successful, once inside the host can overcome innate immune defenses and multiply (Beceiro *et al.,* 2013; Olaitan *et al.,* 2014; Fisher *et al.,* 2017). These processes are facilitated in the case of immunocompromised patients such as those in the Intensive Care Units (Fenton *et al.,* 2006; Nseir *et al.,* 2007).

Multiple Antibiotic Resistance (MAR) Indices, which is the ratio of tested antibiotics to those resisted, have been largely used to determine the pathogenic potentials and the consequent virulent traits of pathogens (Osundiya *et al.,* 2013; Mandal *et al.,* 2018; Davis and Brown, 2019). This compares the ratio at which resistance is exhibited to the number of antibiotics tested, and assigns the possible risks that a particular pathogen bears (Mandal *et al.,* 2018; Davis and Brown, 2019). However, antibiotic resistance alone cannot explain the virulent nature of a pathogen, and its ability to initiate infections or cause diseases (Beceiro *et al.,* 2013; Vadivoo and Usha, 2018). Resistant pathogen marked for virulence must possess traits that allow infection through invasion of the host tissue and translocation through the epithelial cells and also evasion of the responses mounted by the host (Balzan *et al.,* 2007; Pier, 2007). Besides, host-pathogen- interactions require a balance of the interplay of resistance and virulence (Dijkshoorn *et al.,* 2007; Lye *et al.,* 2011; Geisinger and Isberg, 2017).

Resistant bacteria are also associated with opportunistic infections, such as those acquired in the hospitals (Brown *et al.,* 2014; Price *et al.,* 2017). In addition, diverse interactions promoting pathogen survival in critical hospital locations like the ICU are linked directly with virulence (Miller and Diep, 2008; Schwab *et al.,* 2014). For example, direct and indirect contacts by sick people with air-contaminated and fomites-resident endotoxins elicit pathological responses

(Beceiro *et al.,* 2013; Pendleton *et al.,* 2013).The presence and distribution of these potentially virulent agents on fomites and in air can increase the prevalence of these strains in hospital environments and can pose serious dangers to all the hospital users (Miller and Diep, 2008). In Ghana, the ecology of pathogenic bacteria and virulence genes in the hospital environments is still not well understood. However, the prevalence of clinically relevant Gram-negative bacteria, their frequent multiple antibiotic resistance challenge, and their apparently limitless capacity for gene transfer as mediated by mobile genetic elements, makes virulence determination in hospital environment a necessity (Bereket *et al.,* 2012; Park *et al.,* 2013; Agyepong *et al.,* 2018).

Bacterial strains from hospital environments exhibiting strong biofilm producing phenotypes have been essential to antimicrobial resistance (Patel, 2005; Phillips and Schultz, 2012; Zheng *et al.,* 2017). Other studies have also reported that swarm motility enhances the pathogenic strains to bypass the immune systems (Kearns, 2011). While the interplay of these relevant phenotypic characteristics (biofilm, motility and cytotoxicity) increase virulence, also other properties involving cell wall alterations (Gooderham *et al.,* 2008; Juan *et al.,* 2008) and modifications of the LPS structure are important in the adaptation of bacteria in the host (Olaitan *et al.,* 2014; Maldonado *et al.,* 2016).

LPS is a major virulent factor of the Gram-negative bacteria (Pendleton *et al.,* 2013). While it maintains the integrity of the membrane (Olaitan *et al.,* 2014), LPS accounts for the induction of septic shock (Wilson *et al.,* 2002). The lipid A component of the LPS has been established as a toxic molecule that is recognized by the innate immune system, especially by surface receptors on the epithelial and phagocytic cells. This encounter generates different pathological responses including cytokines release and profuse inflammation (Futoma-kołoch, 2016; Rosadini and Kagan, 2017). The host produces antimicrobial peptides that bind the LPS. Pathogens from the ESKAPE

group, such as *Acinetobacter, Klebsiella, Pseudomonas,* and members of the carbapenem-resistant Enterobacteriaceae for example *Salmonella* and *Proteus*, displayed high level of resistance to antimicrobial peptides, thus cause a lot of damage to the host tissues (Ganz, 2003; Pasupuleti *et al.,* 2012; Bahar and Ren, 2013; Tsai *et al.,* 2016; Rudkin *et al.,* 2017). This has been attributed to the presence of LPS molecules with modification in their structure, as they enhance pathogenicity and virulence of the Gram-negative pathogens (Futoma-kołoch, 2016; Maldonado *et al.,* 2016; Price *et al.,* 2017; Rosadini and Kagan, 2017). In addition, other emerging virulent factors like the *KPC* associated with carbapenemase resistant *Klebsiella* species are raising global alarms (Leung *et al.,* 2017). *Klebsiella* carbapenemase producing bacteria have been reported to potentially initiate the LPS modification using the *KPC* as the virulent factor (Chopra *et al.,* 2012; Leung *et al.,* 2017).

The wax moth, *Galleria mellonella,* has been used for host-pathogen interactions of bacteria such as *Pseudomonas aeruginosa, Burkholderia cepacia, Proteus mirabilis, Bacillus cereus, Francisella tularensis,* and some pathogenic fungi (Jander and Rahme, 2000; Junqueira, 2012; Nale *et al.,* 2016). The utility of non-mammalian model system such as the Galleria has also been demonstrated for reliable and accurate studying of pathogenesis of bacterial infection (Peleg *et al.,* 2009). GMI model has been recommended to be effective in facilitating *in vivo* study of virulence in bacteria, and more specifically as they produce similar Antimicrobial Peptides (AMPs) and has a complex innate immune systems as found in humans or other experimental mammalian models (Jander and Rahme, 2000; Ramarao *et al.,* 2012). Furthermore, the Galleria usage is cheap and no ethical approvals are required, reducing the use of mammalian model such as rat and mice (Wodja, *2016*). *Galleria mellonella* infection model is well suited for the study of human pathogens, as it can be maintained at temperatures of 37^0C (Beceiro *et al.,* 2013).

To ascertain that bacterial strains isolated from fomites and air from Ghanaian hospitals is pathogenic and virulent, GMI model system was utilized in this study (**Figure 1a: Appendix IIIA**). This helps in answering interesting questions as to the prevalence of these pathogens in healthcare settings in Ghana. Data on bacterial virulent clinical impacts will go a long way in providing necessary information on the overall public health of the hospital users, and consequently the general community; since there is always hospital-community interactions and vice versa.

4.3 Methods

4.3.1 Study design and strain collection

Strains used in this study were collected from AbiMosi Bacterial Culture (ABC©) Library, Microbiology and Molecular Biology Lab, Department of Biochemistry, Cell and Molecular Biology, University of Ghana. These strains were isolated from fomites and air from selected hospitals in Ghana. The majority of strains selected is very motile, strong biofilm producers, extensively resistant to polymyxin B and are multiple antibiotic resistant phenotypes (**Table 4.1**).

4.3.2 Bacterial culture and growth conditions

Bacterial strains were maintained at -80ºC as 50% (v/v) glycerol stocks. Unless otherwise indicated, all the strains were cultured at 37ºC in Luria-Bertani broth (LB; 15 g/L yeast extract, 7.5 g/L NaCl, 10 g/L tryptone) with vigorous shaking at 150-180 rpm (version of shaker). For strain recovery growth on media, LB agar or Nutrient agar prepared according to manufacturer's instructions was used; strains were streak-cultured and incubated at 37ºC (**Figure 1b: Appendix IIIB**). Pure colonies were picked and used for all the experiments.

4.3.4 Galleria mellonella larva in vivo virulence assay

Larval infection assays were performed as previously described (Ramarao *et al.,* 2012) with some modifications. *Galleria mellonella* larvae were acquired from UK Waxworms Ltd., stored in wood shavings in the dark at 16ºC prior to infection, and used within 2 weeks of receipt. Larvae of approximate weights of 250 to 350 mg were used. Bacteria were grown in 5 ml LB until mid-exponential growth phase. The bacterial cells were harvested at 8000 rpm for 4-5 minutes and re-suspended in sterile Phosphate Buffered Saline (10 mM PBS, pH 7.5). The cultures were adjusted to 0.5 (OD_{600}), re-suspended in sterile PBS, and serially diluted. For each bacterial strain, ten randomly chosen, healthy *Galleria mellonella* larvae were injected with 10µl of the 10^3

CFU/larvae (10 cell/larvae) into the haemocoel of the rear left proleg with a 100µl Hamilton syringe and a 20-G needle. The larvae were kept in a petri dish at 37^{o}C in the dark alongside a group of 10 controls larvae, injected with 10µl of PBS. The viability and mortality were monitored over a period of 72 hours using lack of movement upon stimuli, loss of legs and visual color change. Three independent experiments were performed on different days. Colony forming units were enumerated by plating appropriate 10-fold dilution on LB agar plates.

4.3.5 Lipopolysaccharide preparation and extraction

LPS was extracted as previously described with some modifications (Marolda *et al.*, 1990; Loutet *et al.*, 2006; Rezania *et al.*, 2011; Naguib, 2018). Bacterial strains were grown overnight on LB agar plates at 37^{o}C and the colonies were harvested from the plate and suspended in sterile 3 ml of PBS solution. The culture was adjusted to optical density 3.0 (OD_{600}) and 1.5 ml of the bacterial suspension was transferred to a micro-centrifuge Epperndorf tube and centrifuged at 10, 000 g for 1 min to pellet the cells. The pellet was re-suspended in 150µl of lysis buffer (0.5 M Tris HCl, pH 6.8), 2% sodium dodecyl sulfate (SDS) and 4% β-mercaptoethanol). The mixture was boiled for 10 min and cooled on ice for another 10 min. Ten microliters of 20 mg/ml solution of proteinase K dissolved in 10 mM Tris-HCl buffer (pH 8.0), then, 1 mM $CaCl_2$ and 30% glycerol was added, vortexed and incubated overnight at 60^{0}C in thermo-heat block. After the overnight incubation, 150µl of 90% phenol solution (90% phenol, 0.1 % β-mercaptoethanol and 0.2% 8-hydroxyquinloline) and incubated at 70^{o}C for 15 minutes, with intermittent vortexing every 5 minutes. The separation of the aqueous and phenolic phases was done by ice-cooling for 10 minutes, and then the top aqueous phase was transferred into a new tube. Five hundred microliters (500µl) of ethyl ether saturated with 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA was added and vortexed at 14, 000 rpm for 1 min. The top ether phase was then aspirated and further purified for

the GMI experiment. Kaplan Meier plots were generated with Graphad 6.0 and Mantel cox log-rank tests were used to determine the bacterial virulence level of the bacterial strains in GMI.

Table 4.1: Phenotypic characteristics of strains selected for GMI experiment

	Strains	MIC of [a]PmB (ug/ml)	Motility	Biofilms	Multiple Antibiotic Resistant	MAR Index	Class of Antibiotics resistance to
ENAB1	*E. cloacae complex*	1024	[b]+++	Strong	[c]+	1.0	[d]B-Lac, [e]Mac, [f]Sun, [g]CH, [h]TE, [i]Amin, [j]Nitro, [k]Qui
ENAB2	*E. cloacae*	>1024	+++	Strong	+	1.0	B-Lac, Mac, Sun, CH, TE, Amin, Nitro, Qui
PGAB1	*P. aeruginosa*	512	+++	Strong	+	1.0	B-Lac, Mac, Sun, CH, TE, Amin, Nitro, Qui
PGAB2	*Pseudomonas* spp.	>1024	+++	Strong	+	1.0	B-Lac, Mac, Sun, CH, TE, Amin, Nitro, Qui
CTAB1	*C. freundii*	1024	+++	Strong	+	1.0	B-Lac, Mac, Sun, CH, TE, Amin, Nitro, Qui
CTAB2	*C. freundii*	1024	+++	Strong	+	1.0	B-Lac, Mac, Sun, CH, TE, Amin, Nitro, Qui
KBAB1	*K. pneumoniae*	1024	+++	Strong	+	1.0	B-Lac, Mac, Sun, CH, TE, Amin, Nitro, Qui
KBAB2	*K. oxytoca*	1024	+++	Strong	+	1.0	B-Lac, Mac, Sun, CH, TE, Amin, Nitro, Qui
SRAB1	*S. marcescens*	1024	+++	Strong	+	1.0	B-Lac, Mac, Sun, CH, TE, Amin, Nitro, Qui
SRAB2	*S. marcescens*	>1024	+++	Strong	+	1.0	B-Lac, Mac, Sun, CH, TE, Amin, Nitro, Qui
ACNAB 1	*A. baumannii*	512	+++	Strong	+	1.0	B-Lac, Mac, Sun, CH, TE, Amin, Nitro, Qui
ACNAB 2	*A. baumannii*	1024	+++	Strong	+	1.0	B-Lac, Mac, Sun, CH, TE, Amin, Nitro, Qui
ECAB01	*E. coli*	>1024	+++	Strong	+	1.0	B-Lac, Mac, Sun, CH, TE, Amin, Nitro, Qui
ECAB02	*E. coli*	>1024	+++	Strong	+	1.0	B-Lac, Mac, Sun, CH, TE, Amin, Nitro, Qui
PRMAB 1	*P. mirabilis*	1024	+++	Strong	+	1.0	B-Lac, Mac, Sun, CH, TE, Amin, Nitro, Qui
PRMAB 2	*P. mirabilis*	1024	+++	Strong	+	1.0	B-Lac, Mac, Sun, CH, TE, Amin, Nitro, Qui

[a]PmB – Polymyxin B; [b]+++ = very motile; [c]+ = Positive for Multiple antibiotic resistance (at least to 2 classes of antibiotics); [d] β-Lac – β-Lactam, [e]Mac – Macrolides, [f]Sun – Sulfonamides, [g]CH – Chloramphenicol, [h]Amin – Aminoglycosides, [i]Nitro – Nitrofurantoin, [j]Qui – Quinolones; Multiple Antibiotic Resistant (MAR) Index – Ratio of antibiotic resisted to antibiotic tested

4.4 Results

4.4.1 Extremely virulent strains in GMI model

The data presented here showed the representation of three different independent experiments done in replicates. All the selected strains were MAR resistant phenotypes, biofilm-producers and very motile as described by their characterization profiles (ABC©) (**Table 4.1**). All these strains were used to challenge *Galleria* at a minimal concentration of 10 cells per larvae. The majority of the strains showed virulence in GMI model with 100% killing in less than 24 hours and at most 24 hours (**Table 4.2** and, **Figure 2: Appendix IIIC**). This is relative to the negative (PBS) and positive (ATCC3047 *E. coli* and ATCC29527 *E. cloacae*) controls that were able to survive for the period of the experiments (24-72 hours). This indicated that the strains tested are highly virulent and can pose serious health challenges in the hospital environments.

4.4.2 Different degrees of virulence with LPS in GMI model

Lipolysaccharide (LPS), especially the lipid A component is considered the major virulent factor of all the strains tested in this study. To confirm further if it is only the LPS that is actually responsible for the level of virulence observed; the strains were challenged with serially diluted LPS, at a concentration of 0.001/Larvae as previously described, killing between 24-72 hours at different level and degree was observed. One hundred percent killing was observed in species of *Enterobacter and Klebsiella in* < 24 hours (**Figure 4.1**), which might indicate that LPS is the key virulent factor. Species of *Pseudomonas* showed different degree of virulence 100% in *PGAB1* at 24 hours, while *PGAB2* 80% killing in 24-48 hours and complete killing in hours 72 hours.

Table 4.2: Percentage survival of *Galleria mellonella* Larvae

Strains		Percentage Survival (%) in hours					
		Strains			PBS control		
		24	48	72	24	48	72
ENAB1	*Enterobacter cloacae complex*	0	0	0	100	100	100
ENAB2	*Enterobacter cloacae*	0	0	0	100	100	100
PGAB1	*Pseudomonas aeruginosa*	20	0	0	100	100	100
PGAB2	*Pseudomonas spp.*	0	0	0	100	100	100
CTAB1	*Citrobacter freundii*	0	0	0	100	100	100
CTAB2	*Citrobacter freundii*	20	10	0	100	100	100
KBAB1	*Klebsiella pneumoniae*	0	0	0	100	100	100
KBAB2	*Klebsiella oxytoca*	0	0	0	100	100	100
SRAB1	*Serratia marscenses*	0	0	0	100	100	100
SRAB2	*Serratia marscenses*	0	0	0	100	100	100
ACNAB1	*Acinetobacter baumannii*	0	0	0	100	100	100
ACNAB2	*Acinetobacter baumannii*	20	0	0	100	100	100
ECAB01	*Escherichia coli*	0	0	0	100	100	100
ECAB02	*Escherichia coli*	0	0	0	100	100	100
PRMAB1	*Proteus mirabilis*	0	0	0	100	100	100
PRMAB2	*Proteus mirabilis*	0	0	0	100	100	100
ATCC3047	***Escherichia coli***	**100**	**100**	**80**	**100**	**100**	**100**
ATCC29527	***Enterobacter cloacae***	**100**	**100**	**70**	**100**	**100**	**100**

0 = 100% killing of Galleria, 10 = one out of ten tested Galleria survived, 20 = two out of the ten tested Galleria survived, 100 = No killing

Acinetobacter species (*ACNAB1)* with 100% killing at 24 hours, *ACNAB2 with* 60% in 24-48 hours and complete killing at 72 hours (**Figure 4.2**). In species of *Citrobacter, Serratia, Proteus* and *E. coli*, different levels of virulence were observed ranging from 24-72 hours with killings at different percentages. This indicated that although, LPS might be the virulent factors, however there are other factors in association with or complementary to LPS that might be responsible for the level of virulence observed.

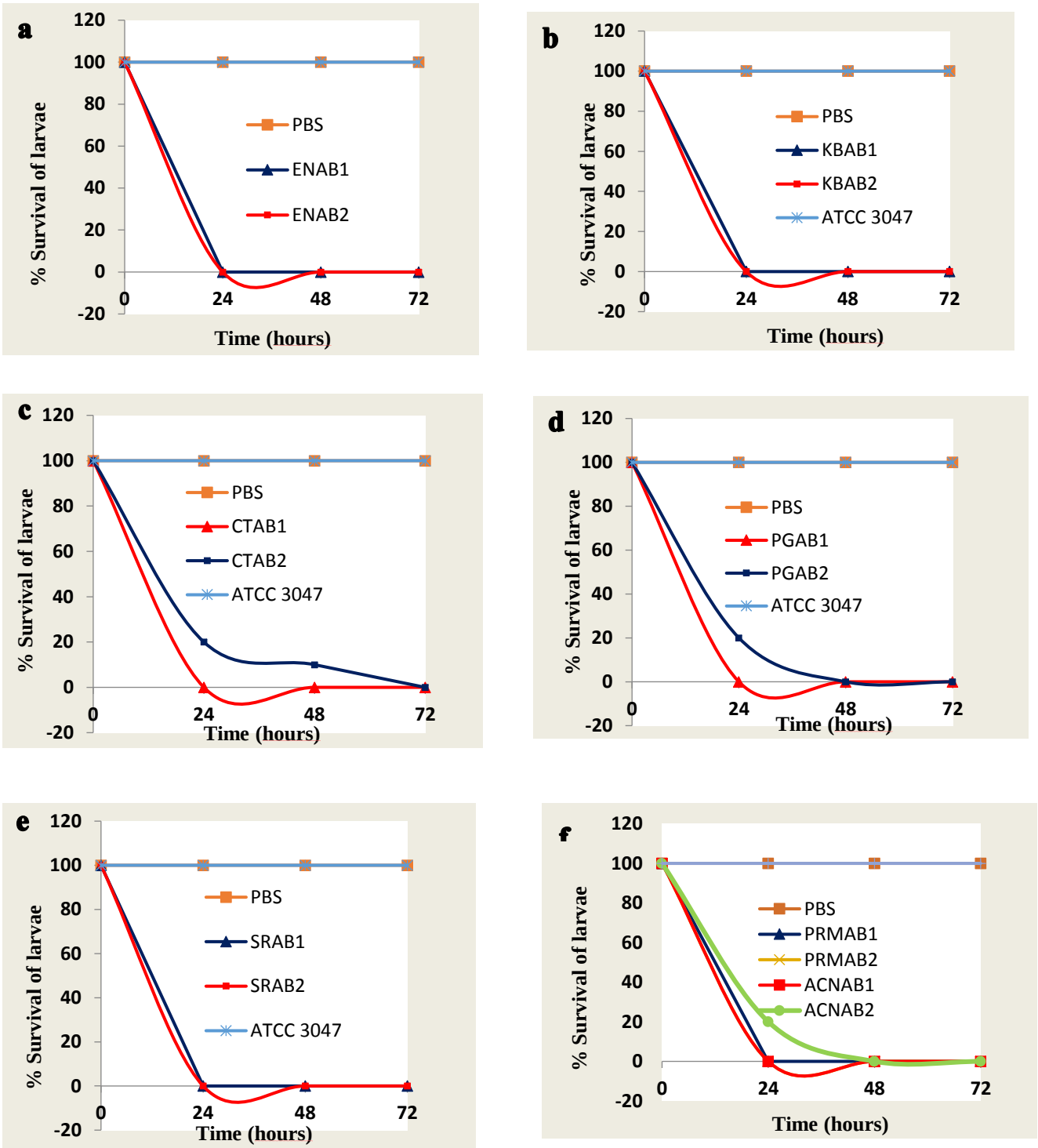


Figure 4.1: Extreme virulence (10^3 cells/larvae) of strains in GMI model system relative to PBS and ATCC positive controls

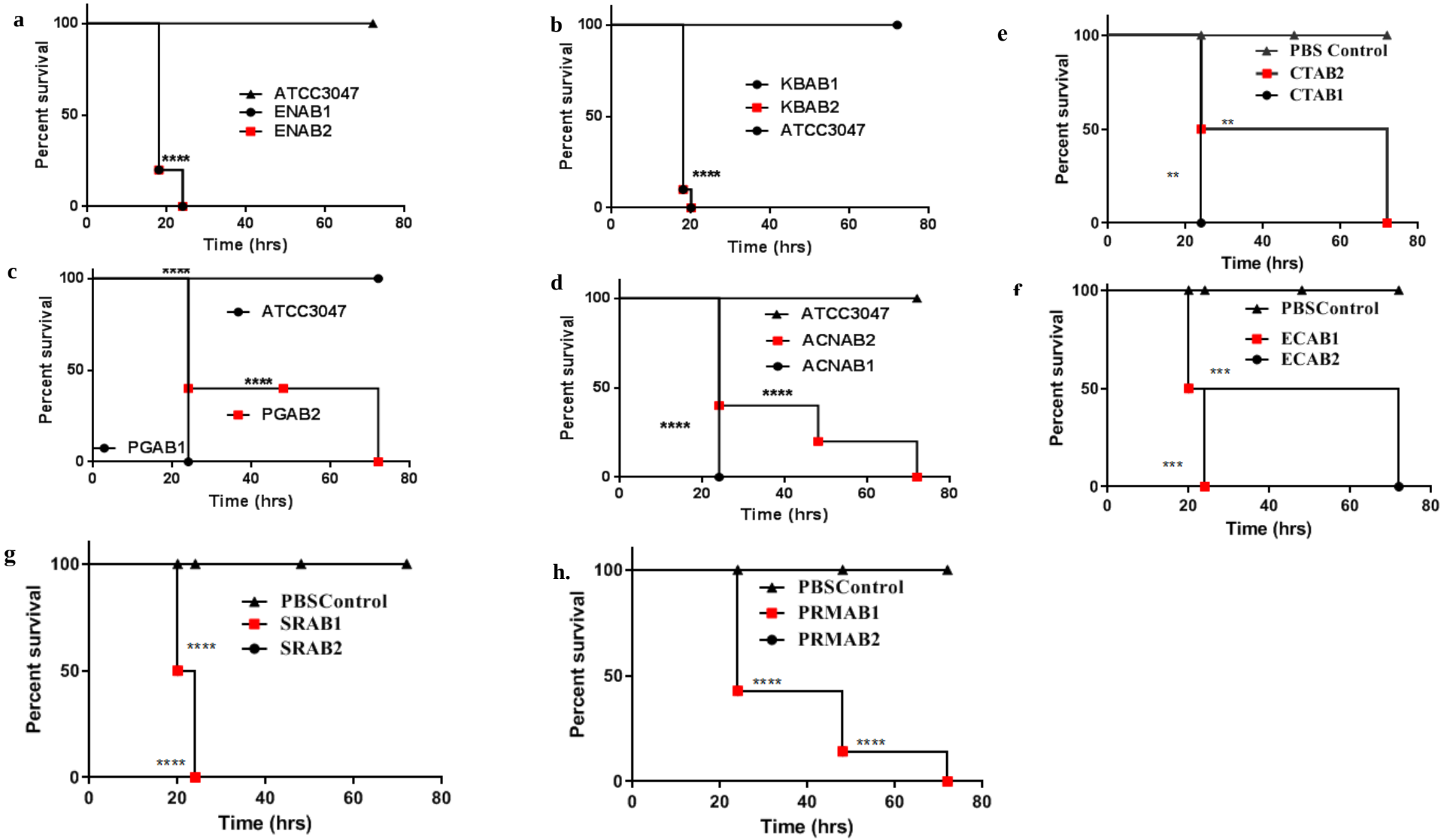


Figure 4.2: Extreme virulence (1:1000 dilutions) of LPS in selected *strains* in GMI model system relative to PBS and ATCC positive controls (Mantel cox log-rank tests: ***p* < 0.0001), ATCC3047 positive/PBS negative controls**

4.5 Discussion

In this study, non-mammalian *Galleria mellonella* infection model system was utilized to investigate the virulence profiles of selected Gram-negative bacteria isolated from hospital air and fomites from ICU, surgical wards and NICU. Determining the virulent nature of these strains is important to understand the role and explore the mechanism by these pathogens to cause HAIs.

All the tested strains in this study are highly virulent in *Galleria mellonella* infection, as just 10 bacterial cells per larvae were sufficient to kill the host in less than 24 hours post-infection. Also, survival of these strains in GMI at 37ºC to an extent of causing death implies their tendency to survive in human macrophages or human cells. Studies have indicated extreme level of resistance and virulence in *Pseudomonas aeruginosa, Burlkholderia cepacia* and *Achromobacter xyloxidans* in *Galleria mellonella* infection, although from cystic fibrosis and bloodstream infections patients (Leitão *et al.,* 2010; Bradbury *et al.,* 2019; Pereira and Leão, 2019).

Bacterial strains from the ESKAPE pathogens group (*Klebsiella, Acinetobacter, Pseudomonas* and *Entreobacter*) tested in this study were extremely virulent. By speculation, the level of virulence observed reflects the increased resistance of these strains to antimicrobial peptides. Antimicrobial peptides is one of the main key components of the immune-systems in Galleria and also in other hosts including human (Pasupuleti *et al.*, 2012; Bahar and Ren, 2013). This agrees with the high level of resistance of these isolates against the polymyxin, as previously identified in Chapter 4. Other studies have established that resistance to antimicrobial peptides is a component of bacterial virulence (Groisman *et al.,* 1992; Taylor and Ramachandran, 2014; Louhi *et al.*, 2015; Wang *et al.,* 2015; Joo *et al.,* 2016), pathogens benefit from their inherent/natural resistance to peptide antibiotics like polymyxin and colistin (Bahar and Ren, 2013; Olaitan *et al.*, 2014).

LPS is a key virulent factor of the Gram-negative bacteria (Cross, 2008; Taylor and Ramachandran, 2014) and has been associated with different cases of infections caused by pathogenic strains (Cross, 2008).The levels of virulence observed when *Galleria* was challenged with purified LPS was similar to some strains, while slightly different from others relative to when the whole organism was used. This indicated that other factors in association with LPS are responsible for the level of virulence displayed by the pathogens tested in this study. Other factors that might be responsible for this would be possible modifications in the LPS, as this has been associated with the ability of a strain to exhibit virulence (Matsuura, 2013; Maldonado *et al.*, 2016). LPS modifications are specific, and this specificity determines the level of virulence (Matsuura, 2013; Maldonado *et al.*, 2016). In conclusion, virulent-like factors, which also have compensatory effects as resistance mediators are possible contributors to virulence.

CHAPTER 5

Investigating the mechanisms of virulence and antimicrobial resistance of highly resistant bacterial strains from hospital environments

5.0 Abstract

Resistance mechanisms of multidrug selected bacterial strains were determined. Common resistant markers associated with bacterial resistance to conventional antibiotics were detected and their relevant proposed roles in resistance discussed. Polymyxin sensitive assay provided a backdrop on which resistance mechanisms can be based. Detection and profiling of LPS components (lipid A and O-antigen) in the selected bacteria further confirms their possible roles in resistance. Identification of LPS-modifying genes in the selected strains highlights their possible roles in Ghanaian resistant Gram-negative bacteria.

5.1 Specific Aims:

1. Developing sensitive assays to understand resistance mechanisms,
2. Identification of resistance markers encoding resistance,
3. Profiling the Lipopolysaccharides (LPS),
4. Characterization of SNPs and determination of TCS roles in resistance mechanism,
5. Investigate and establish AMR mechanisms of bacteria from some Ghanaian hospitals.

5.2 Introduction

Emerging pathogenic bacteria account for major causes of death in the developing world, due to the increase in the incidence of antimicrobial resistance (Kapoor *et al.*, 2017). Prolonged admissions of patients demands lengthy therapy with antibiotics, leading to plausible antimicrobial resistance (Ruppé *et al.*, 2015). Resistant bacteria are responsible for 45-70% of HAI-pneumonia cases, 20-30% bloodstream and catheter infections (Dalhoff and Ewig, 2013). They are also common cause of surgical site and urinary tract infections (Khan *et al.*, 2017). Studies have also reported a rise of antimicrobial resistance in the NICU (Patel *et al.*, 2015), and established almost exclusively this with Gram-negative bacteria. For example, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, carbapenem-resistant Enterobacteriaceae are responsible for the major parts of the resistance problem (Pendleton *et al.*, 2013).

Gram-negative bacteria have various mechanism (**Figure 5.1**) to circumvent antimicrobial agents (Blair *et al.*, 2014). They can express enzymes that can inactivate antimicrobial molecules through chemical modification or hydrolysis (Blair *et al.*, 2014). In other instances, they engaged other non-enzymatic mechanisms such as target site mutations or antibiotic efflux pumps (Joo *et al.*, 2016). Also, they exhibit circumstantial resistance (Admassie, 2018), a situation that makes them more dangerous as they take advantage of compromised situations in the host, especially in a case where the immune system is compromised. Furthermore, bacteria build-up a biofilm capsular matrix as a mechanism to resist antimicrobial agents (Patel, 2005). Antibiotic selection pressure is also complementary, as more resistant bacteria population are selected as drivers to determine the overall resistance of the bacteria (Jayol *et al.*, 2015). All these forms of resistance mechanisms have been associated with opportunistic bacteria from the hospital (Price *et al.*, 2017).

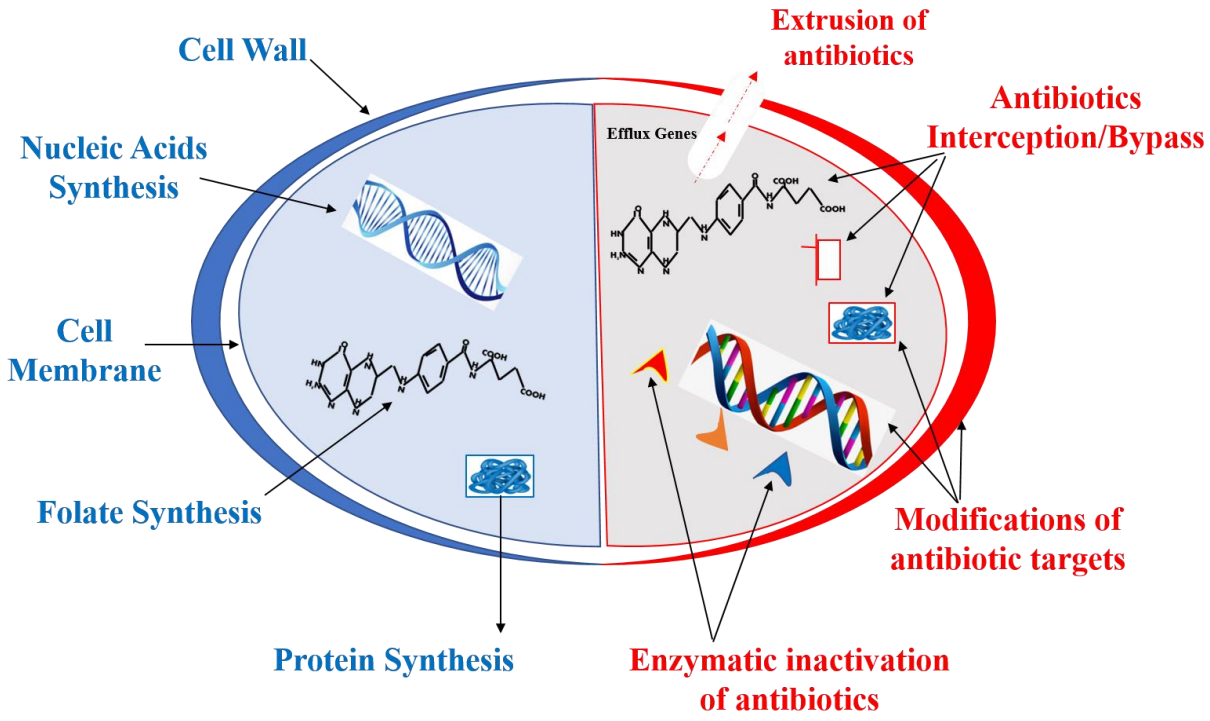


Figure 5.1: General targets of common antibiotics (Left side with blue labels) and resistance mechanisms (Right side with red labels); (Abiola Isawumi and Lydia Mosi, 2019)

There are also growing evidences that bacteria are capable of surviving diverse exposure to antibiotics by releasing molecules into the extracellular milieu (Sabnis *et al.*, 2018). This enables them to sequester or capture antibiotics before they can reach the bacterial cells (Sabnis *et al.*, 2018). Antibiotic interception is another strategy employed by bacteria to resist antibiotics (Benoun *et al.*, 2016). Here, the interceptor acts as a decoy that mimics the antibiotic target molecules (Sabnis *et al.*, 2018), this therefore leads to therapeutic failure. Many of these interceptors also help the bacteria to persist in the presence of antibiotic or function as the structural components enabling them to tolerate antibiotics (Patel, 2005). Bacterial persistence, presents the bacteria with the opportunity to survive lethal antibiotic doses (Maclean *et al.*, 2004).

Phenotypically, bacterial persistence involves a switch from normal/sensitive to a more antibiotic tolerant state. This is a simple bacterial resistance strategy, surprisingly this mechanism also makes

the antibiotic irrelevant (Fisher *et al.,* 2017). Bacteria that survive harsh environmental conditions as those residents on fomites, exhibit this strategy (Cc & Ia, 2018; Miller and Diep, 2008). Bacteria exhibiting this resistance mechanisms survive and actively grow in the presence of antibiotics (Ruppé *et al.,* 2015; Harms *et al.,* 2016;). Most Gram-negative pathogens are favored by this mechanism (Admassie, 2018).

Efflux pumps contribute to the intrinsic resistance against multiple antibiotic classes (Amaral *et al.,* 2014 Admassie, 2018). Members of the ESKAPE group have the ability to resist antibiotics effective against Gram-positive bacteria using the efflux-pumps (Blair *et al.,* 2014; (Sun *et al.,* 2014; Blanco *et al.,* 2016). Another clinical challenge is the overexpression of efflux-pumps, as it confers resistance to previously relevant antibiotics (Blair *et al.,* 2014). *Pseudomonas aeruginosa, Salmonella enterica,* and *E. coli* harbor efflux genes. Gram-positive bacteria exports tetracycline as they express single polypeptide Tet-pump localized in the cytoplasmic membrane (Admassie, 2018). Efflux systems confer cross-resistance to other multiple antibiotics and disinfectants. For example, macrolide efflux genes (*mefA* and *mefE*), multidrug efflux transporter (*mexB*), and inner-membrane antibiotic associated efflux gene(*AcrB*), for macrolide, quinolone, sulfonamides and some β-lactams (Blanco *et al.,* 2016). Mutations in MDR efflux pumps have been described to facilitate resistance to last resort antibiotics (Piddock, 2006; Fernando and Kumar, 2013; Vaez *et al.,* 2019).

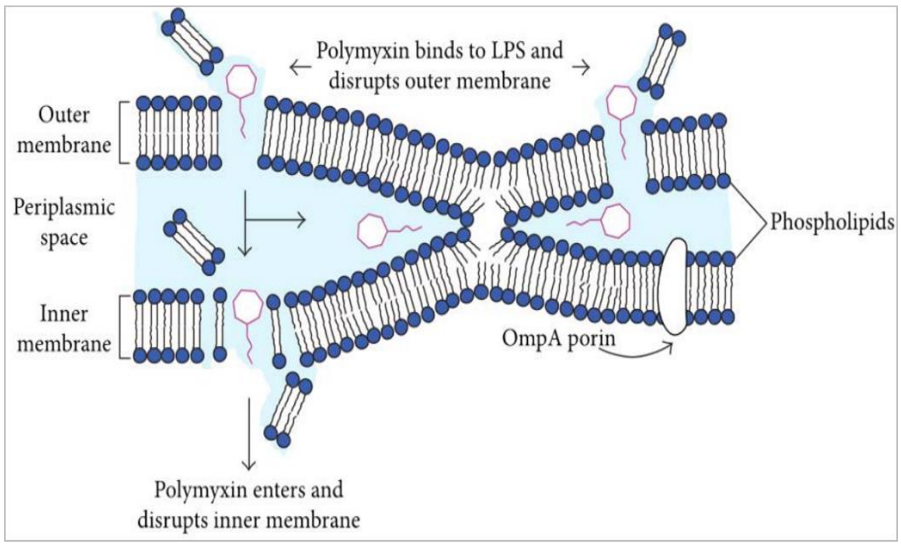


Figure 5.2: Mechanisms of action of polymyxin against the bacterial cell membrane (Deris *et al.*, 2014; Yu *et al.*, 2015)

Polymyxin and colistin, are peptide antibiotics with similar functional activities as cationic antimicrobial peptides (CAMPs) resident in the host (Joo *et al.*, 2016). CAMPs interact with LPS on the surface of the Gram-negative pathogens by outcompeting divalent metal cation and binding to LPS negative charges (**Figure 5.2**) (Blair *et al.*, 2014; Koch *et al.*, 2015). This allows the entry of the CAMPs across the outer membrane, the peptides and finally insert in the inner membrane causing damage to bacterial cell (Srinivas and Rivard, 2017). Polymyxin binds selectively to LPS, particularly the lipid A phosphate groups (Yu *et al.*, 2015). Resistance to these antibiotics is very rare, but can occur through modification in the lipid A that reduce CAMPs binding (Maldonado *et al.*, 2016; Srinivas and Rivard, 2017). Such alterations have been reported for *Pseudomonas, Klebsiella, Salmonella* and newly emerging complex of *Enterobacter cloacae* from hospitals (Pendleton *et al.*, 2013). Alterations in the LPS results from covalent modifications of the lipid A moiety with addition of phosphoethanolamine (PEtN) and 4-amino-4-deoxy-L-arabinose sugar (L-Ara4N) to lipid A phosphates (**Figure 5.3**) (Olaitan *et al.*, 2014).

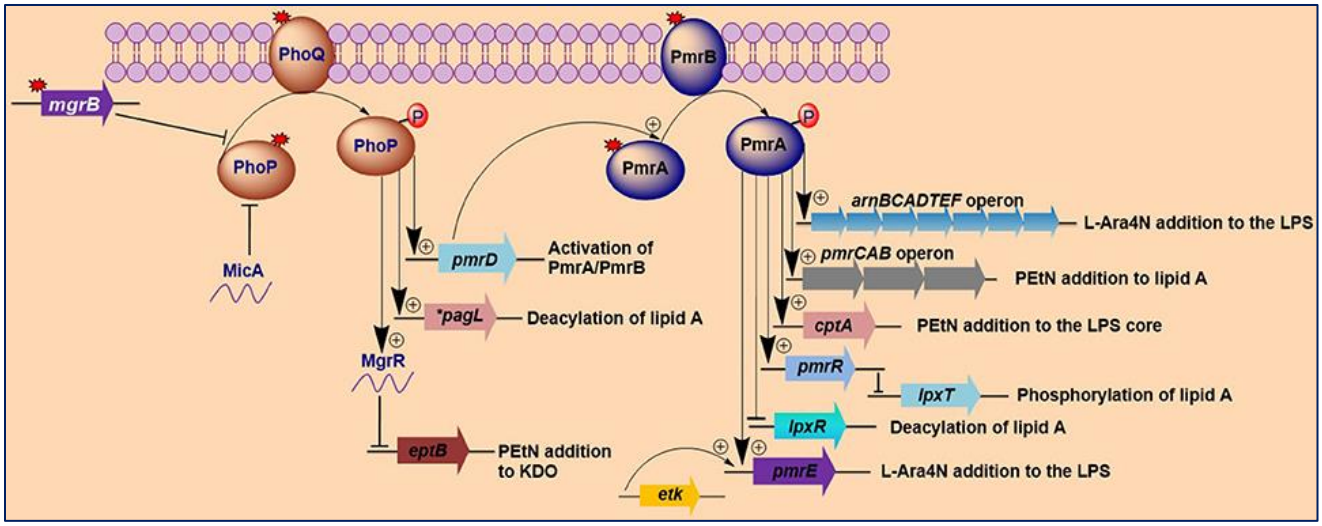


Figure 5.3: General targets of last-resort antibiotics and resistance mechanisms (Olaitan *et al.*, 2014)

Lipid A modifications are regulated in most Gram-negative pathogens via sensory genes (Joo *et al.*, 2016). For example, *PhoP/PhoQ* and *PrmA/PrmB* result in LPS modifications as these proteins regulate expression of lipid A modification enzymes (Olaitan *et al.*, 2014). Bacteria use *PhoP/PhoQ* as histidine kinase sensor and DNA-binding response regulator (Joo *et al.*, 2016). *PrmA/PrmB* together work to regulate the role of lipid A modifications in antimicrobial resistance (Gunn, 2001; Moual and Gruenheid, 2012). Polymyxin resistance is further compounded by the emergence of plasmid-mediated mcr-1 gene, which has spread from China to other parts of the world including Africa (Joo *et al.*, 2016; Liu *et al.*, 2016). This gene encodes a novel phosphoethanolamine transferase (Liu *et al.*, 2016), and studies have indicated rapid dissemination of *mcr-1* between carbapenemase-producing Enterobacteriaceae (e.g., *E. coli* and *Klebsiella pneumoniae*) in hospitals (Olaitan *et al.*, 2014; Liu *et al.*, 2016). The acquisition of *mcr-1* may contribute to AMR and high-risk of untreatable infections caused by mcr-1-produer bacterial populations thriving in hospital environments.

5.3 Methods

5.3.1 Study design and strain collection

Strains used in this study were collected from AbiMosi Bacterial Culture (ABC©) Library, Microbiology and Molecular Biology Lab, Department of Biochemistry, Cell and Molecular Biology, University of Ghana. These strains were isolated from fomites and air from selected hospitals in Ghana. Majority of the strains selected were very motile, strong biofilm producers, extensively resistant to polymyxin B and are multiple antibiotic resistant phenotypes (**Table 5.1**). The experimental approach is described in **Figure 5.4**.

Table 5.1: Antimicrobial resistance profiles of selected strains

Strains		MIC of [a]PmB (ug/ml)	Multiple Antibiotic Resistant	Class of conventional antibiotics
ENAB1	*E. cloacae complex*	1024	[b]+	[c]B-Lac, [d]Mac, [e]Sun, [f]CH, [g]TE, [h]Amin, [i]Nitro, [j]Qui
ENAB2	*E. cloacae*	>1024	+	B-Lac, Mac, Sun, CH, TE, Amin, Nitro, Qui
PGAB1	*P. aeruginosa*	512	+	B-Lac, Mac, Sun, CH, TE, Amin, Nitro, Qui
PGAB2	*Pseudomonas spp.*	>1024	+	B-Lac, Mac, Sun, CH, TE, Amin, Nitro, Qui
CTAB1	*C. freundii*	1024	+	B-Lac, Mac, Sun, CH, TE, Amin, Nitro, Qui
CTAB2	*C. freundii*	1024	+	B-Lac, Mac, Sun, CH, TE, Amin, Nitro, Qui
KBAB1	*K. pneumoniae*	1024	+	B-Lac, Mac, Sun, CH, TE, Amin, Nitro, Qui
KBAB2	*K. oxytoca*	1024	+	B-Lac, Mac, Sun, CH, TE, Amin, Nitro, Qui
SRAB1	*S. marscenses*	1024	+	B-Lac, Mac, Sun, CH, TE, Amin, Nitro, Qui
SRAB2	*S. marscenses*	>1024	+	B-Lac, Mac, Sun, CH, TE, Amin, Nitro, Qui
ACNAB1	*A. baumannii*	512	+	B-Lac, Mac, Sun, CH, TE, Amin, Nitro, Qui
ACNAB2	*A. baumannii*	1024	+	B-Lac, Mac, Sun, CH, TE, Amin, Nitro, Qui
ECAB01	*E. coli*	>1024	+	B-Lac, Mac, Sun, CH, TE, Amin, Nitro, Qui
ECAB02	*E. coli*	>1024	+	B-Lac, Mac, Sun, CH, TE, Amin, Nitro, Qui
PRMAB1	*P. mirabilis*	1024	+	B-Lac, Mac, Sun, CH, TE, Amin, Nitro, Qui
PRMAB2	*P. mirabilis*	1024	+	B-Lac, Mac, Sun, CH, TE, Amin, Nitro, Qui

[a]PmB – Polymyxin B; [b]+ = Positive for Multiple antibiotic resistance (at least to 2 classes of antibiotics); [d] β-Lac – β-Lactam, [e]Mac – Macrolides, [f]Sun – Sulfonamides, [g]CH – Chloramphenicol, [h]Amin – Aminoglycosides, [i]Nitro – Nitrofurantoin, [j]Qui – Quinolones;

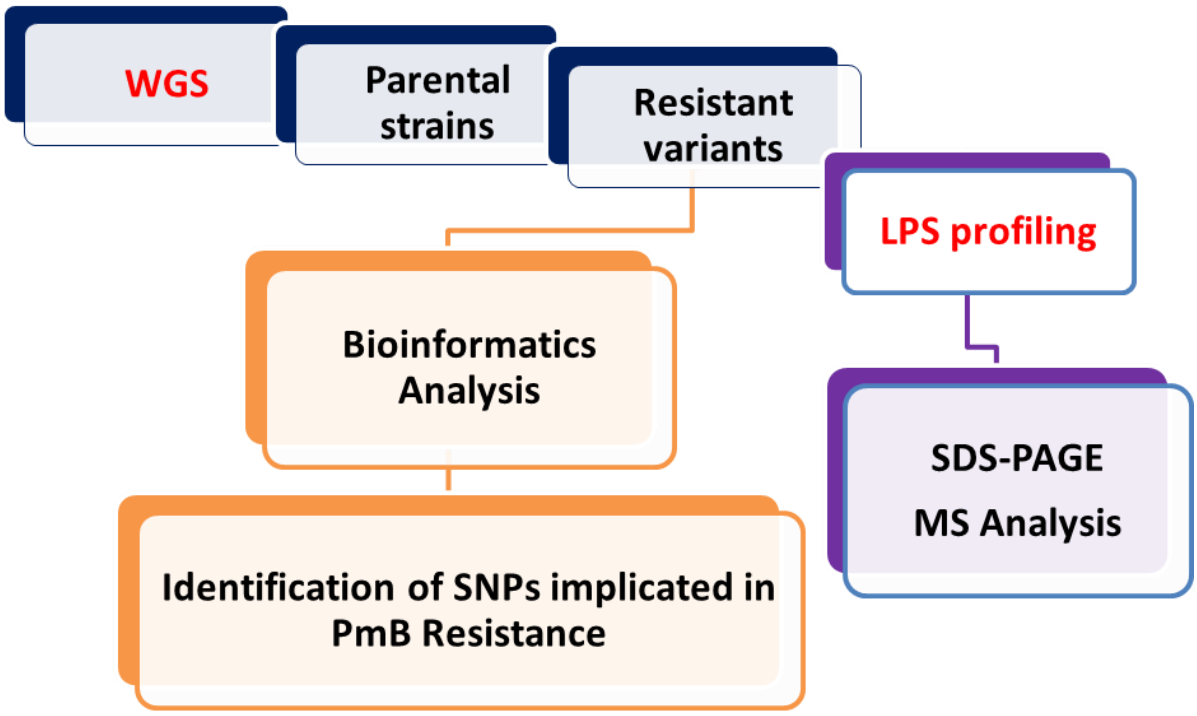


Figure 5.4: Flowchart for polymyxin resistance mechanisms; LPS-Lipopolysaccharides, SNPs- Single Nucleotides Polymorphism, PmB -Polymyxin B, SDS-PAGE – Sodium Dodecyl Sulfate – Pulse Field Acrylamide Gel Electrophoresis, MS – Mass Spectrometry

5.3.2 Bacterial culture and growth conditions

Bacterial strains used in this study were maintained at -80ºC as 50% (v/v) glycerol stocks. Unless otherwise indicated, all the strains were cultured at 37ºC in LB broth with vigorous shaking at 180 rpm. For strain recovery growth on media, LB agar was prepared according to the manufacturer's instructions; strains were streak-cultured and incubated at 37ºC. Pure colonies were picked and used for all the experiments.

5.3.3 Calcium adjusted polymyxin B sensitive bioassay

Calcium adjusted broth dilution polymyxin assay was used to study the response of the selected strains at different concentrations of polymyxin. The assay was developed based on the concept

that the binding of polymyxin to lipid A displace divalent ions (Ca^{2+} and Mg^{2+}) responsible for stabilizing of the bacteria outer membrane (Falagas and Kasiakou, 2005; Fernández *et al.*, 2013). The Mueller-Hinton broth (Oxoid, England) was prepared from the powder and supplemented with Ca^{2+} (20-25 mg/L) according to CLSI guidelines (Koeth *et al.*, 2000; Girardello *et al.*, 2012). The experimental PmB assay was made by dissolving Polymyxin B sulfate salt (12,000 µg/ml; Sigma Aldrich) in buffer solution (0.2% bovine serum albumin, BSA and 0.01% acetic acids) and was filter sterilized. Overnight test bacterial culture was diluted into fresh media and grown to logarithmic-phase in double strength MHB (OD_{600} nm, 0.2 to 0.4). In a Bioscreen special well plates, 100 µl of the culture, 100 µl double concentrations of polymyxin (8.0-2048 µg/ml) in the presence of 100 µl of calcium ion solution and incubated at 37^0C with maximum shaking continuously for 24 hours in a Bioscreen C automated growth curve analyzer (MTX Lab Systems, Vienna, VA, USA). Minimum inhibitory concentration of ≤ 2 µg/ml was determined (S) and ≥ 4 µg/ml as resistant (R) (CLSI 12th edition).

5.3.4 Lipopolysaccharide preparation and extraction

LPS was extracted as previously described with some modifications (Marolda *et al.*, 1990; Su and Ding, 2015). Bacterial strains were grown overnight on LB agar plates at 37°C and the colonies were harvested from the plate and suspended in sterile 3 ml of Phosphate Buffered Saline (PBS) solution. The culture was adjusted to optical density 3.0 (OD_{600}) and 1.5 ml of the bacterial suspension was transferred to a micro-centrifuge Epperndorf tube and centrifuged (10, 000 g, 1 min) to pellet the cells. The pellet was re-suspended in 150µl of lysis buffer (0.5 M Tris HCl, pH 6.8), 2% sodium dodecyl sulfate (SDS) and 4% β-mercaptoethanol). The mixture was boiled for 10 min and cooled on ice for 10 min. Ten microlitres of 20 mg/ml solution of proteinase K dissolved in 10 mM Tris-HCl buffer (pH 8.0), then, 1 mM $CaCl_2$ and 30% glycerol was added,

vortexed and incubated overnight at 60^{0}C on a thermo-heat block. After the overnight incubation, 150µl of 90% phenol solution (90% phenol, 0.1 % β-mercaptoethanol and 0.2% 8-hydroxyquinloline) was added and incubated at 70^{o}C for 15 min, with intermittent vortexing every 5 min. The separation of the aqueous and phenolic phases was done by ice-cooling for 10 min, and then the top aqueous phase was transferred into a new tube. Five hundred microlitres of ethyl ether saturated with 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA was added and vortexed at 14, 000 rpm for 1 min. The top ether phase was aspirated, mixed with loading buffer and stored at -20^{o}C.

5.3.5 SDS-Polyacrylamide Gel Electrophoresis Analysis of LPS

Tris-Glycine-SDS-PAGE gel was casted at acrylamide concentration of 12.5% for resolving gel (5.68 ml of ABA 44:80, 0.35 ml of 10% SDS, 4.73 ml of 1.5 M Tris-HCl pH 8.0), 4.2 g of urea, 4.2 ml of deionized water, 0.035 ml of 10% APS, and 0.025 ml of TEMED, 4.4% for the stacking gel (acrylamide: *bis*-acrylamide ratio 20:1; 0.5 ml ABA 44:0.8, 0.05 ml of 10% SDS, 2.5 ml of 0.25 M Tris-HCl pH 6.8) 1.83 ml deionized water, 0.05 ml of 10% APS and 0.02 ml of TEMED. LPS samples already mixed with protein loading buffer were loaded 1 µl per SDS-PAGE gel well. Conditions for gel running were set at 70 V for 30 min and then changed to 150 V for an approximate of 2 hours with additional 10 min to allow the 3X loading dye (0.187 M of Tris-HCl pH 6.8, 6% SDS, 30% glycerol, 0.03% bromophenol blue and 15% 2-ME) to run-out of the gel.

5.3.6 Detection of LPS by Silver Staining

Lipopolysaccharide was detected using a fixing solution (60% methanol and 10% ethanoic acid) at 4^{o}C overnight. After overnight fixing, the gels were washed in 200 ml of 7.5% ethanoic acid solution for 30 min at room temperature and were rewashed again in 200 ml freshly prepared 0.2% (w/v) periodic acid solution for another 30 min. The gels were then washed in Milli-Q water for another 1 hour with the water being changed every 15 min, 500 ml at a time. Freshly prepared

Silver nitrate solution (0.5 g of Sliver nitrate dissolved in 10 ml of Milli-Q water) was added in drops to 190 ml solution of 1.4% sodium hydroxide and 0.7% ammonia, swirling the solution after each drop. Then, the gels were soaked in the prepared Silver staining solution and strictly left for 15 min and further washed for 1 hour with 500 ml Milli-Q water, replacing it every 15 min. Then the gels were further developed in 200 ml of 1.25% sodium carbonate solution with 36 µl of 36% formaldehyde. Gels were photographed after they fully developed and stored in 300-500 ml of Milli-Q water or 50 mM EDTA to prevent overdeveloping.

5.3.7 LPS Hydrolyses and Mass Spectrometry (MS) Analysis of Lipid A

Lipid A component of the LPS was further confirmed using MS analysis as previously described (Hamidi *et al.,* 2005). Isobutyric acid and ammonium hydroxide extraction method was used (Hamidi *et al.,* 2005). Briefly, overnight bacterial culture was normalized to desired optical density (1.0-1.2 OD_{600}). The bacteria cells (pellets at 4000 rpm, 20 min) was washed (2-3 times) with equal volume of PB (1.15 g/L di-sodium hydrogen and 0.2 g/L potassium di-hydrogen orthophosphate VWR™) and centrifuged (4000 rpm, 20 min). The bacterial cells were then washed twice with 400 µl fresh, single-phase chloroform-methanol (1:2 v/v) and with 400 µl of chloroform: methanol: water mixture (3:2:0.25, v/v); centrifugation was used to recover the pellets (2000xg, 15 min). The recovered freshly washed cells were treated with 400 µl of isobutyric acid: 1 M ammonium hydroxide (5:3, v/v), thoroughly vortex and place on thermo-heat block in a screw-cap tube (100^0C, 2 hours). Intermittently, the sample was vortex every 15 min to cleave the KDO (Ketodeoxyoctonate) to release lipid A. Then, the samples were cooled on ice (15 min); 400 µl of the recovered supernatant by centrifugation (2000xg, 15 min) was diluted with 400 µl (1:1) deionized water and lyophilized overnight. The lyophilized powder was washed twice with 400 µl (100% methanol) and centrifuged (2000xg, 15 min). About 100 µl mixture of chloroform:

methanol: water (3:1.5:0.25, v/v) was used to solubilized the insoluble lipid A for 4 hours at room temperature. Twenty microliters of the lipid A suspension were desalted with a few grains of Dowex® and the mixture centrifuged (13000, rpm, 1 min). Matrix dyes (2, 5-Dihydroxybenzoic acid in 100 mM citric and acetonitrile: 0.1% trifluoroacetic acids), vortex vigorously and matrices centrifuged (13000, rpm, 5 min). One microliter of the lipid A was spotted on the polished sheet target plate, allowed to dry, and then an equal volume of matrix was added and air-dried (15-20 min). Mass Spectrometry analysis was performed with negative ion matrix-assisted laser desorption ionization time of flight (MALDI-TOF) (Autoflex Speed, Bruker). Autoflex speed analyzer was used for spectra generation and FlexAnalysis (version 3.0), Graphad 6.0, was used to process the spectra and mass spectroscopy was used to analyze the lipid A structures. The negative spectra were scanned between 1200 to 2400 m/z ratio.

5.3.8 Preparation of Genomic DNA and PCR Amplification of resistant markers

Genomic chromosomal DNA of the selected strains was recovered using a commercially available column-based kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions. The sensory and two-component regulatory system genes, *phoQ, phoP, prmA* and *prmB* were amplified with specific oligonucleotide primers (**Figure 1: Appendix IVA**). Primers were also designed for detection of other resistant markers which include quinolone, macrolide, carbapenems, β-lactams, methicillin aminoglycosides, fluoroquinolones, penicillins, chloramphenicol, sulfonamides and cephalosporins, (*mefA, qnrA, mefE, blaCTX, blaKPC, ermB, gyrA, gyrB, TetA, mecA, ampC, and qnrB*) implicated in resistance (**Figure 5.5**).

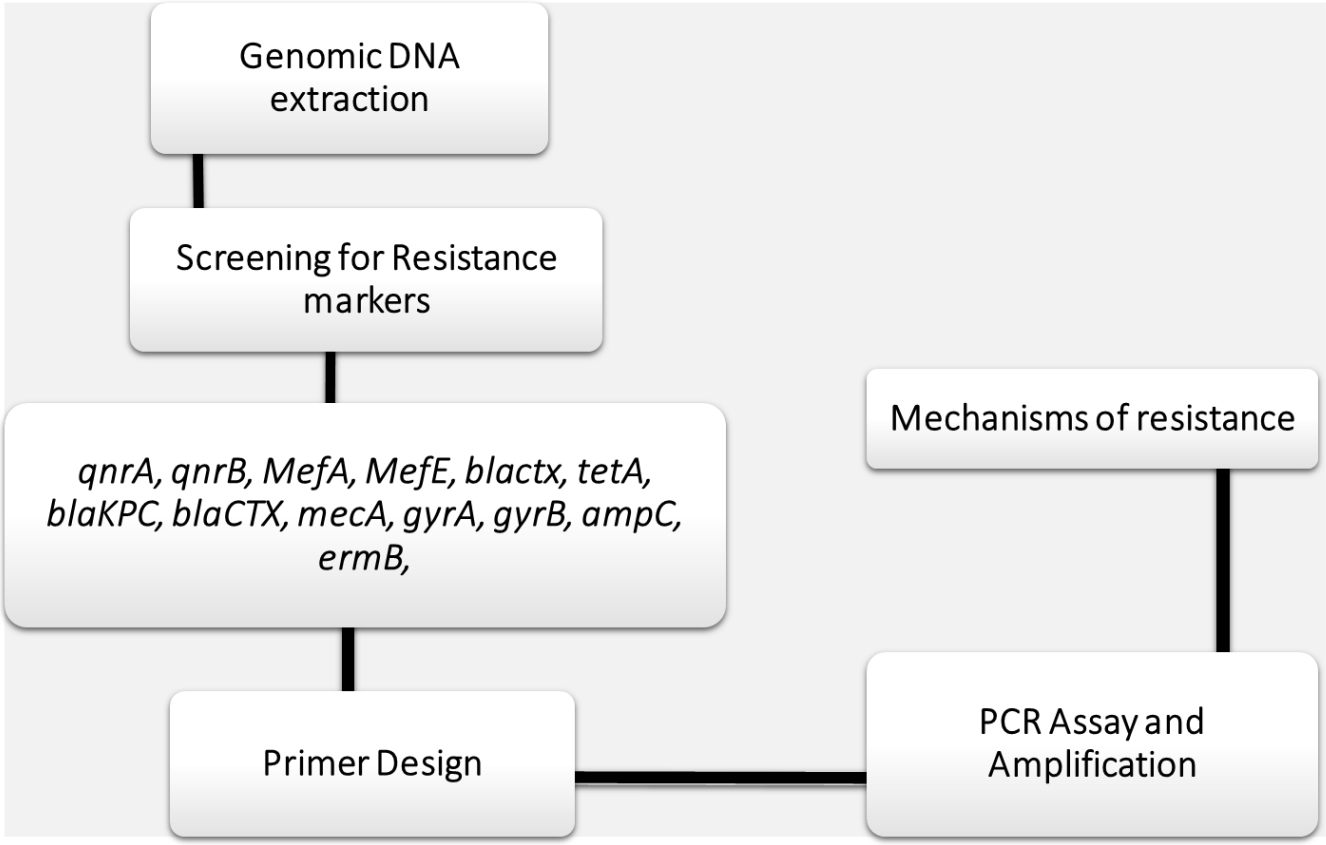


Figure 5.5: Flowchart for detection of resistance markers

5.4 Results

5.4.1 Calcium-adjusted assay in resistance mechanism

The effects of calcium on the polymyxin-resistant bacterial strains ability to show sensitivity or maintained resistance to polymyxin B using the CLSI guidelines was tested (Koeth *et al.,* 2000; Girardello *et al.*, 2012; Sahalan *et al.*, 2013). Responses of the strains in the presence of calcium varies, as some showed some levels of sensitivity at the initial incubation time, but later expressed some significant level of resistance. For the first 4 hours of incubation, species of *Enterobacter* in the presence of calcium was sensitive, but started building resistance at 5^{th} hour and maintained it increasingly for 24 hours. This trend was also observed in species of *Klebsiella KBAB1*, *Acinetobacter ACNAB2*, *Serratia SRAB1*, *Citrobacter CTAB1* and *Pseudomonas PGAB2* (**Figure 5.6**). There is possibility that the polymyxin-resistant abilities of the strains were compromised within the first few hours of contact with the supplemented calcium, but later expressed some resistance mechanisms. Species of *Proteus*, *Pseudomonas (PGAB1), Citrobacter (CTAB2), Acinetobacter (ACNAB1)* and *Klebsiella (KBAB2)* maintained resistance irrespective of calcium. Contrary to what has been described in previous modeled studies, it was observed that extreme resistance to polymyxin after few hours of sensitivity might signify strains displaying some mechanisms of resistance.

5.4.2 Detection of resistance markers as basis for resistance mechanisms

Mechanisms of bacterial resistance to conventional antibiotics have been based on resistant genes they harbored. In this study, different resistant markers in the selected strains tested were detected.

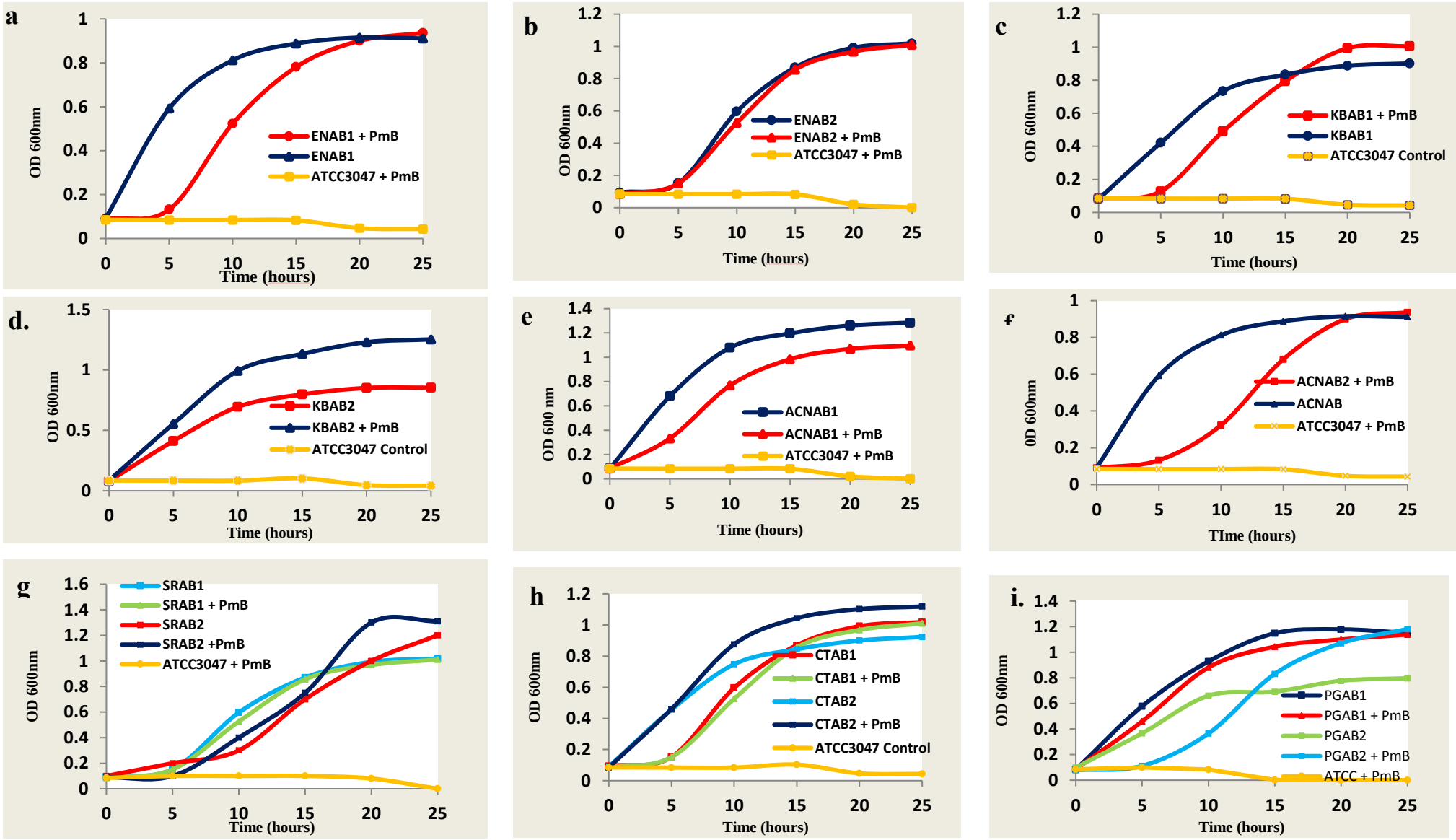


Figure: 5.6: Polymyxin B AMR mechanisms profile of strains with Calcium adjusted sensitive assay

With regards to the selected antibiotics, the following resistant markers in the strains: β-lactamase (*blaCTX*), macrolide efflux (*mefA and mef E*), methicillin (*mecA*), erythromycin (*ermB*) quinolone (*qnrA and qnrB*), tetracycline (*tetA*), aminoglycoside, DNA gyrase (*gyrA and gyrB*) and carbapenamase (*KPC*) were detected (**Table 5.2 and Appendix IVA**). This study shows that the mechanisms of resistance exhibited by the selected strains are diverse and the detected resistant markers are key players in their extensive antibiotic resistance as observed in this study.

Table 5.2: Detection of resistant markers in selected strains

S No	Code	Strains	Resistant Markers
1	ENAB1	*E. cloacae complex*	*qnrA gyrA blaCTX, mefE,*
2	ENAB2	*E. cloacae*	*mefA, qnrA, blaCTX,*
3	PGAB1	*P. aeruginosa*	*qnrA, tetA, qnrB*
4	PGAB2	*Pseudomonas* spp	*qnrA, ermB, qnrB*
5	CTAB1	*C. freundii*	*blaCTX, ampC, mefE*
6	CTAB2	*C. freundii*	*gyrA, blaCTX*
7	KBAB1	*K. pneumoniae*	*mefA, blaKPC, blaCTX,*
8	KBAB2	*K. oxytoca*	*ermB, blaCTX*
9	SRAB1	*S. marscenses*	*ampC, gyrA, mefE*
10	SRAB2	*S. marscenses*	*qnrA, ermB, mefE*
11	ACNAB1	*A. baumannii*	*gyrB, blaCTX*
12	ACNAB2	*A. baumannii*	*blaCTX, mefA*
13	ECAB01	*E. coli*	*gyrB, gyrA, blaCTX*
14	ECAB02	*E. coli*	*tetA, blaCTX*
15	PRMAB1	*P. mirabilis*	*gyrB, gyrA, blaCTX*
16	PRMAB2	*P. mirabilis*	*ermB, blaCTX*
17	STAB1	*S. aureus*	*mecA, ermB*

5.4.3 LPS profiling and LPS-modifying genes

The surface membrane localized LPS in most Gram-negative bacteria is the key virulent factor and it easily interacts with polymyxin (cationic AMP). Modification in LPS is the main factor responsible for resistance. LPS of the selected polymyxin resistant bacterial strains were profiled. Lipid A and O-antigen, the main components of LPS were detected in more than 95% percent of the strains by SDS-PAGE (**Figure 5.7**).

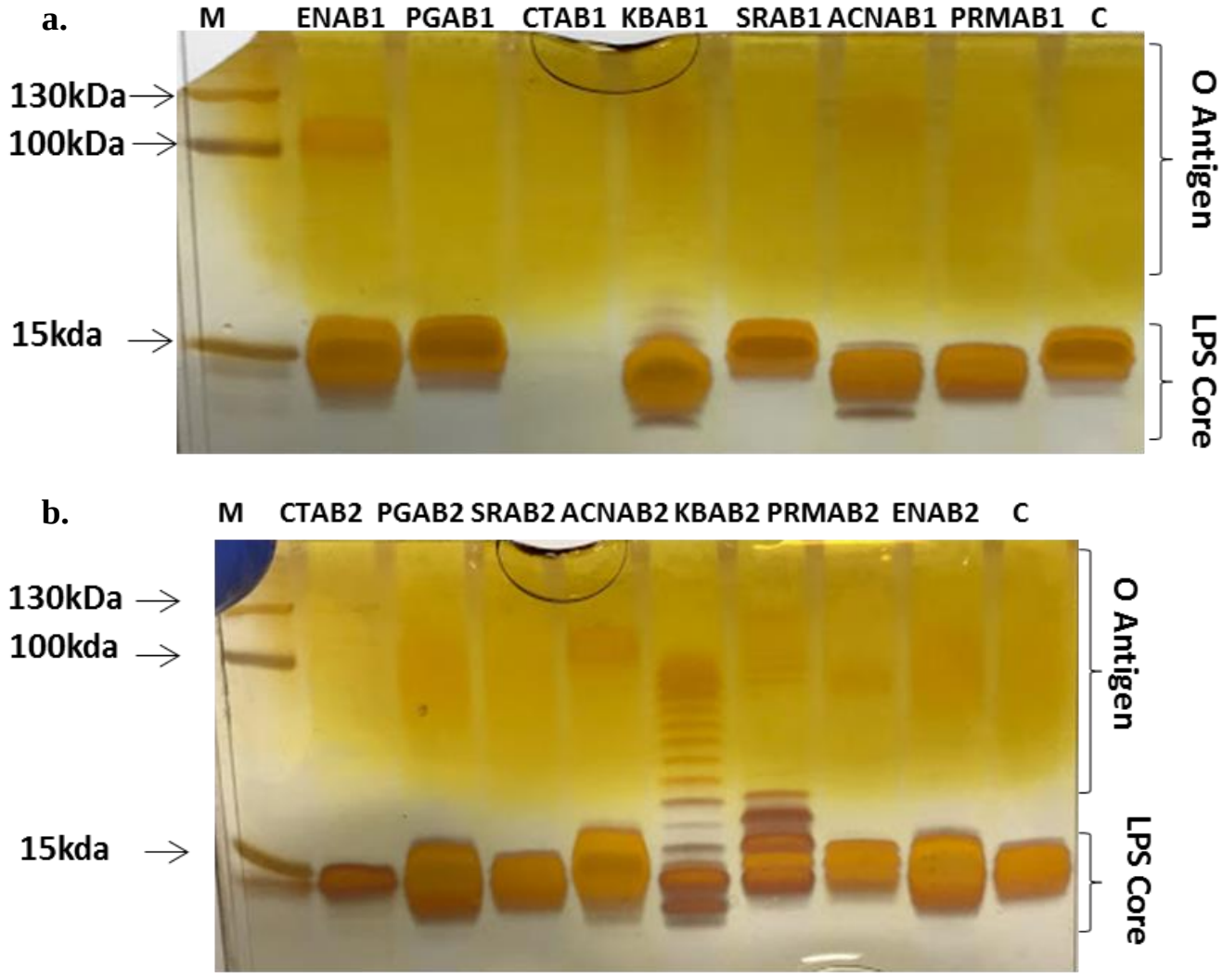


Figure 5.7: Glycine SDS-PAGE (12.5%) LPS profile of strains showing O-Antigen and LPS core a) ENAB1, PGAB1, CTAB1, KBAB1, SRAB1, ACNAB1, PRMAB1; b) CTAB2, PGAB2, SRAB2, ACNAB2, KBAB2, PRMAB2, ENAB2

The Lipid A component was further ascertained using the MS analysis. The generated mass spectra corresponding to the respective lipid A of the tested strains was detected. Spectra with peaks and intensity compatible with *Enterobacter, Klebsiella, Citrobacter, E coli* were identified. While majority of the spectra correlates with spectra of some already profiled Gram-negative bacterial strains, others were different (**Table 5.3**). This indicated a possible follow-up on the uniqueness of these strains, especially as environmental bacteria. As observed in this study, the strains have different structural analogues. Based on their dominant ion peaks, *Serratia marscenses* has two unique lipid A structures at m/z 1825 and m/z 1850, two structures at m/z 1824 and m/z 1850 for *Proteus mirabilis*, m/z 1797 peaks for *Klebsiella oxytoca* lipid A structure, *E. coli* at m/z 1388 lipid A structure, m/z 1850 and m/z 1824 lipid A structures for *Enterobacter cloacae* and m/z 1797 lipid A structure for *Citrobacter freundii.* Some strains of *Serratia marscenses, Proteus mirabilis* and *Enterobacter cloacae* are *hexa*-acylated, *bis*-phosphorylated lipid A structures containing four C14:0 3-OH and two C14:0. The peaks characterizing diphosphryl lipid A structures of some other strains of *Serratia, Enterobacter, Citrobacter, Proteus* and *Klebsiella oxytoca* contains four C14 3-OH, one C14:0 and one C16:0, a deviation from the conventional lipid A. Uniquely different, the peak characterizing *E. coli* corresponds to tetra-acyl diphosphoryl lipid A structure containing three C14:0 3-OH and one C14:0 (**Figure 5.8 a-f**). The lipid A structures appears unmodified, variation in the number of carbons as displayed by the strains play some roles in the resistance observed in this study. This defines strain unique Lipid A identity and we are reporting them in association with bacteria from Ghanaian hospital for the first time.

Table 5.3: Ion peaks observed from MALDI-TOF MS analysis of lipid A of the selected polymyxin resistant strains and proposed structure (fatty acid and phosphate)

Peaks (m/z)	Proposed Structure	Bacterial Strain
1824	Hexa-acyl (4x C14:0(3-OH), 2x C14:0), 2P	*S. marscenses (SRAB2)*
1850	Hexa-acyl (4x C14:0(3-OH), 1x C14:0, 1x C16:0), 2P	*S. marscenses (SRAB2)*
1824	Hexa-acyl (4x C14:0(3-OH), 2x C14:0), 2P	*P. mirabilis (PRMAB1)*
1850	Hexa-acyl (4x C14:0(3-OH), 1x C14:0, 1x C16:0), 2P	*P. mirabilis (PRMAB1)*
1797	Hexa-acyl (4x C14:0(3-OH), 1x C14:0, 1x C12:0), 2P	*K. oxytoca (KBAB2)*
1388	Tetra-acyl (3x C14:0(3-OH), 1x C14:0), 2P	*E. coli (ECAB1)*
1825	Hexa-acyl (4x C14:0(3-OH), 2x C14:0), 2P	*E. cloacae (ENAB1/2)*
1797	Hexa-acyl (4x C14:0(3-OH), 1x C14:0, 1x C12:0), 2P	*C. freundii (CTAB2)*
1850	Hexa-acyl (4x C14:0(3-OH), 1x C14:0, 1x C16:0), 2P	*E. cloacae (ENAB1)*

Peaks (m/z) = mass to charge ratio determining the ions peak and possible structure of the LPS of the identified strains

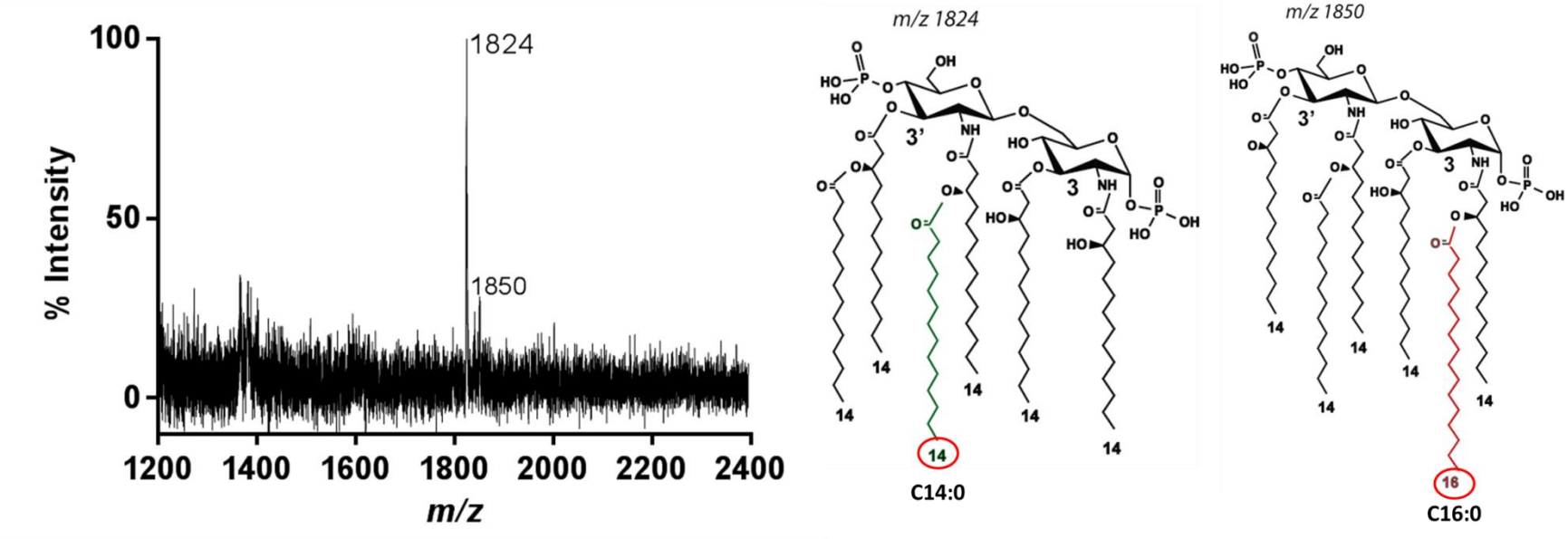


Figure 5.8a: MALDI-TOF MS analysis of lipid A of *Serratia marscenses SRAB2* (ion peaks, 1824, 1850) and proposed structures

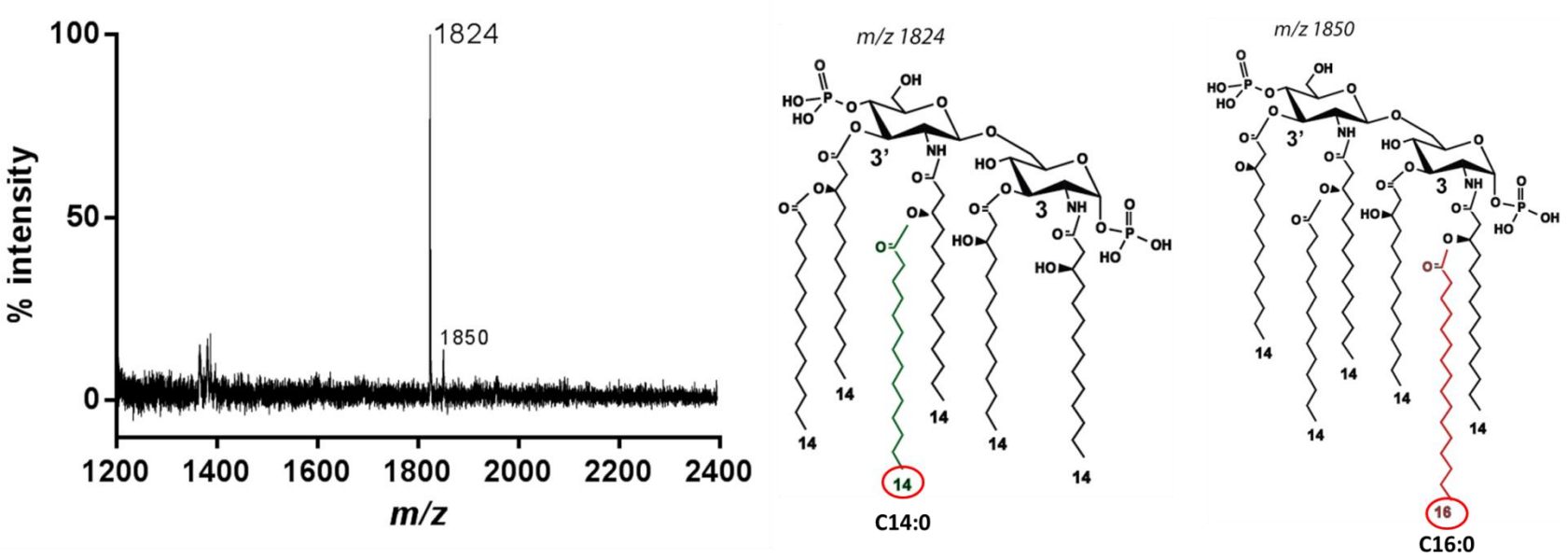


Figure 5.8b: MALDI-TOF MS analysis of lipid A of *Proteus mirabilis PRMAB2* (ion peaks, 1824, 1850) and proposed structures

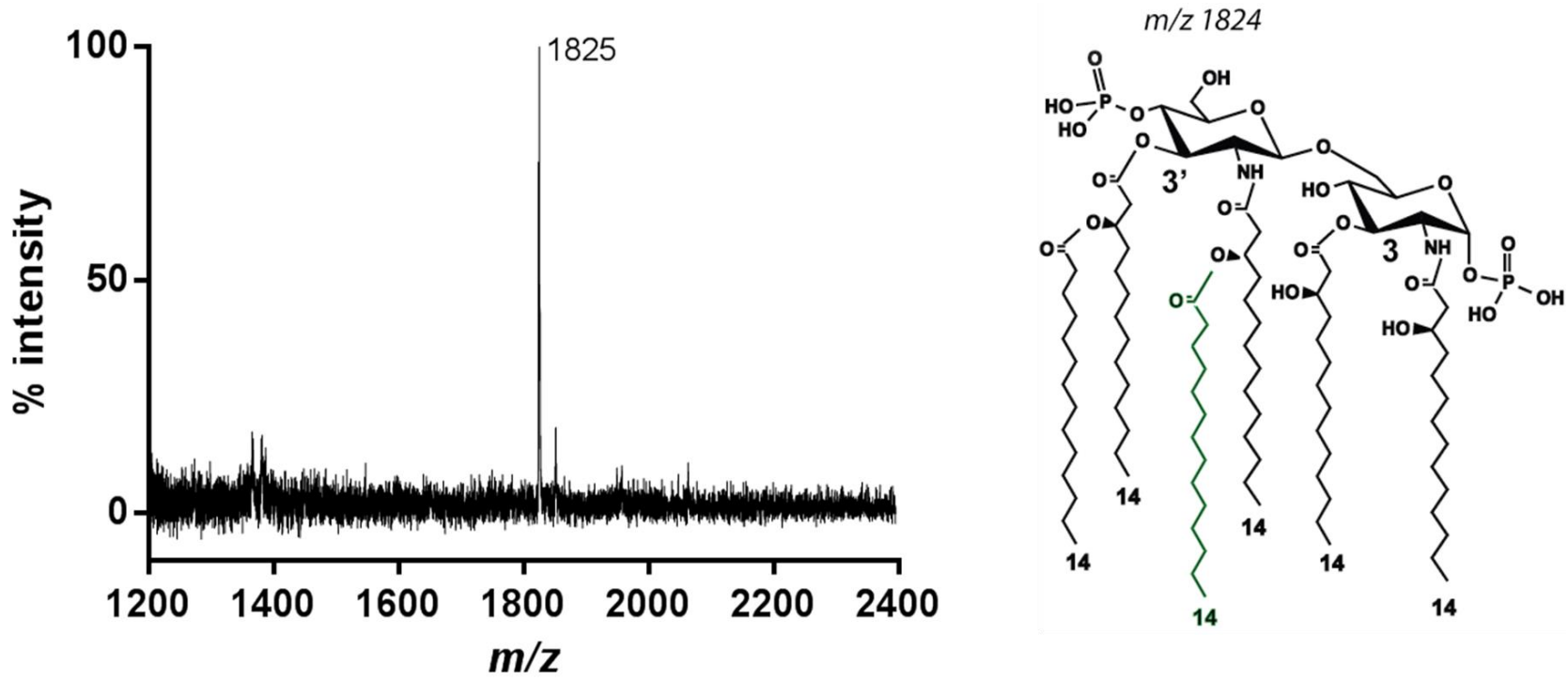


Figure 5.8c: MALDI-TOF MS analysis of lipid A of *Proteus mirabilis ENAB1/2* (ion peak, 1825) and proposed structure

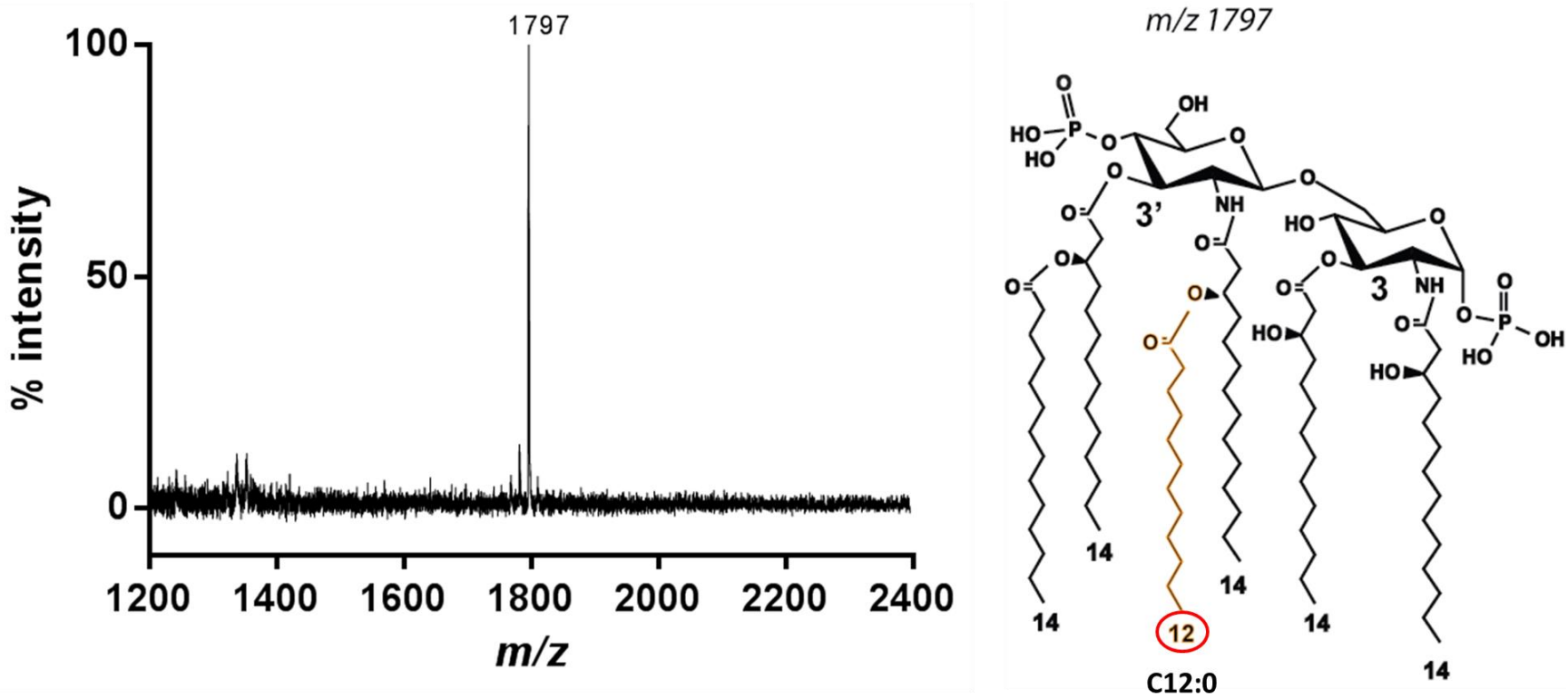


Figure 5.8d: MALDI-TOF MS analysis of lipid A of *Klebsiella oxytoca KBAB2* (ion peak, 1797) and proposed structure

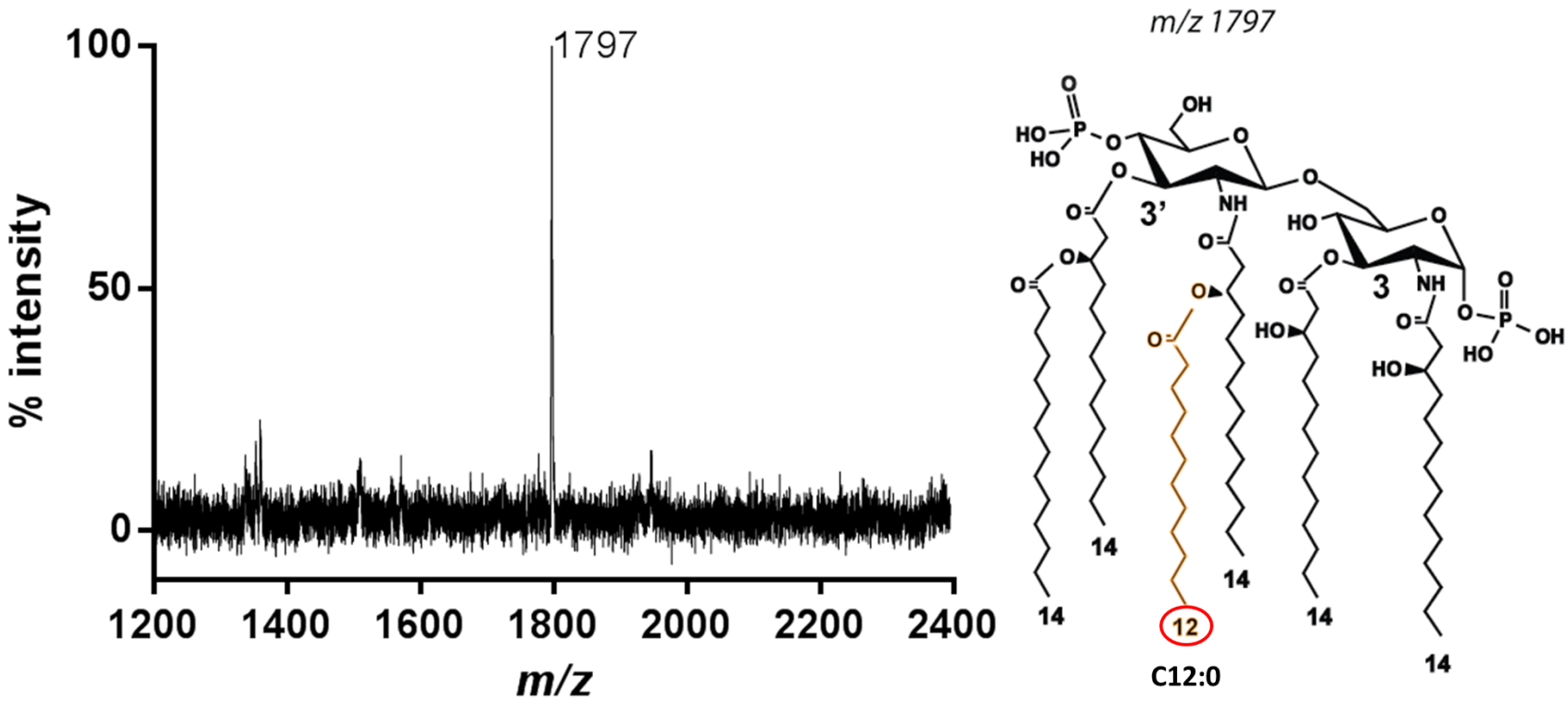


Figure 5.8e: MALDI-TOF MS analysis of lipid A of *Citrobacter freundii CTAB2* (ion peak, 1797) and proposed structure

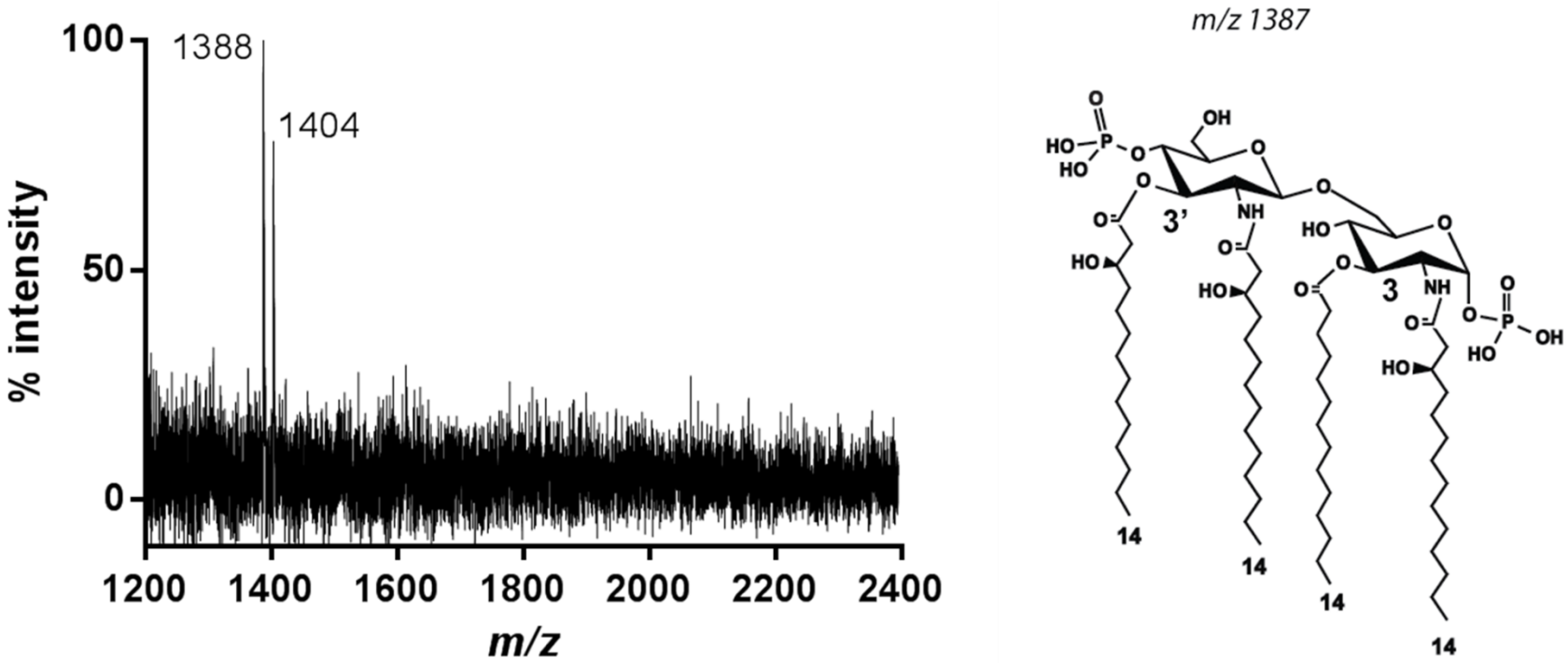


Figure 5.8f: MALDI-TOF MS analysis of lipid A of *E. coli ECAB1* (ion peak, 1388) and proposed structure

LPS-modifying genes that have been implicated in resistance to polymyxin using specific primers listed on **Table 1 in Appendix IVB** were further screened. The detected LPS-modifying genes are listed on **Table 5.4**. Out of the sixteen selected, *prmA* was detected in fifteen and eleven of the strains harbored *prmB*, *phoP* and *phoQ* respectively (**Figure 5.9**). Detection of the most important part of LPS (lipid A) and the LPS-modifying genes are the possible drivers of extreme resistance to polymyxin as observed in this study.

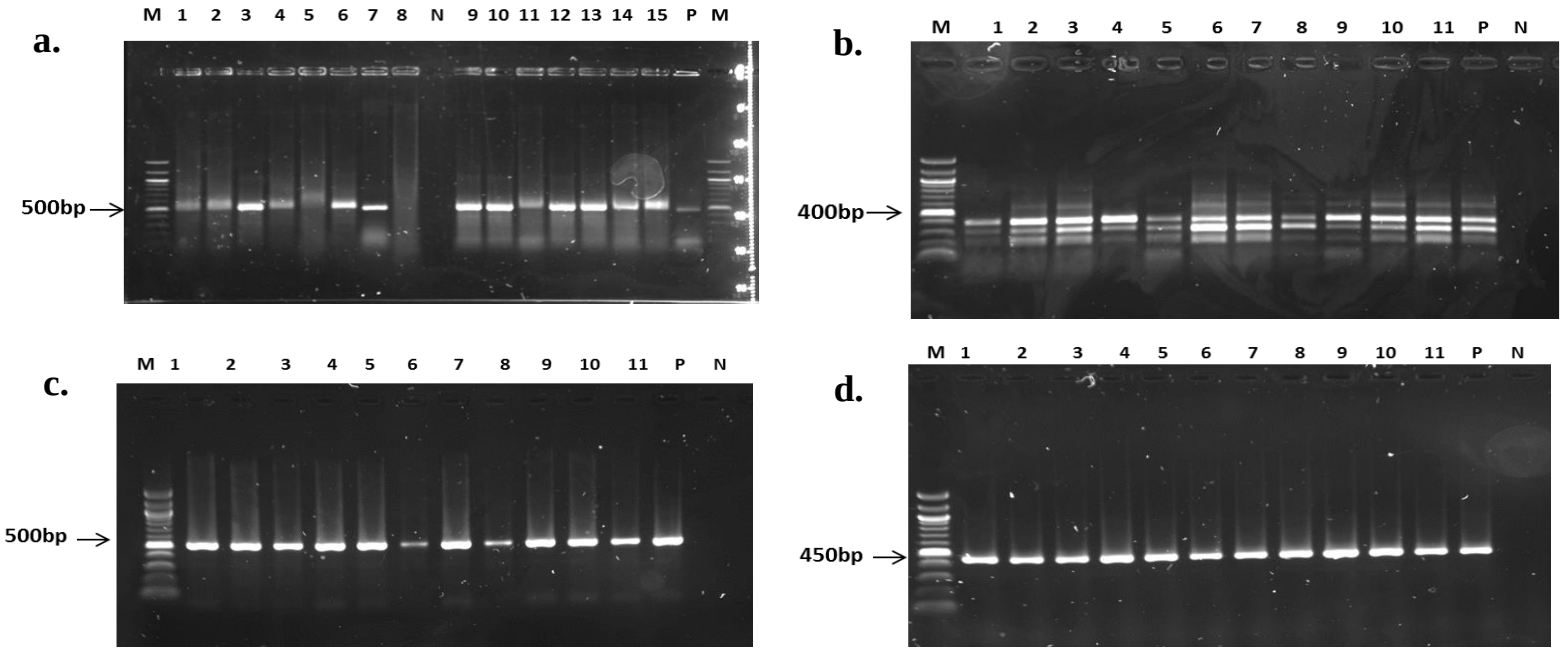


Figure 5.9: PCR detection of LPS-Modifying gene in selected strains (1-15), M (100 bp molecular marker, P/N (Positive/Negative controls); (a.) *prmA* 500 bp, (b.) *prmB* 400 bp, (c.) *phoP* 500 bp, (d.) *phoQ* 450 bp (1 – ENAB1, 2 – ENAB2, 3 – KBAB1, 4 – KBAB2, 5 – PGAB1, 6 - PGAB2, 7 – CTAB1, 8 – CTAB2, 9 – SRAB1, 10 – SRAB2, 11 – PRMAB1, 12 – PRMAB2, 13 – ACNAB1, 14 – ACNAB2, 15 ECCO1)

Table 5.4: Summary of LPS profiling and Detection of LPS-modifying Genes

Strains	LPS		LPS-Modifying Genes			
	O-Antigen	**LPS Core**	***phoP***	***phoQ***	***prmA***	***prmB***
E. cloacae complex	[a]+	+	+	+	+	+
E. cloacae	+	+	+	+	+	+
K. pneumoniae	+	+	+	+	+	+
K. oxytoca	+	+	+	+	+	+
Acinetobacter spp. *(ACNAB1/2)*	+	+	ND	ND	+	ND
C. freundii (CTAB2)	+	+	+	+	+	+
P. mirabilis (PRMAB1/2)	+	+	+	+	+	+
P. aeruginosa	+	+	+	+	+	+
Pseudomonas spp.	+	+	+	+	+	+
S. marscenses (SRAB1/2)	+	+	+	+	+	+
E. coli (EC01)	ND	ND	ND	ND	+	ND

ND – Not detected; [a]+ = Positive for the specific marker

5.5 Discussion

As pathogenic bacteria are emerging from hospital environment with extreme levels of resistance to antibiotics, focusing on Ghana, this present study investigated the possible mechanisms of antimicrobial resistance. These strains possibly engaged persistence as approach of resistance mechanisms to polymyxin based on their sensitive response to the antibiotic in the first few hours of challenge, before they built resistance at an extreme rate. Studies conducted on establishing bacterial resistance mechanisms associated with HAIs have indicated antibiotic interception and selection of more resistant subpopulation through differential gene expression and epigenetic modifications as possible mechanisms of resistance (Depardieu *et al.,* 2007; Adam *et al.,* 200 8; Wecke and Mascher, 2011; Motta *et al.,* 2015). In line with this, we posit that these strains adapt to polymyxin exposure in the presence of calcium ion and build resistance through tolerance thereafter. Other studies have also reported changes in response to polymyxin when assayed with calcium supplements (Falagas and Kasiakou, 2005; Conly and Johnston, 2006; Zavascki *et al.,* 2007; Yu *et al.,* 2015). While this is an indulgent escape of bacteria from cationic antibiotics, it might also indicate that displacement of divalent ions as a result of LPS interaction with cationic polymyxin may be complementary or not to the resistance of bacteria.

Species of *Klebsiella, Enterobacter, Citrobacter, Acinetobacter, Proteus and Escherichia coli* in this present study harbors *blaCTX* and have been associated with resistance to β-lactams, and most especially carbapenemes, penicillin and cephalosporin. Studies conducted on clinical bacterial isolates have reported that *blaCTX* enhance antimicrobial resistance (Literacka *et al.,* 2009; Moghaddam *et al.,* 2014; Chen *et al.,* 2012). This marker encodes an Extended Spectrum β-lacatamse (ESBL) gene and it is most times responsible for therapeutic failure (Moghaddam *et al.,*

2014; Zhao *et al.*, 2010; Chen *et al.*, 2012). Majority of these strains are ESBL-producers, hence the observed resistance in this study.

Also, strains of *Klebsiella pneumonia, Enterobacter cloacae complex, Citrobacter freundii, Serratia marscenses* and *Acinetobacter baumannii* can extrude antibiotics from their cells using multidrug efflux pumps (*mefA* and *mefE*), and more specifically macrolide and quinolones class of antibiotics. This mechanism of resistance has been reported in clinical bacterial strains accompanied with serious health issues in the ICU (Masi *et al.*, 2005; Pagès *et al.*, 2011). Also, efflux genes such as *mefA* and *mefE* enable bacteria to survive higher concentrations of antibiotics, thus lowering the intracellular concentration of such antibiotics (Blair *et al.*, 2014).

The mechanisms of resistance exhibited as investigated in this study also include those to quinolone with the detection of *qnrA and qnrB* markers in species of *Pseudomonas, Enterobacter* and *Serratia*. These genes have been reported in association with resistance in clinically isolated *S. marscenses* (Yang *et al.*, 2014) and as a causative agent of recurrent infections in implantable cardioverter defibrillator (Hawkey and Choy, 2015). Studies reported quinolone-resistant genes as factors for extensive hospital-wide spread of multidrug-resistant *E. cloacae and P. aeruginosa* (Hall *et al.*, 2006; Cabot *et al.*, 2012). Although most of these resistant markers facilitating resistance have been detected in isolates from patients, the mechanisms of resistance are not different from that of multidrug resistant strains resident on fomites and in the air. This study presents that resistance mechanisms exhibited is directly linked with resistant markers resident in strains, though some of this may be intrinsic as in *S. marscenses* (Olaitan *et al.*, 2014) and others horizontally transferred as in *E. coli* (Messerer and Fischer, 2017). In this study, DNA-gyrase (*gyrA and gyrB*), *ampC* and *ermB* resistant markers as mediators of resistance to ampicillin,

erythromycin, amoxicillin, ciprofloxacin, ofloxacillin, norfloxacin and gentamicin were detected. It is interesting to state that this is similar to what have been previously described and reported, particularly in association with HAIs in other parts of Africa, and the world (Okamoto *et al.*, 2002; García-fernández *et al.*, 2015; Arjomandzadegan *et al.*, 2016; Ingle *et al.*, 2017; Tadesse *et al.*, 2018).

Klebsiella pneumoniae carbapenemase (*KPC*) detected in this study is a key player in resistance of *K. pneumoniae* and other bacteria like *E. coli* to β-lactamases. *KPC* producing bacteria are now emerging from hospital environment as highly drug-resistant Gram-negative bacteria causing severe hospital infections (Arnold *et al.*, 2012). *KPC* have been associated with outbreak in the northeastern US and since, it has spread to other parts of the world as a key pathogen set for global nosocomial dominance (Pitout and Nordmann, 2015). *KPC* represents an enzyme that possesses exceptional antimicrobial resistance mechanisms especially to carbapenem, cephalosporins and penicillins (Paterson and Bonomo, 2005; Pitout and Nordmann, 2015). *KPC* is major drivers of resistance in hospital associated bacterial strains as observed in this study. The detection of *tetA* gene in *E. coli* in this study indicated a known antimicrobial resistance mechanism to broad-spectrum tetracycline. Studies in other parts of Africa have associated this resistant marker with multidrug-resistant *E. coli* mostly in clinical samples as compared to fomites and air in this study (Zhang *et al.*, 2010; Olowe *et al.*, 2013). We also report the occurrence of methicillin resistance gene (*mecA)* in *S. aureus*, as this indicated a mechanism of resistance that has been globally reported (Wielders *et al.*, 2002; Zhang *et al.*, 2010).

Profiling of LPS, lipid A and O-antigen of the selected strains play significant role during infection in resistance to host antimicrobial peptides and some other immune factors (Gunn, 2001; Rosenfeld and Shai, 2006). In particular, lipid A profiling showed different unique signatures and

peaks. This is a possible pointer to novel signature that can possibly provide information on the extreme resistance of the strains to polymyxin as reported in this study. The structural differences in the lipid A moieties reported in this study are possible pointers to mechanisms of resistance exhibited by these strains. For the first time from Ghanaian hospital environmental strains, differences in structural analogue of lipid A were reported. It is interesting to also mention that unique structural differences were detected in lipid A of not commonly reported strains in association with fomites and air from the hospital. *Citrobacter, Serratia, E. coli, Proteus, Klebsiella* and the newly emerging *Enterobacter* strains have unique lipid A structures. Structural differences in the same species of *Enterobacter* strains with respect to the number of attached carbon and peak of ionization was observed, though the lipid A may be functionally different but they both play some special roles in AMR mechanisms. Some of the strains share similar structural differences; indicating that the resistance mechanisms are unique to each strain. Most of the lipid A structures analyzed in this study are different from the conventional lipid due to minor deviation with respect to the number of hydroxyl and carbon. It is also good to note that the structural differences do not correspond to the already established extra lipid modifications, for instance the addition of L-arabinose and ethnolamine to the left/right acyl chains of lipid A. Detection of these peaks could potentially be used to define the resistance mechanisms presented by the polymyxin resistant bacterial strains.

Also, the random detection of previously described and reported LPS-associated modifying genes, *prmA, prmB, phoP* and *phoQ* in the extensively resistant polymyxin strains in this study indicated other potential resistance mechanisms. The roles of these genes in resistance have been described (Hoare, 2011; Yu *et al.*, 2015; Maldonado *et al.*, 2016) and more specifically as they influence modifications in LPS (Schnaitman and Klenat, 1993; Zavascki *et al.*, 2007; Deris *et al.*, 2014). In

this study, specific possible modifications caused by these LPS modifying genes were not identified, but our findings was based on other studies that have reported their association with bacteria isolated from hospital in-patients especially in *Pseudomonas aeruginosa*, *Burkholderia cepacia* and *Klebsiella pneumoniae* (Olaitan *et al.*, 2014; Khodai-kalaki *et al.*, 2015; Maldonado *et al.*, 2016; Liu *et al.*, 2017). Studies have also reported possible modifications in LPS with characterization of polymyxin genome and resistome of *Pseudomonas aeruginosa* and *Burkholderia cepacia* (Fernández *et al.*, 2013; Zhang *et al.*, 2000; Bengoechea *et al.*, 2019).

The data generated in this study serves as pointers and relevant background to the likely resistance mechanisms that is obtainable in Gram-negative bacteria from Ghanaian hospitals, as the presence of the resistant genes agree with already characterized resistance mechanism. Future studies and further characterizations of the detected LPS-modifying genes would provide other specific information on the mechanisms of resistance expressed.

CHAPTER 6

General Conclusions and Future Directions

6.0 General conclusions

The overall focus of this doctoral research was to characterize bacterial strains isolated from fomites and air in selected Ghanaian hospitals and more specifically, to identify these bacteria and establish their antimicrobial and pathogenic profiles. This is very important since there is little information in Ghana about the state of bacterial contamination in the hospital environment and its relevance for HAIs.

Three hypotheses were addressed 1.) Ghanaian hospitals are dominated by ***'diverse pathogenic and virulent bacterial strains'*** 2.) These bacterial strains are ***'extensively resistant'*** to common and last resort antibiotics 3.) Unique ***'resistant signatures'*** are drivers of their resistance mechanisms.

In Chapter 2, the study demonstrated that Hospital environments in Ghana are dominated with diverse bacteria

The data shows that hospital environments in Ghana serve as a hub for Gram-negative and Gram-positive bacteria of pathogenic relevance. These bacteria are resident on fomites and are also dispersed in the air, mostly in the ICU, NICU, surgical ward, waiting rooms, maternity departments and other sensitive locations within the hospital. This is somehow worrying as fomites like door/toilet handles, waste-bins, faucets, sinks, table/chairs and beddings are daily and consistently engaged and play special roles in infection transmission. This comes with serious concerns especially in the ICU and NICU as most of the in-patients are immunosuppressed and are vulnerable to infections. Isolation and identification of especially Gram-negative bacteria, most of which are opportunistic pathogens poses a potential risk to immunocompromised patients in the hospitals. This is accompanied by the isolation of similar bacteria in the air environment. On the

basis of our findings, Ghanaian hospital houses a mixture of common and also other bacteria that are not commonly reported. It was also observed that strains recovered from fomites and air in Ghanaian hospitals is motile and produces biofilms. These are two specific phenotypic properties of potential pathogens especially biofilm-producing bacteria have been associated with increased resistance to antibiotics and thereby cause infections that are difficult to treat.

Chapter 3 shows that bacterial strains from Ghanaian hospitals are extensively multidrug resistant

Multidrug resistance was noticed in the ESKAPE pathogen and also other not commonly reported strains in hospital environments. Remarkably, these strains were highly resistant to the last-option of antibiotic treatments, carbapenems and polymyxin. This means that hospital users in Ghana might be exposed to a growing burden of untreatable infections if appropriate interventions to cut transmission are not implemented.

The clinical relevance of heteroresistance is still been understudied, but there are indications that they contribute largely to therapeutic failures. Besides, they can respond differently to the same antibiotic under the same conditions. They have also been qualified as more resistant subpopulation of bacteria that results from antibiotic selection pressure, thus leading to overall resistant state of the general population of bacteria. While it is known that they slow infection healing process in the ICU, they can also differentially express different phenotypic traits to frustrate antibiotic effectiveness. The bacterial strains in this study were identified as heteroresistant phenotypes, this may account for the extreme level of resistance observed.

Chapter 4 reveals that the strains are highly virulent and display resistance to antimicrobial peptides *in vivo*

The *Galleria mellonella* Infection (GMI) model was employed to establish the virulent nature of the modeled bacteria strains in this study. These strains showed unusual level of virulence at lower concentration (MOI = 10 cells/larvae) in Galleria. Majority of the strains showed 100% mortality rate in *Galleria* in less than 24 hours. By implication, it means that the strains are extensively resistant to antimicrobial peptides, which is a major component of the innate immune system as expressed by *Galleria*. This indicated that, the tendency is higher that these bacteria might show almost the same level of virulence in human, as the immune system in *Galleria* is similar to that of human and at the same time, they produce almost equal amount of antimicrobial peptides.

In Chapter 5, the data shows that the model bacteria express diverse resistance mechanisms

It was confirmed that bacteria harbor different resistant markers to facilitate their resistance to antibiotics. From efflux pumps to extrude antibiotics from their cells, to production of β-lactamases and DNA-gyrases to inactivate the activities of antibiotics as enzymes, to fluoroquinolone, macrolide, tetracycline and methicillin resistant genes, to genes inhibiting the activities of the antibiotics from damaging their cell walls and protein synthesis, to sensory genes that can capture or intercept antibiotic activities, to LPS-modifying genes as basis of bacterial resistance to polymyxin and colistin. For the first time, different lipid A structural modifications from bacteria associated with hospital fomites and air in Ghana were reported. These modifications are potential resistant mechanisms that the bacteria employed in crippling antibiotic effectiveness. These bacterial strains, if by any means implicated in any infection associated with hospitals in Ghana, they are capable of expressing multidrug resistance to mechanisms. By implication, it means they

will engage diverse strategies to display resistance to any form of antibiotics, even when two or more than one classes of antibiotics are combined for treatment purpose.

6.1 Future directions

6.1.1 Whole Genome Sequencing to uncover other resistance mechanisms

The data generated in this study has provided relevant information on the likely resistance mechanisms that is obtainable in bacteria from Ghanaian hospitals. However, further studies, and more specifically, the characterization of the detected resistant markers and LPS-modifying genes would help identify possible mutations in the genome of these bacteria that might explain more the mechanisms of resistance. Further characterization of the lipid A with NMR will further provide insights into the roles they play in bacterial resistance to Polymyxin B. Whole genomic information would confirm other possible bacterial approaches to resistance.

6.1.2 RNA Sequencing to uncover mechanisms of heteroresistance

Analysis of the bacterial RNA through sequencing would be a good follow-up on the acquired genome information. This will not only uncover regulatory markers mediating resistance, but also genetic expressions of these markers in association with resistance and heteroresistance.

6.1.3 Identification of other virulent markers

In this study, it was observed and established that other virulent factors are in association with LPS as possible key players in GMI model *in vivo* virulence studies. This can be further understood using human macrophages or animal model.

6.1.4 Comparing environmental with clinical bacterial strains

Though, the focus of this present study was to characterize bacterial strains in hospital environments, a follow-up project on analyzing clinical (blood, urine, sputum and nasal) samples

from in-patients in the selected hospital environments is ongoing. This will help us to further establish the environment-human transmission interplay (and vice versa) of infectious bacterial strains.

6.1.5 Phage-therapeutic approach

It would also be interesting to devise means on how to handle bacterial resistance within environments, and also control their spread to the community. In an attempt to achieve this, phage-therapeutic approach to killing these bugs have been initiated. Further, collaborations on how to develop other antibiotic sensitive assays is ongoing; of such is to engage pentamidine to first sensitize these strains before antibiotic challenge.

6.1.6 Public health measures and finding a lasting solution to AMR in Ghanaian hospital

This study was approved by Ghana Health Service (GHS) (GHS-ERC01/02/17); there would be a need to team-up with this agency on how the practicality of our findings can be incorporated into public health orientations program. A different approach to the use of hospital, and its facilities would go a long way in subverting infections. Proper disinfection practice especially of medical handy tools and equipment should be made a priority. Bacteria resistant gloves, if possible, will minimize the spread of infectious agents. Basic hygiene, particularly hand hygiene through consistent washing should be intensified, encouraged and recommended to all hospital users. Inappropriate prescription of antibiotics should be discouraged; prevention over treatment should be a priority (**Figure 6.1a**). In Ghana, the use of antibiotics is still not properly regulated and they are available over the counter without a prescription. Indoor air quality monitoring is a necessity in most of our hospitals, as this will provides information on infectious agents, endotoxins and pollutants in circulation. Periodic surveillance (**Figure 6.1b**) of diverse bacteria and consistent

determination of their antimicrobial profiles is encouraged, as this will uncover emerging bacteria and their response to new and old antibiotics.

6.2 Study Limitations

This study has presented a concise account of the presence of multiple-antibiotic resistant and potential high-risk pathogens in some Ghanaian hospital environments. However, these findings demonstrate a need for further studies as indicated in section 6 to understand the full scope of this problem in Ghana. As noted, data from three hospitals representing three regions of Ghana is presented in this study, there is a need to sample more hospital across Ghana to further confirm the prevalence of these potential bacterial pathogens. While this study confirms that there might be more bacterial strains than what has been reported from some Ghanaian hospitals; however, there is a need for more sensitive bacterial resistance surveillance and profiling, which would be complementary to the robust methods used in the present study. There is limited local data from Ghana on bacteria circulating in Ghanaian hospital air and those resident on fomites; availability of this data would have made the study rationale stronger. One limitation that could facilitate further study is the predicted structures of endotoxin represent one of numerous polymyxin B resistance possibilities, unfortunately, the proposed structures could not be further confirmed in this study.

b.

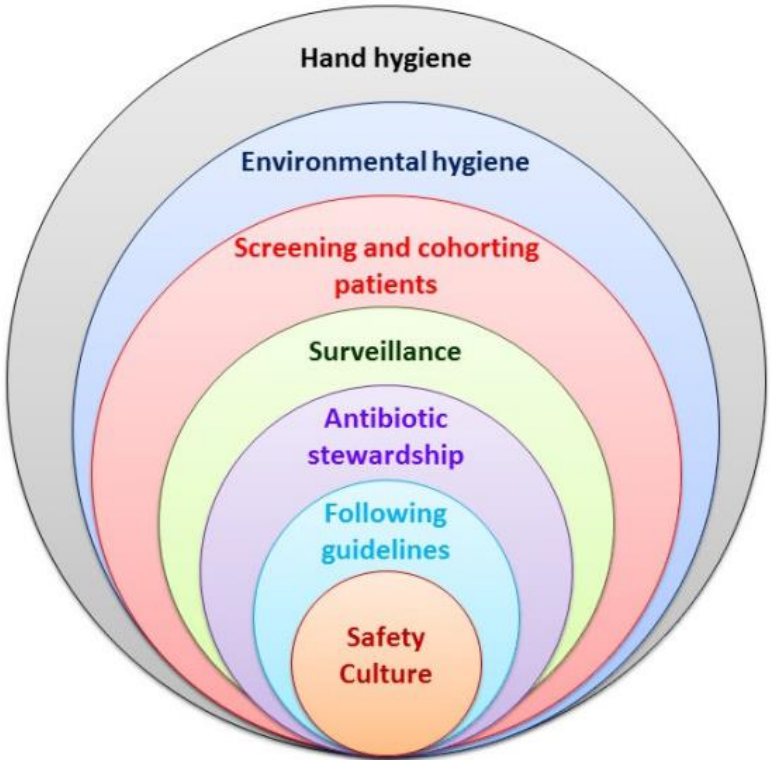


Figure 6.1:

Possible recommendations for antibiotic stewardship and strategies to prevent healthcare associated infections

https://infectionsinsurgery.org/core-elements-of-antibiotic-stewardship/; https://infectionsinsurgery.org/7-strategies-to-prevent-healthcare-associated-infections/

References

Abdallah, F., Mijouin, L., & Pichon, C. (2017). Review Article Skin Immune Landscape : Inside and Outside the Organism. *Mediators of Inflammation*. *2017*(1). https://doi.org/10.1155/2017/5095293.

Abiola, I., Abass, A., Duodu, S., & Mosi, L. (2018). Characterization of culturable airborne bacteria and antibiotic susceptibility profiles of indoor and immediate-outdoor environments of a research institute in Ghana. *AAS Open Research*. *1*(17). doi:10.12688/aasopenres.12863.2

Adam, M., Murali, B., Glenn, N. O., & Potter, S. S. (2008). Epigenetic inheritance based evolution of antibiotic resistance in bacteria. *BMC Evolutionary Biology*. *12*:1–12. doi:10.1186/1471-2148-8-52

Adams, R. I., Bateman, A. C., Bik, H. M., & Meadow, J. F. (2015). Microbiota of the indoor environment : a meta-analysis. *Microbiome*. *3*:49 doi:10.1186/s40168-015-0108-3

Adams, R. I., Miletto, M., Taylor, J. W., & Bruns, T. D. (2013). Dispersal in microbes : fungi in indoor air are dominated by outdoor air and show dispersal limitation at short distances. *The ISME Journal*. *7*(7):1262–1273. doi:10.1038/ismej.2013.28

Admassie, M. (2018). Current Review on Molecular and Phenotypic Mechanism of Bacterial Resistance to Antibiotic. *Science Journal of Clinical Medicine.* *7*(2):13–19. doi:10.11648/j.sjcm.20180702.11

Afshinnekoo, E., Meydan, C., Chowdhury, S., Jaroudi, D., Boyer, C., Bernstein, N., … Mason, C. E. (2015). Geospatial Resolution of Human and Bacterial Diversity with City-Scale Metagenomics. *Cell Systems*, *1*(1), 72–87. doi:10.1016/j.cels.2015.01.001

Agyepong, N., Govinden, U., & Owusu-ofori, A. (2018). Multidrug-resistant gram-negative

bacterial infections in a teaching hospital in Ghana. *Antimicrobial Resistance Infection Control*. *9*(7):371-8. doi: 10.1186/s13756-018-0324-2

Aiken, A. M., Mutuku, I. M., Sabat, A. J., Akkerboom, V., Mwangi, J., Scott, J. A., Morpeth, S. C., Friedrich, A. W., & Grundmann, H. (2014). Carriage of Staphylococcus aureus in Thika Level 5 Hospital, Kenya: a cross-sectional study. *Antimicrobial resistance and infection control*, *3*, 22. https://doi.org/10.1186/2047-2994-3-22

Alonso-, N. M., Bocanegra-garcia, V., Juárez-enríquez, S. R., Luna-herrera, J., Martínez, C. M., & Ma, A. (2015). Identification and Typing Methods for the Study of Bacterial Infections : a Brief Review and Mycobacterial as Case of Study Abstract, *Biochemistry and Molecular Biology Journal*. 1–10.

Allegranzi, B., Nejad, S. B., Combescure, C., Graafmans, W., Attar, H., Donaldson, L., & Pittet, D. (2011). Burden of endemic health-care-associated infection in developing countries : systematic review and meta-analysis. *The Lancet*. *377*(9761):228–241. doi:10.1016/S0140-6736(10)61458-4

Almagor, J., Temkin, E., Benenson, I., & Fallach, N. (2018). The impact of antibiotic use on transmission of resistant bacteria in hospitals: Insights from an agent-based model. *PLOS Pathog*. 1–14. doi: 10.1371/journal.pone.0197111

Amaral, L., Martins, A., Spengler, G., & Molnar, J. (2014). Efflux pumps of Gram-negative bacteria: What they do, how they do it, with what and how to deal with them. *Frontiers in Pharmacology*. *4:*1–11. doi:10.3389/fphar.2013.00168

Anderson, A., & Elmi, A. (2017). Evaluation of microbial contamination in frequently used Fomites in Kuwait. *Biodiversity Int J*. *1*(3), 80–86. doi:10.15406/bij.2017.01.00013

Apisarnthanarak, A., Mundy, L. M., Tantawichien, T., & Leelarasamee, A. (2017). Infection

Prevention and Control in Asia: Current Evidence and Future Milestones. *Clinical Infectious Diseases.64*(2):49–50. doi:10.1093/cid/cix071

Arjomandzadegan, M., Titov, L., Farnia, P., & Owlia, P. (2016). Molecular detection of fluoroquinolone resistance- associated gyrA mutations in ofloxacin-resistant clinical isolates of Mycobacterium tuberculosis. *International Journal of Mycobacteriology*. *5*(3): 299–305. doi:10.1016/j.ijmyco.2016.07.004

Armelagos, G. J., Barnes, K. C., & Lin, J. (1991). The Changing Disease-Scape in the Third Epidemiological *Transition. Int J Environ Res Public Health. 7*(2): 675–697.

Arnold C. (2014). Rethinking sterile: the hospital microbiome. *Environmental health perspectives*. *122*(7):A182-7. doi: 10.1289/ehp.122-A182

Arnold, R. S., Thom, K. A., Sharma, S., Phillips, M., Kristie Johnson, J., & Morgan, D. J. (2011). Emergence of *Klebsiella pneumoniae* carbapenemase-producing bacteria. *Southern medical journal*. *104*(1):40-45. doi: 10.1097/SMJ.0b013e3181fd7d5a

Arredondo-garcía, J. L., Soriano-becerril, D., Solórzano-santos, F., & Arbo-sosa, A. (2007). Resistance of Uropathogenic Bacteria to First-Line Antibiotics in Mexico City: A Multicenter Susceptibility Analysis. *Current Therapeutic Research, and Clinical Experimental. 68*(2):120–126. doi:10.1016/j.curtheres.

Ashbolt, N. J., Amézquita, A., Backhaus, T., Borriello, P., Brandt, K. K., Collignon, P., Coors, A., Finley, R., Gaze, W. H., Heberer, T., Lawrence, J. R., Larsson, D. G., McEwen, S. A., Ryan, J. J., Schönfeld, J., Silley, P., Snape, J. R., Van den Eede, C., & Topp, E. (2013). Human Health Risk Assessment (HHRA) for environmental development and transfer of antibiotic resistance. *Environmental health perspectives*, *121*(9), 993–1001. https://doi.org/10.1289/ehp.1206316

Ashgar, S. S., & El-said, H. M. (2012). Pathogenic Bacteria Associated with Different Public Environmental Sites in Mecca City. *Open Journal of Medical Microbiology*. *2*(4):133–137. doi: 10.4236/ojmm.2012.24020

Bahar, A. A., & Ren, D. (2013). Antimicrobial Peptides. *Pharmaceuticals*. *6*(12):1543–1575. doi:10.3390/ph6121543

Baker, L. Y., Hobby, C. R., Siv, A. W., Bible, W. C., Glennon, M. S., Anderson, D. M., Symes, S. J., & Giles, D. K. (2018). Pseudomonas aeruginosa responds to exogenous polyunsaturated fatty acids (PUFAs) by modifying phospholipid composition, membrane permeability, and phenotypes associated with virulence. *BMC microbiology*, *18*(1), 117. https://doi.org/10.1186/s12866-018-1259-8

Bakkali, M. E. L., Hmid, K., Kari, K. El, Zouhdi, M., Mzibri, M. E. L., & Laglaoui, A. (2015). Characterization of Bacterial Strains and their Resistance Status in Hospital Environment. *Journal of Tropical Diseases*. *4*(1), 1–6. doi:10.4172/2329-891X.1000180

Balzan, S., Quadros, C. D. A., Cleva, R. De, Zilberstein, B., & Cecconello, I. (2007). Bacterial translocation: Overview of mechanisms and clinical impact. *Journal of Gastroenterology and Hepatology*. *22*(4):464–471. doi:10.1111/j.1440-1746.2007.04933.x

Barker, J., & Jones, M. V. (2005). The potential spread of infection caused by aerosol contamination of surfaces after flushing a domestic toilet. *Journal of Applied Microbiology*. *99*(2):339–347. doi:10.1111/j.1365-2672.2005.02610.x

Bartlett, J. G., Gilbert, D. N., & Spellberg, B. (2013). Seven Ways to Preserve the Miracle of Antibiotics, *56*, 1445–1450. doi:10.1093/cid/cit070

Bazinet, A. L. (2017). Pan-genome and phylogeny of Bacillus cereus sensu lato, *BMC Evolutionary Biology*. ***17***(176):1–16. doi:10.1186/s12862-017-1020-1

Beceiro, A., Tomás, M., & Bou, G. (2013). Antimicrobial Resistance and Virulence : a Successsful or Deleterious Association in the Bacterial World ?. *Clinical Microbiology Reviews. 26*(2): 185–230. doi:10.1128/CMR.00059-12

Beggs, C. B. (2003). The Airborne Transmission of Infection in Hospital Buildings: Fact or Fiction? *Indoor and Built Environment*. *12*(1–2):9–18. doi:10.1177/1420326X03012001002

Bengoechea, J. A., Llobet, E., & Toma, J. M. (2008). Capsule polysaccharide is a bacterial decoy for antimicrobial peptides. *Microbiology 154:3877–3886.* doi:10.1099/mic.0.2008/02230-0

Bengtsson-Palme, J., Kristiansson, E., & Larsson, D. (2018). Environmental factors influencing the development and spread of antibiotic resistance. *FEMS microbiology reviews*. *42*(1):fux053. doi:10.1093/femsre/fux053

Benoun, J. M., Labuda, J. C., & Mcsorley, S. J. (2016). Collateral Damage : Detrimental Effect of Antibiotics on the Development of Protective Immune Memory. *mbio. 7*(6):4–9. doi:10.1128/mBio.01520-16.

Bereket, W., Hemalatha, K., Getenet, B., Wondwossen, T., Solomon, A., Zeynudin, A., & Kannan, S. (2012). Update on bacterial nosocomial infections. *European Review for Medical and Pharmacological Sciences*. *16*(8):1039–1044.

Bertolini, V., Gandolfi, I., Ambrosini, R., Bestetti, G., Innocente, E., Rampazzo, G., & Franzetti, A. (2012). Temporal variability and effect of environmental variables on airborne bacterial communities in an urban area of Northern Italy. *Applied Microbiology and Biotechnology*. *97*(14):6561-70. doi:10.1007/s00253-012-4450-0

Best, E., Parnell, P., Couturier, J., Barbut, F., Bozec, A. Le, Arnoldo, L., Madia, A., Brusaferro, S. M.H. & Wilcox, M. H. (2018). Environmental contamination by bacteria in hospital washrooms according to hand-drying method: a multi-centre study. *Journal of Hospital*

Infection. *100*(4):469–475. doi:10.1016/j.jhin.2018.07.002

Bhatta, D. R., Hamal, D., Shrestha, R., Subramanya, S. H., & Baral, N. (2018). Bacterial contamination of frequently touched objects in a tertiary care hospital of Pokhara , Nepal : how safe are our hands? *Antimicrob Resist Infect Control.* *6*(7):97. doi: 10.1186/s13756-018-0385-2

Blair, J. M. A., Webber, M. A., Baylay, A. J., Ogbolu, D. O., & Piddock, L. J. V. (2014). Molecular mechanisms of antibiotic resistance. *Nature Review Microbiology*. *13*(1):42–51. doi:10.1038/nrmicro3380

Blanco, P., Hernando-Amado, S., Reales-Calderon, J., Corona, F., Lira, F., Alcalde-Rico, M., … Martinez, J. (2016). Bacterial Multidrug Efflux Pumps: Much More Than Antibiotic Resistance Determinants. *Microorganisms*. *4*(1):14. doi:10.3390/microorganisms4010014

Bloom, G., Merrett, G. B., Wilkinson, A., Lin, V., & Paulin, S. (2017). Antimicrobial resistance and universal health coverage. *BMJ Global Health. 2*:1–6. doi:10.1136/bmjgh-2017-000518

Boone, S. A., & Gerba, C. P. (2007). Significance of Fomites in the Spread of Respiratory and Enteric Viral Disease, *Journal of Applied & Environmental Microbiol. 73*(6), 1687–1696. doi:10.1128/AEM.02051-06

Bridier, A., Briandet, R., Thomas, V., Briandet, R., & Thomas, V. (2011). Resistance of bacterial biofilms to disinfectants: a review. *Biofouling.* *27*(9):1017-1032. doi:10.1080/08927014.2011.626899

Bradbury, R. S., Roddam, L. F., Merritt, A., Reid, D. W., & Champion, A. C. (2019). Virulence gene distribution in clinical , nosocomial and environmental isolates of Pseudomonas aeruginosa. *Journal of Medical Microbiology.* 881–890. doi:10.1099/jmm.0.018283-0

Brown, S. P., Cornforth, D. M., & Mideo, N. (2014). Evolution of virulence in opportunistic

pathogens: generalism, plasticity and control. *Trends in Microbiology*. *20*(7):336–342. doi:10.1016/j.tim.2012.04.005

Butler, M. T., Wang, Q., & Harshey, R. M. (2010). Cell density and mobility protect swarming bacteria against antibiotics. *Proc Natl Acad Sci. 107*(8):3776-3781. doi:10.1073/pnas.0910934107

Cabot, G., Ocampo-Sosa, A. A., Domínguez, M. A., Gago, J. F., Juan, C., Tubau, F., Rodríguez, C., Moyà, B., Peña, C., Martínez-Martínez, L., Oliver, A., Spanish Network for Research in Infectious Diseases (REIPI) (2012). Genetic markers of widespread extensively drug-resistant Pseudomonas aeruginosa high-risk clones. *Antimicrobial agents and chemotherapy*. *56*(12):6349-57.

Cai, Y., Lee, W., Kwa, A. L., Cai, Y., Lee, W., & Kwa, A. L. (2015). Polymyxin B versus colistin: an update. *Expert Review of Anti-infective Therapy. 13*(12):1481-97. doi:10.1586/14787210.2015.1093933

Campanile, F., Bongiorno, D., Falcone, M., Vailati, F., Pasticci, M. B., Perez, M., … Stefani, S. (2012). Changing Italian nosocomial-community trends and heteroresistance in Staphylococcus aureus from bacteremia and endocarditis. *European Journal of Clinical Microbiology of Infectious Disease. 31*(5):739–745. doi:10.1007/s10096-011-1367-y

Cantas, L., & Suer, K. (2014). Review: the important bacterial zoonoses in "one health" concept. *Frontiers in public health*. *2*:144. doi:10.3389/fpubh.2014.00144

Cappitelli, F., Fermo, P., Vecchi, R., Piazzalunga, A., Valli, G., & Zanardini, E. (2009). Chemical – physical and Microbiological Measurements for Indoor Air Quality Assessment at the Ca' Granda Historical Archive, Milan (Italy). *Water Air & Soil Pollution. 201*(1-4):109-120. doi:10.1007/s11270-008-9931-5

Cattoir V. (2016). Mechanisms of Antibiotic Resistance. In: Ferretti JJ, Stevens DL, Fischetti VA, editors. Streptococcus pyogenes: Basic Biology to Clinical Manifestations [Internet]. Oklahoma City (OK): University of Oklahoma Health Sciences Center; 2016-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK333414/access date 2019

CDC. (2015). *Antibiotic Resistance (AR) Solutions Initiative: AR Lab Network ARLABnetwork AR Lab Network*. Retrieved from https://www.cdc.gov/drugresistance/pdf/cdc-ar-lab-network-final.pdf accessed in 2019

Celandroni, F., Salvetti, S., Gueye, S. A., & Mazzantini, D. (2016). Identification and Pathogenic Potential of Clinical Bacillus and Paenibacillus Isolates. *PLOS ONE*. 11(3):1–13. doi:10.1371/journal.pone.0152831

Champs, C. De, Bonnet, R., Sirot, D., Chanal, C., & Sirot, J. (2000). JAC Clinical relevance of Proteus mirabilis in hospital patients: a two year survey. *Journal of Antimicrobial Chemotherapy*. *45*(4):537–539.

Chen, Y., Zhao, J., Ding, F., Wang, B., Zhang, W., Gu, J., … Zhang, F. (2012). The bla CTX-M gene independently enhances drug resistance level to ampicillin in clinical isolates of Klebsiella pneumoniae. *Journal of Antibiotics (Tokyo).* *65*(9):479–481. doi:10.1038/ja.2012.44

Chopra, T., Rivard, C., Awali, R. A., Krishna, A., Bonomo, R. A., Perez, F., & Kaye, K. S. (2012). Epidemiology of Carbapenem-Resistant Enterobacteriaceae at a Long-term Acute Care Hospital. *Open Forum Infectious Disease.* 1–6. doi:10.1093/ofid/ofy224

Clare, S., Huett, A., & Dougan, G. (2002). Host / pathogen interactions at mucosal surfaces: Immune consequences. *153*:455–459.

CLSI. (2009). *Performance Standards for Antimicrobial Susceptibility Testing; Nineteenth*

Informational Supplement; CLSI document M100-S19.

Codjoe, F. S., Donkor, E. S., Smith, T. J., & Miller, K. (2019). Phenotypic and Genotypic Characterization of Carbapenem-Resistant Gram-Negative Bacilli Pathogens from Hospitals in Ghana. *Microbial drug resistance (Larchmont, N.Y.)*, *25*(10), 1449–1457. https://doi.org/10.1089/mdr.2018.0278

Coll, F., Harrison, E. M., Toleman, M. S., Reuter, S., Raven, K. E., Blane, B., Palmer, B., Kappeler, A., Brown, N. M., Török, M. E., Parkhill, J., & Peacock, S. J. (2017). Longitudinal genomic surveillance of MRSA in the UK reveals transmission patterns in hospitals and the community. *Science translational medicine*, *9*(413), eaak9745. https://doi.org/10.1126/scitranslmed.aak9745

Conly, J., & Johnston, B. (2006). Colistin: the phoenix arises. *The Canadian journal of infectious diseases & medical microbiology. 17*(5):267-9.

Contie, V., Defibaugh, A., Steinberg, D., & Wein, H. (2014). Stop the Spread of Superbugs. *Nih news in health*. *1*. Retrieved from https://newsinhealth.nih.gov/issue/feb2014/feature1

Crank, C. W. (2015). Vancomycin-resistant enterococcal infections: epidemiology, clinical manifestations, and optimal management. *Infection and drug resistance*. *8*:217-30. doi:10.2147/IDR.S54125

Cross, A. S. (2008). What is a virulence factor ?. *Critical Care. 12*(6):196. doi: 10.1186/cc7127.

Da Fonseca, T. A. P., Pessôa, R., Felix, A. C., & Sanabani, S. S. (2016). Diversity of bacterial communities on four frequently used surfaces in a large Brazilian teaching hospital. *International Journal of Environmental Research and Public Health*. *13*(2):1–11. doi:10.3390/ijerph13020152

Dalhoff, K., Ewig, S., Gideline Development Group, Abele-Horn, M., Andreas, S., Bauer, T. T.,

von Baum, H., Deja, M., Gastmeier, P., Gatermann, S., Gerlach, H., Grabein, B., Höffken, G., Kern, W., Kramme, E., Lange, C., Lorenz, J., Mayer, K., Nachtigall, I., Pletz, M., … Welte, T. (2013). Adult patients with nosocomial pneumonia: epidemiology, diagnosis, and treatment. *Deutsches Arzteblatt international*, *110*(38), 634–640. https://doi.org/10.3238/arztebl.2013.0634

David, M. D., & Gill, M. J. (2008). Potential for underdosing and emergence of resistance in Acinetobacter baumannii during treatment with colistin. *Journal of Antimicrobial Chemotherapy*. *61*(4):962–964. doi:10.1093/jac/dkn009

Davies, J., & Davies, D. (2010). Origins and Evolution of Antibiotic Resistance. *Microbiology and Molecular Biology Reviews. 74*(3):417–433. doi:10.1128/MMBR.00016-10

Davis, R., & Brown, P. D. (2019). Multiple antibiotic resistance index , fitness and virulence potential in respiratory Pseudomonas aeruginosa from Jamaica. *Journal of Medical Microbiology. 65*:261–271. doi:10.1099/jmm.0.000229

Depardieu, F., Podglajen, I., Leclercq, R., Collatz, E., & Courvalin, P. (2007). Modes and Modulations of Antibiotic Resistance Gene Expression. *Clinical Microbiolgy Reviews. 20*(1):79–114. doi:10.1128/CMR.00015-06

Deresinski, S. (2009). Vancomycin Heteroresistance and Methicillin-Resistant Staphylococcus aureus. *The Journal of Infectious Diseases. 199*:605–609. doi:10.1086/596630

Deris, Z. Z., Swarbrick, J. D., Roberts, K. D., Azad, M. A., Akter, J., Horne, A. S., Nation, R. L., Rogers, K. L., Thompson, P. E., Velkov, T., & Li, J. (2014). Probing the penetration of antimicrobial polymyxin lipopeptides into Gram-negative bacteria. *Bioconjugate chemistry*, *25*(4), 750–760. https://doi.org/10.1021/bc500094d

Dickmann, P. (2017). Communicating the Risk of MRSA: The Role of Clinical Practice,

Regulation and Other Policies in Five European Countries. *Frontiers of Public Health*. *5*:44. doi:10.3389/fpubh.2017.00044

Dijkshoorn, L., Nemec, A., & Seifert, H. (2007). An increasing threat in hospitals: multidrug-resistant Acinetobacter baumannii. *Nature Reviews Microbiology*. *5*(12):939-51. doi:10.1038/nrmicro1789

Duckro, A. N., Blom, D. W., Lyle, E. A., Weinstein, R. A., & Hayden, M. K. (2015). Transfer of Vancomycin-Resistant Enterococci via Health Care Worker Hands. *Archieves of Internal Medicine*. *165*(3):302-307.

Dulon, M., Haamann, F., Peters, C., Schablon, A., & Nienhaus, A. (2011). MRSA prevalence in european healthcare settings: a review. *BMC Infectious Diseases*. *11*(1):138. doi:10.1186/1471-2334-11-138

Duncan, E. J. S., Kournikakis, B., Ho, J., & Hill, I. (2009). Pulmonary Deposition of Aerosolized Bacillus Atrophaeus in a Swine Model Due to Exposure from a Simulated Anthrax Letter Incident. *Inhalation Toxicology*. 141–152. doi:10.1080/08958370802412629

Eames, I., Tang, J. W., Li, Y., & Wilson, P. (2009). Airborne transmission of disease in hospitals. *Journal of Royal Society Interface. 6:697-702.*

Edmonds-wilson, S. L., Nurinova, N. I., Zapka, C. A., Fierer, N., & Wilson, M. (2015). Review of human hand microbiome research. *Journal of Dermatological Science*, *80*(1):3–12. doi:10.1016/j.jdermsci.2015.07.006

Eggleston, K., Zhang, R., & Zeckhauser, R. J. (2010). The Global Challenge of Antimicrobial Resistance: Insights from Economic Analysis. *International Journal of Environmental Research and Public Health.* 7(8):3141–3149. doi:10.3390/ijerph7083141

El-halfawy, O. M., & Valvano, A. (2015). Antimicrobial Heteroresistance : an Emerging Field in

Need of Clarity. *Clinical Microbiology Reviews. 28*(1), 191–207. doi:10.1128/CMR.00058-14

El-Halfawy, O. M., & Valvano, M. A. (2013). Chemical Communication of Antibiotic Resistance by a Highly Resistant Subpopulation of Bacterial Cells. *PLOS ONE*. *8*(7). doi:10.1371/journal.pone.0068874

Ekrami, A., Kayedani, A., & Jahangir, M. (2011). Isolation of common aerobic bacterial pathogens from the environment of seven hospitals, Ahvaz, Iran. *Jundishapur J Microbiol. 4*(2): 75-82.

Essack, S. Y., Desta, A. T., Abotsi, R. E., & Agoba, E. E. (2016). Antimicrobial resistance in the WHO African region: current status and roadmap for action. *Journal of public health (Oxford, England)*. *39*(1):8-13. doi: 10.1093/pubmed/fdw015

European Commission. (2008). Public consultation on strategies for improving patient safety by prevention and control of healthcare-associated infections. *Public Health and Risk Assessment*. 16.

Eze, U. A. (2012). Bacterial contamination of door handles / knobs in selected public conveniences in Abuja metropolis , Nigeria : A public health threat. *Continental J. Medical Research. 6*(1):7 -11

Fair, R. J., & Tor, Y. (2014). Antibiotics and bacterial resistance in the 21st century. *Perspectives in medicinal chemistry*. *6*:25-64. doi:10.4137/PMC.S14459

Falagas, M. E., Karageorgopoulos, D. E., Leptidis, J., & Korbila, I. P. (2013). MRSA in Africa : Filling the Global Map of Antimicrobial Resistance. *PLOS ONE*. *8*(7). doi:10.1371/journal.pone.0068024

Falagas, M. E., & Kasiakou, S. K. (2005). Colistin : The Revival of Polymyxins for the Management of Multidrug-Resistant Gram-Negative Bacterial Infections. *Clinical Infectious*

Diseases. 40(9):1333–1342.

Falagas, M. E., Makris, G. C., Dimopoulos, G., & Matthaiou, D. K. (2007). Heteroresistance : a concern of increasing clinical significance ? *Clinical Microbiology and Infection. 14*(2):101-4. 10.1111/j.1469-0691.2007.01912.x

Fang, Z., Gong, C., Ouyang, Z., Liu, P., Sun, L., & Wang, X. (2014). Characteristic and Concentration Distribution of Culturable Airborne Bacteria in Residential Environments in Beijing, China. *Aerosol and Air Quality Research*, *14*(3), 943–953. doi:10.4209/aaqr.2013.04.0109

Fenton, A., Lello, J., & Bonsall, M. B. (2006). Pathogen responses to host immunity: the impact of time delays and memory on the evolution of virulence. *Proc Biol Sci. 273*(1597): 2083–2090. doi:10.1098/rspb.2006.3552

Fernández, L., Alvarez-Ortega, C., Wiegand, I., Olivares, J., Kocíncová, D., Lam, J. S., Martínez, J. L., & Hancock, R. E. (2013). Characterization of the polymyxin B resistome of Pseudomonas aeruginosa. *Antimicrobial agents and chemotherapy*, *57*(1), 110–119. https://doi.org/10.1128/AAC.01583-12

Fernando, D. M., & Kumar, A. (2013). Resistance-Nodulation-Division Multidrug Efflux Pumps in Gram-Negative Bacteria: Role in Virulence. *Antibiotics (Basel, Switzerland). 2*(1):163-81. doi:10.3390/antibiotics2010163

Fernstrom, A., & Goldblatt, M. (2013). Aerobiology and Its Role in the Transmission of Infectious Diseases. *Journal of Pathogens. 2013*(13):e493960. https://doi.org/10.1155/2013/493960

Finley, R. L., Collignon, P., Larsson, D. G., McEwen, S. A., Li, X. Z., Gaze, W. H., Reid-Smith, R., Timinouni, M., Graham, D. W., & Topp, E. (2013). The scourge of antibiotic resistance: the important role of the environment. *Clinical infectious diseases: an official publication of*

the Infectious Diseases Society of America, *57*(5), 704–710. https://doi.org/10.1093/cid/cit355

Fisher, R. A., Gollan, B., & Helaine, S. (2017). Persistent bacterial infections and persister cells. *Nature Reviews Microbiology*. *15*(8):453-464. doi:10.1038/nrmicro.2017.42

Frca, K. I., & Frca, A. P. (2005). Nosocomial infections. *International Journal of Pharmacy*. *5*(1), 14–17. doi:10.1093/bjaceaccp/mki006

Fletcher, L.A., Noakes, C.J., Beggs, C.B. & Sleigh, P.A. (2002). The Importance of Bioaerosols in Hospital Infections and the Potential for Control using Germicidal Ultraviolet Irradiation. *1st seminar on Applied Aerobiology*.1-12

French, G. L., Otter, J. A., Shannon, K. P., Adams, N. M. T., Watling, D., & Parks, M. J. (2004). Tackling contamination of the hospital environment by methicillin-resistant Staphylococcus aureus (MRSA): a comparison between conventional terminal cleaning and hydrogen peroxide vapour decontamination. *Journal of Hospital Infection*. *57*(1):31-7. doi:10.1016/j.jhin.2004.03.006

Futoma-kołoch, B. (2016). Immune Response against Bacterial Lipopolysaccharide. *Journal of Molecular Immunology*. *2*(1), 1–2. doi:10.4172/jmi.Page

Ganz, T. (2003). The Role of Antimicrobial Peptides in Innate Immunity. *Integrative Comparative Biology*. *43*(2):300-304. doi: 10.1093/icb/43.2.300

García-Fernández, A., Gallina, S., Owczarek, S., Dionisi, A. M., Benedetti, I., Decastelli, L., & Luzzi, I. (2015). Emergence of Ciprofloxacin-Resistant Salmonella enterica Serovar Typhi in Italy. *PLOS ONE*. *10*(6):e0132065. doi:10.1371/journal.pone.0132065

Gaynes, R., & Edwards, J. R. (2005). Overview of nosocomial infections caused by gram-negative bacilli. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases*

Society of America. *41*(6):848–54. doi:10.1086/432803

Ge, M., Kuo, A., Liu, K., Lee, M., Wu, T., & Chang, S. (2017). ScienceDirect Routine identification of microorganisms by matrix-assisted laser desorption ionization time-of-flight mass spectrometry : Success rate, economic analysis and clinical outcome. *Journal of Microbiology, Immunology and Infection*. *50*(5):662–668. doi:10.1016/j.jmii.2016.06.002

Geisinger, E., & Isberg, R. R. (2017). Interplay Between Antibiotic Resistance and Virulence During Disease Promoted by Multidrug-Resistant Bacteria. *Journal of Infectious Disease. 215*:9–17. doi:10.1093/DOI

Getachew, H., Derbie, A., & Mekonnen, D. (2018). Surfaces and Air Bacteriology of Selected Wards at a Referral Hospital, Northwest Ethiopia: A Cross-Sectional Study. *International Journal of Microbiology. 2018*:6413179. doi: 10.1155/2018/6413179.

Gibbons, H. S., Broomall, S. M., Mcnew, L. A., Daligault, H., Chapman, C., Bruce, D., … Nicole, C. (2011). Genomic Signatures of Strain Selection and Enhancement in Bacillus atrophaeus var. globigii, a Historical Biowarfare Simulant. *PLOS ONE*. *6*(3). doi:10.1371/journal.pone.0017836

Girardello, R., Bispo, P. J., Yamanaka, T. M., & Gales, A. C. (2012). Cation concentration variability of four distinct Mueller-Hinton agar brands influences polymyxin B susceptibility results. *Journal of clinical microbiology*. *50*(7):2414-8.

Gizaw, Z., Gebrehiwot, M., & Yenew, C. (2016). High bacterial load of indoor air in hospital wards: the case of University of Gondar teaching hospital, Northwest Ethiopia. *Multidisciplinary Respiratory Medicine*. *11*(24):1–7. doi:10.1186/s40248-016-0061-4

Gooderham, W. J., Bains, M., Mcphee, J. B., Wiegand, I., & Hancock, R. E. W. (2008). Induction by Cationic Antimicrobial Peptides and Involvement in Intrinsic Polymyxin and

Antimicrobial Peptide Resistance , Biofilm Formation , and Swarming Motility of PsrA in Pseudomonas aeruginosa. *Journal of Bacteriol. 190*(16), 5624–5634. doi:10.1128/JB.00594-08

Graber, C. J. (2017). Route of transmission of *Staphylococcus aureu. The Lancet Infectious Diseases*. *17*(2):124–125. doi:10.1016/S1473-3099(16)30512-6

Groisman, E. A., Parra-lopez, C., Salcedo, M., Lippst, C. J., & Heffron, F. (1992). Resistance to host antimicrobial peptides is necessary for Salmonella virulence. *Proc Natl Acad Sci U S A. 89*:11939–11943.

Gupta, S., Govil, D., Kakar, P. N., Prakash, O., Arora, D., & Das, S. (n.d.). Colistin and polymyxin B : A re-emergence, 49–54. doi:10.4103/0972-5229.56048

Gupte, S., Kaur, T., & Kaur, M. (2015). Journal of Tropical Diseases Virulence Factors of Environmental Microbes in Human Disease. *Journal of Tropical Disease and Public Health. 3*(2):2–4. doi:10.4172/2329-891X.1000161

Gunn, J. S. (2001). Bacterial modification of LPS and resistance to antimicrobial peptides. *Journal of Endotoxin Research. 7*(1):57-62

Leverstein-van Hall, M. A., Blok, H. E., Paauw, A., Fluit, A. C., Troelstra, A., Mascini, E. M., Bonten, M. J., & Verhoef, J. (2006). Extensive hospital-wide spread of a multidrug-resistant enterobacter cloacae clone, with late detection due to a variable antibiogram and frequent patient transfer. *Journal of clinical microbiology*, *44*(2), 518–524. https://doi.org/10.1128/JCM.44.2.518-524.

Hackman, H., K., Arhin, R., E., Gordon, A., & Mensah, S., N., B. (2017). Emergence of Carbapenem-resistant Enterobacteriaceae among Extended-spectrum Beta-lactamase Producers in Accra, Ghana. *Journal of Natural Sciences Research*

7: 24

Hamidi, A. El, Tirsoaga, A., Novikov, A., Hussein, A., & Caroff, M. (2005). Microextraction of bacterial lipid A: easy and rapid method for mass spectrometric characterization Journal of Lipids Research. *46*(8):1773–1778. doi:10.1194/jlr.D500014-JLR200

Harms, A., Maisonneuve, E., & Gerdes, K. (2016). Mechanisms of bacterial persistence during stress and antibiotic exposure. *Science (New York, N.Y.)*. *354*(6318):aaf4268. doi:10.1126/science.aaf4268

Hawkey, S., & Choy, A. (2015). Serratia marcescens: A Rare Cause of Recurrent Implantable Cardioverter Defibrillator Site Infection. *Case reports in cardiology*, *2015*, 641297. doi: 10.1155/2015/641297

Hermes, D. M., Pormann Pitt, C., Lutz, L., Teixeira, A. B., Ribeiro, V. B., Netto, B., Martins, A. F., Zavascki, A. P., & Barth, A. L. (2013). Evaluation of heteroresistance to polymyxin B among carbapenem-susceptible and -resistant Pseudomonas aeruginosa. *Journal of medical microbiology*, *62*(Pt 8), 1184–1189. https://doi.org/10.1099/jmm.0.059220-0

Hibberd, P. L., Hansen, N. I., Wang, M. E., Goudar, S. S., Pasha, O., Esamai, F., Chomba, E., Garces, A., Althabe, F., Derman, R. J., Goldenberg, R. L., Liechty, E. A., Carlo, W. A., Hambidge, K. M., Krebs, N. F., Buekens, P., McClure, E. M., Koso-Thomas, M., & Patel, A. B. (2016). Trends in the incidence of possible severe bacterial infection and case fatality rates in rural communities in Sub-Saharan Africa, South Asia and Latin America, 2010-2013: a multicenter prospective cohort study. *Reproductive health*, *13*(1), 65. https://doi.org/10.1186/s12978-016-0177-1

Hoare, A. (2011). Contribution of the Lipopolysaccharide to Resistance of *Shigella flexneri* 2a to Extreme Acidity. *PLOS ONE 6*(10): e25557. doi: 10.1371/journal.pone.0025557

Hu, H., Johani, K., Gosbell, I. B., Jacombs, A. S. W., & Almatroudi, A. (2015). Intensive care unit environmental surfaces are contaminated by multidrug-resistant bacteria in biofilms: combined results of conventional culture, pyrosequencing, scanning electron microscopy, and confocal laser microscopy. *Journal of Hospital Infections.* *91*(1):35-44. doi:10.1016/j.jhin.2015.05.016

Huttunen, R., & Aittoniemi, J. (2011). New concepts in the pathogenesis, diagnosis and treatment of bacteremia and sepsis. *Journal of Infection*. *63*(6):407–419. doi:10.1016/j.jinf.2011.08.004

Ii, A. J. P., & Marr, L. C. (2015). Sources of airborne microorganisms in the built environment. *Microbiome*. 1–10. doi:10.1186/s40168-015-0144-z

Ingle, D. J., Levine, M. M., Kotloff, K. L., & Holt, K. E. (2017). Drivers of antimicrobial resistance amongst intestinal *Escherichia coli* isolated from children in South Asia and sub-Saharan Africa. *Nature Microbiology*. *3*(9). doi: 10.1038/s41564-018-0217-4

Jander, G., & Rahme, L. G. (2000). Positive Correlation between Virulence of Pseudomonas aeruginosa Mutants in Mice and Insects. *Journal of Bacteriology*. *182*(13), 3843–3845

Jayol, A., Nordmann, P., Brink, A., & Poirel, L. (2015). Heteroresistance to colistin in *Klebsiella pneumoniae* associated with alterations in the *PhoPQ* regulatory system. *Antimicrobial agents and chemotherapy*. *59*(5):2780-2784

Jiang, L., Ng, I., Hou, Y., Li, D., Tan, L., Ho, H., & Chen, M. I. (2018). Infectious disease transmission: survey of contacts between hospital-based healthcare workers and working adults from the general population. *The Journal of hospital infection*, *98*(4), 404–411. https://doi.org/10.1016/j.jhin.2017.10.020

Johnson, A. P. (2011). Methicillin-resistant *Staphylococcus aureus*: the European landscape. *Journal of Antimicrobial Chemotherapy*. *66*:43–48. doi:10.1093/jac/dkr076

Joo, H. S., Fu, C. I., & Otto, M. (2016). Bacterial strategies of resistance to antimicrobial peptides. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, *371*(1695), 20150292. https://doi.org/10.1098/rstb.2015.0292

Jung, M. Y., Kim, J. S., Paek, W. K., Lim, J., Lee, H., Kim, P. I., Ma, J. Y., Kim, W., & Chang, Y. H. (2011). Bacillus manliponensis sp. nov., a new member of the Bacillus cereus group isolated from foreshore tidal flat sediment. *Journal of microbiology (Seoul, Korea)*, *49*(6), 1027–1032. https://doi.org/10.1007/s12275-011-1049-6

Junqueira, J. C. (2012). *Galleria mellonella* as a model host for human pathogens Recent studies and new perspectives. *Virulence. 3*(6):474–476. doi: 10.4161/viru.22493

Kanamori, H., Rutala, W. A., & Weber, D. J. (2017). The Role of Patient Care Items as a Fomite in Healthcare-Associated Outbreaks and Infection Prevention. *Clinical Infectious Diseases*. *65*(8):1412-1419. doi:10.1093/cid/cix462

Kapoor, G., Saigal, S., & Elongavan, A. (2017). Action and resistance mechanisms of antibiotics: A guide for clinicians Basic Anatomy of Bacterial Cell. *Journal of Anaesthesiology Pharmacology. 33*(3):300-305. doi:10.4103/joacp.

Karki, S. (2015). Should we continue to isolate patients with vancomycin-resistant enterococci in hospitals? *The Medical Journal of Australia*. *202*:234–236. doi:10.5694/mja14.00672

Karo, B., Herfst, S., Bo, M., Lawrence, P., Lewis, N. S., Mina, M. J., & Menge, C. (2017). Drivers of airborne human-to-human pathogen transmission. *Science Direct.* 22–29. doi: 10.1016/j.coviro.2016.11.006

Kavanagh, K. T., Abusalem, S., & Calderon, L. E. (2017). The incidence of MRSA infections in the United States: is a more comprehensive tracking system needed? *Antimicrobial Resistance & Infection Control. **6***(34):1–6. doi:10.1186/s13756-017-0193-0

Kearns, D. B. (2011). A field guide to bacterial swarming motility. *Nature Review Microbiology*. *8*(9):634–644. doi:10.1038/nrmicro2405.

Kelley, S. T., & Gilbert, J. A. (2013). Studying the microbiology of the indoor environment. *Genome Biology*. *14*(2):202. doi:10.1186/gb-2013-14-2-202

Khan, H. A., Baig, F. K., & Mehboob, R. (2017). Nosocomial infections: Epidemiology, prevention, control and surveillance. *Asian Pacific Journal of Tropical Biomedicine*. *7*(5), 478–482. doi:10.1016/j.apjtb.2017.01.019

Khodai-kalaki, M., Andrade, A., & Mohamed, F. (2015). Burkholderia cenocepacia Lipopolysaccharide Modification and Flagellin Glycosylation Affect Virulence but Not Innate Immune. *mbio. 6*(3):1–11. doi:10.1128/mBio.00679-15.

Kramer, A., Schwebke, I., & Kampf, G. (2006). How long do nosocomial pathogens persist on inanimate surfaces? A systematic review. *BMC Infectious Disease 8*:1–8. doi:10.1186/1471-2334-6-130

King, M., Noakes, C. J., & Sleigh, P. A. (2013). Bioaerosol deposition in single and two-bed hospital rooms: A numerical and experimental study. *Building and Environment 59*:436–447. doi: 10.1016/j.buildenv.2012.09.011

Kleef, E., Robotham, J. V., Jit, M., Deeny, S. R., & Edmunds, W. J. (2013). Modeling the transmission of healthcare associated infections: a systematic review. *BMC infectious diseases*. *13*: 294. doi:10.1186/1471-2334-13-294

Koch, G., Yepes, A., Förstner, K. U., Wermser, C., Stengel, S. T., Modamio, J., Ohlsen, K., Foster, K. R., & Lopez, D. (2014). Evolution of resistance to a last-resort antibiotic in *Staphylococcus aureus* via bacterial competition. *Cell*, *158*(5), 1060–1071. https://doi.org/10.1016/j.cell.2014.06.046

Koeth, L. M., King, A., Knight, H., May, J., Miller, L. A., Phillips, I., & Poupard, J. A. (2000). Comparison of cation-adjusted Mueller – Hinton broth with Iso-Sensitest broth for the NCCLS broth microdilution method. *Journal of Antimicrobial Chemotherapy. 46*(3):369–376

Kov, L. (2017). Infectious Hospital Agents: A Hospital Acquired Infections Spreading Simulation Framework. Acta *Polytechnica Hungarica. 14*(1):95–110

Kramer, A., & Assadian, O. (2011). Paper to Hands: Survival of Bacterial Pathogens on Paper and Bacterial Retrieval from Paper to Hands: Preliminary Results. *American Journal of Nursing. 111*(12):30-34. 10.1097/01.NAJ.0000408181.37017.82

Kulengowski, B., Ribes, J. A., & Burgess, D. S. (2019). Polymyxin B Etest® compared with gold-standard broth microdilution in carbapenem-resistant Enterobacteriaceae exhibiting a wide range of polymyxin B MICs. *Clinical microbiology and infection: the official publication of the European Society of Clinical Microbiology and Infectious Diseases*, *25*(1), 92–95. https://doi.org/10.1016/j.cmi.2018.04.

Kurashige, E. J. O., Oie, S., & Furukawa, H. (2016). Contamination of environmental surfaces by methicillin-resistant Staphylococcus aureu s (MRSA) in rooms of inpatients with MRSA-positive body sites. *Brazilian Journal of Microbiology*. *47*(3):703–705. doi:10.1016/j.bjm.2016.04.002

Labi, A., Obeng-nkrumah, N., Owusu, E., & Bjerrum, S. (2019). Multi-centre point-prevalence survey of hospital- acquired infections in Ghana. *Journal of Hospital Infection*, *101*(1):60–68. doi:10.1016/j.jhin.2018.04.019

Labler, L., & Trentz, O. (2007). energy pelvic trauma The use of vacuum assisted closure (VAC™) in soft tissue injuries after high energy pelvic trauma, *392*, 601–609. doi:10.1007/s00423-

006-0090-0

Lawson, P. A., Latre, M. V, Falsen, E., Vela, A. I., & Ferna, E. (2018). *Streptococcus entericus* sp. nov., isolated from cattle intestine. *International Journal of Systematic and Evolutionary Microbiology*. *52*(2):665-9. doi:10.1099/00207713-52-2-665

Lax, S., Sangwan, N., Smith, D., Larsen, P., Handley, K. M., Richardson, M., Guyton, K., Krezalek, M., Shogan, B. D., Defazio, J., Flemming, I., Shakhsheer, B., Weber, S., Landon, E., Garcia-Houchins, S., Siegel, J., Alverdy, J., Knight, R., Stephens, B., & Gilbert, J. A. (2017). Bacterial colonization and succession in a newly opened hospital. *Science translational medicine*, *9*(391), eaah6500. doi: 10.1126/scitranslmed.aah6500

Lee, C.-M., Yeh, S.-C., Lim, H.-K., Liu, C.-P., & Tseng, H.-K. (2009). High prevalence rate of multidrug resistance among nosocomial pathogens in the respiratory care center of a tertiary hospital. *Journal of Microbiology Immunology and Infection*, *42*(5), 401–404.

Lerner, A., Adler, A., Meitus, I., Carmeli, Y., Aviv, T., & Diagnostics, C. (2013). Environmental Contamination by Carbapenem-Resistant. *Journal of Clinical Microbiology*. *51*(1):177–181. doi:10.1128/JCM.01992-12

Leitão, J. H., Sousa, S. A., Ferreira, A. S., & Moreira, L. M. (2010). Pathogenicity, virulence factors and strategies to fight against *Burkholderia cepacia* complex pathogens and related species. *Appl Microbiol Biotechnol*. *87*(1)31–40. doi:10.1007/s00253-010-2528-0

Leung, L. M., Cooper, V. S., Rasko, D. A., Guo, Q., Pacey, M. P., Mcelheny, C. L., … Doi, Y. (2017). Structural modification of LPS in colistin-resistant, KPC-producing *Klebsiella pneumoniae*. *Journal of Antimicrobial Chemotherapy*. *11*(72):3035–3042. doi:10.1093/jac/dkx234

Leung, M. H. Y., & Lee, P. K. H. (2016). The roles of the outdoors and occupants in contributing to a potential pan-microbiome of the built environment: a review. *Microbiome*. *4*(21):1–15. doi:10.1186/s40168-016-0165-2

Levin, B. R., & Rozen, D. E. (2006). Non-inherited antibiotic resistance. *Nature Reviews*. *4*(7):556-562. doi: 10.1038/nrmicro1445

Levy, S. B., & Marshall, B. (2004). Antibacterial resistance worldwide: causes, challenges and responses. *Nature Reviews*. *10*(12):122–129. doi:10.1038/nm1145

Li, J., Zhou, L., Zhang, X., Xu, C., Dong, L., & Yao, M. (2016). Bioaerosol emissions and detection of airborne antibiotic resistance genes from a wastewater treatment plant. *Atmospheric Environment*, *124*, 404–412. doi:10.1016/j.atmosenv.2015.06.030

Literacka, E., Bedenic, B., Baraniak, A., Fiett, J., Tonkic, M., Jajic-Bencic, I., & Gniadkowski, M. (2009). blaCTX-M genes in *Escherichia coli* strains from Croatian Hospitals are located in new (blaCTX-M-3a) and widely spread (blaCTX-M-3a and blaCTX-M-15) genetic structures. *Antimicrobial agents and chemotherapy*. *53*(4):1630-5. doi: 10.1128/AAC.01431-08

Liu, Y. Y., Wang, Y., Walsh, T. R., Yi, L. X., Zhang, R., Spencer, J., Doi, Y., Tian, G., Dong, B., Huang, X., Yu, L. F., Gu, D., Ren, H., Chen, X., Lv, L., He, D., Zhou, H., Liang, Z., Liu, J. H., & Shen, J. (2016). Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *The Lancet. Infectious diseases*, *16*(2), 161–168. doi:10.1016/S1473-3099(15)00424-7

Lodise, T., Ye, M. J., & Zhao, Q. (2017). Prevalence of Invasive Infections Due to Carbapenem-Resistant Enterobacteriaceae among Adult Patients in U.S. Hospitals. *Antimicrobial agents*

and chemotherapy. *61*(8):e00228-17. doi:10.1128/AAC.00228-17

Louhi, K., Sundberg, L., Jokela, J., & Karvonen, A. (2015). Interactions among bacterial strains and fluke genotypes shape virulence of co-infection. *Proceedings of the Royal Society B: Biological Sciences. 282:*1–8. doi: 10.1098/rspb.2015.2097

dependent on relative humidity and temperature. *PLOS pathog*. *3*(10):1470-6.

Loutet, S. A., Flannagan, R. S., Kooi, C., Sokol, P. A., & Valvano, M. A. (2006). A Complete Lipopolysaccharide Inner Core Oligosaccharide Is Required for Resistance of Burkholderia cenocepacia to Antimicrobial Peptides and Bacterial Survival In Vivo. *Journal of Bacteriology*. *188*(6): 2073–2080. doi:10.1128/JB.188.6.2073

Loutet, S. A., Mussen, L. E., Flannagan, R. S., & Valvano, M. A. (2011). A two-tier model of polymyxin B resistance in Burkholderia cenocepacia. *Environmental Microbiology Reports. 3*:278–285. doi:10.1111/j.1758-2229.2010.00222.x

Loutet, S. A., & Valvano, M. A. (2011). Extreme antimicrobial peptide and polymyxin B resistance in the genus Burkholderia, *Frontiers of Microbiology. 2*(159):1–8. doi:10.3389/fmicb.2011.00159

Lowen, A. C., Mubareka, S., Steel, J., & Palese, P. (2007). Influenza virus transmission is dependent on relative humidity and temperature. *PLoS pathogens, 3*(10), 1470–1476. https://doi.org/10.1371/journal.ppat.0030151

Luyt, C., Bréchot, N., Trouillet, J., & Chastre, J. (2014). Antibiotic stewardship in the intensive care unit. *Critical Care. 18*(480):1–12. doi:10.1186/s13054-014-0480-6

Lye, D. C., Earnest, A., Ling, M. L., Lee, T. E., Yong, H. C., Fisher, D. A., Krishnan, P., & Hsu, L. Y. (2012). The impact of multidrug resistance in healthcare-associated and nosocomial Gram-negative bacteraemia on mortality and length of stay: cohort study. *Clinical*

microbiology and infection: the official publication of the European Society of Clinical Microbiology and Infectious Diseases, *18*(5), 502–508. https://doi.org/10.1111/j.1469-0691.2011.03606.x

Ma, M. F., & Bchir, M. B. (2004). Methicillin-resistant *Staphylococcus aureus* (MRSA) in the UK. *Australian Infection Control*. *9*(1):5-8-11. doi:10.1071/HI04005

Magiorakos, A. P., Srinivasan, A., Carey, R. B., Carmeli, Y., Falagas, M. E., Giske, C. G., Harbarth, S., Hindler, J. F., Kahlmeter, G., Olsson-Liljequist, B., Paterson, D. L., Rice, L. B., Stelling, J., Struelens, M. J., Vatopoulos, A., Weber, J. T., & Monnet, D. L. (2012). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clinical microbiology and infection: the official publication of the European Society of Clinical Microbiology and Infectious Diseases*, *18*(3), 268–281. doi:10.1111/j.1469-0691.2011.03570.x

Maldonado, R. F., Isabel, S., & Valvano, M. A. (2016). Lipopolysaccharide modification in Gram-negative. *FEMS Microbiology Reviews. 40*(4):480–493. doi:10.1093/femsre/fuw007

Mandal, S. & Mandal Shyamapada (2018). Multiple Antibiotic Resistance Indices of Potential Pathogenic Bacteria Isolated from Street, Vended Fruit and Sugarcane Juices, Malda Town, India. *Acta Scientific Pharmaceutical Sciences. 2*(10):89–94.

Maina, S. W., & Sitoni, T. (2012). Ghana loses GHC420 million annually due to poor sanitation. Ghana Sanitiation Coverage. *499*:1–6.

Martín, J., Soraya, L., & Adrian, M. (2013). One-Year Surveillance of ESKAPE Pathogens in an Intensive Care Unit of Monterrey, Mexico. *Chemotherapy. 64460*, 475–481. doi:10.1159/000346352

Martínez, J. L. (2008). Antibiotics and Antibiotic Resistance. Science. *321*(5887):365–368. doi: 10.1126/science.1159483.

Marolda, C. L., Welsh, J., Dafoe, L., & Valvano, M. A. (1990). Genetic Analysis of the 07-Polysaccharide Biosynthesis Region from the Escherichia coli 07 : K1 Strain VW187, *172*(7), 3590–3599.

Masi, M., Barbe, J., & Page, J. (2005). Inhibitors of efflux pumps in Gram-negative bacteria. *Trends in Molecular Medicine. 11*(8). doi:10.1016/j.molmed.2005.06.006

Matsuura, M. (2013). Structural modifications of bacterial lipopolysaccharide that facilitate Gram-negative bacteria evasion of host innate immunity. *Frontiers Immunology. 4*:1–9. doi:10.3389/fimmu.2013.00109

Mcdermott, H., Skally, M., Rourke, J. O., Humphreys, H., & Fitzgerald-hughes, D. (2017). Vancomycin-Resistant Enterococci (VRE) in The Intensive Care Unit in a Nonoutbreak Setting: Identification of Potential Reservoirs and Epidemiological Associations Between Patient and Environmental VRE. *Infection Control and Hospital Epidemiology. 39*(1):1–6. doi:10.1017/ice.2017.248

McOrist, A. L., McOrist, A. L., Jackson, M., Jackson, M., Bird, A. R., & Bird, A. R. (2002). A comparison of five methods for extraction of bacterial DNA from human faecal samples. *Journal of Microbiological Methods*. *50*(2):131–9. doi:10.1016/S0167-7012(02)00018-0

Mehrad, B., Clark, N. M., Zhanel, G. G., & Lynch, J. P. (2015). Antimicrobial resistance in hospital-acquired gram-negative bacterial infections. *Chest*. *147*(5):1413-1421.

Mehta, Y., Gupta, A., Todi, S., Myatra, S. N., & Samaddar, D. P. (2014). Guidelines for prevention of hospital acquired infections. *Indian Journal of Critical Care Medicine: Peer-Reviewed, Official Publication of Indian Society of Critical Care Medicine. 18*(3):149–164.

doi:10.4103/0972-5229.128705

Messerer, M., & Fischer, W. (2017). Investigation of horizontal gene transfer of pathogenicity islands in Escherichia coli using next-generation sequencing. *PLOS Pathog.* 1–17. 10.1371/journal.pone.0179880

Michael, C. A., Dominey-Howes, D., & Labbate, M. (2014). The antimicrobial resistance crisis: causes, consequences, and management. *Frontiers in public health*. *2*:145. doi:10.3389/fpubh.2014.00145

Miller, L. G., & Diep, B. A. (2008). Colonization, Fomites, and Virulence: Rethinking the Pathogenesis of Community-Associated Methicillin-Resistant *Staphylococcus aureus* Infection. *Clinical Infectious Diseases. 46*(5):752-760. doi:10.1086/526773

Morse S.S. (2001) Factors in the Emergence of Infectious Diseases. In: Price-Smith A.T. (eds) Plagues and Politics. Global Issues Series. Palgrave Macmillan, London. doi: 10.1057/9780230524248_2

Moghaddam, M. N., Beidokhti, M. H., Jamehdar, S. A., & Ghahraman, M. (2014). Genetic properties of blaCTX-M and blaPER β-lactamase genes in clinical isolates of Enterobacteriaceae by polymerase chain reaction. *Iranian journal of basic medical sciences*, *17*(5), 378–383.

Moradigaravand, D., Boinett, C. J., Martin, V., Peacock, S. J., & Parkhill, J. (2016). Recent independent emergence of multiple multidrug-resistant *Serratia marcescens* clones within the United Kingdom and Ireland. *Genome Research. 26*(8):1101–1109. doi:10.1101/gr.205245.116

Morales-soto, N., Anyan, M. E., Mattingly, A. E., Madukoma, C. S., Harvey, C. W., Alber, M., … Shrout, J. D. (2015). Preparation, Imaging and Quantification of Bacterial Surface Motility

Assays. *J. Vis. Exp.* (98):e52338. doi:10.3791/52338

Morand, B., & Mühlemann, K. (2007). Heteroresistance to penicillin in *Streptococcus pneumoniae*. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(35), 14098–14103. https://doi.org/10.1073/pnas.0702377104

Motta, S. S., Cluzel, P., & Aldana, M. (2015). Adaptive resistance in bacteria requires epigenetic inheritance, genetic noise, and cost of efflux pumps. *PLOS ONE*. *10*(3):e0118464. doi:10.1371/journal.pone.0118464

Moual, L., & Gruenheid, S. (2012). Resistance to antimicrobial peptides in Gram-negative bacteria. *FEMS Microbiology Letters*. *330*(2):81-89. doi:10.1111/j.1574-6968.2012.02528.x

Naguib, M. M., & Valvano, M. A. (2018). Vitamin E Increases Antimicrobial Sensitivity by Inhibiting Bacterial Lipocalin Antibiotic Binding. *mSphere*. *3*(6):e00564-18. doi:10.1128/mSphere.00564-18

Nale, J. Y., Chutia, M., Carr, P., Hickenbotham, P. T., & Clokie, M. R. (2016). 'Get in Early'; Biofilm and Wax Moth (*Galleria mellonella*) Models Reveal New Insights into the Therapeutic Potential of Clostridium difficile Bacteriophages. *Frontiers in microbiology*. *7*: 1383. doi:10.3389/fmicb.2016.01383

Napoli, C., Marcotrigiano, V., & Montagna, M. T. (2012). Air sampling procedures to evaluate microbial contamination: a comparison between active and passive methods in operating theatres. *BMC Public Health*. *12*(1):1. doi:10.1186/1471-2458-12-594

Narvaez-bravo, C., Taboada, E. N., Mutschall, S. K., & Aslam, M. (2017). retail meats in Canada. *International Journal of Food Microbiology*, *253*:43–47. doi:10.1016/j.ijfoodmicro.2017.04.019

Naylor, N. R., Atun, R., Zhu, N., Kulasabanathan, K., Silva, S., Chatterjee, A., Knight, G. M., &

Robotham, J. V. (2018). Estimating the burden of antimicrobial resistance: a systematic literature review. *Antimicrobial resistance and infection control*, *7*, 58. https://doi.org/10.1186/s13756-018-0336-y

Neely, A. N., & Maley, M. P. (2000). Survival of Enterococci and Staphylococci on Hospital Fabrics and Plastic. *Journal of Clinical Microbiology. 38*(2):724–726.

Newman, M. (2017). Nosocomial and Community Acquired Infections in Korle Bu Teaching Teaching Hospital, Accra. *West African journal of medicine. 28*(5):300-3. doi:10.4314/wajm.v28i5.55005

Newman, M. J., Frimpong, E., Donkor, E. S., Opintan, J. A., & Asamoah-Adu, A. (2011). Resistance to antimicrobial drugs in Ghana. *Infection and drug resistance*, *4*, 215–220. https://doi.org/10.2147/IDR.S21769

Ngonda, F. (2017). Assessment of bacterial contamination of toilets and bathroom doors handle / knobs at Daeyang Luke hospital. *Pharmaceutical and Biological Evaluations*. *4*(4):193–197.

Nicas, M., & Sun, G. (2006). An Integrated Model of Infection Risk in a Health-Care Environment. *Risk Analysis. 26*(4):1085-1096. doi:10.1111/j.1539-6924.2006.00802.x

Nseir, S., Pompeo, C. Di, Diarra, M., & Brisson, H. (2007). Relationship between immunosuppression and intensive care unit-acquired multidrug-resistant bacteria: A case-control study. *Critical Care Medicine*. *35*(5):1318-1323. doi:10.1097/01.CCM.0000261885.50604.20

Nunes, Z. G., Martins, A. S., Altoe, A. L. F., Nishikawa, M. M., Leite, M. O., Aguiar, P. F., & Fracalanzza, S. E. L. (2005). Indoor air microbiological evaluation of offices, hospitals, industries and shopping centers. doi:10.1590/S0074-02762005000400003

Nuñez, L., & Moretton, J. (2007). Disinfectant-Resistant Bacteria in Buenos Aires City Hospital Wastewater. *Brazillian J of Miocrobiol.* 38(4):644–648. 10.1590/S1517-83822007000400012

Nwankwo E. (2012). Isolation of pathogenic bacteria from fomites in the operating rooms of a specialist hospital in Kano, North-western Nigeria. *The Pan African medical journal*. *12*:90.

Odigie, A. B., Ekhiase, F. O., Orjiakor, P. I., & Omozuwa, S. (2017). The Role of Door Handles in the Spread of Microorganisms of Public Health Consequences in University of Benin Teaching Hospital (UBTH), Benin City, Edo State. *Pharmaceutical Science and Technology*. *2*(2), 15–21. doi:10.11648/j.pst.20170202.12

Okamoto, H., Tateda, K., Ishii, Y., Matsumoto, T., & Kobayashi, T. (2002). High frequency of erythromycin A resistance and distribution of *mefE* and *ermB* genes in clinical isolates of Streptococcus pneumoniae in Japan. *Journal of Infection Chemotherapy*. *8*(1):28–32. 10.1007/s101560200002

Olaitan, A. O., Morand, S., & Rolain, J. (2014). Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. *Frontiers in Microbiology*. *5*(643):1–18. doi:10.3389/fmicb.2014.00643

Olaitan, A. O., Morand, S., & Rolain, J. (2015). Emergence of colistin-resistant bacteria in humans without colistin usage: a new worry and cause for vigilance. *International Journal of Antimicrobial Agents*. *47(*1):1-3. doi:10.1016/j.ijantimicag.2015.11.009

Olise, C. C. & Simon-Oke, I. (2018). Fomites: Possible vehicle of nosocomial infections. *J pub health catalog. 1*(1):16-16.

Olowe, O. A., Idris, O. J., & Taiwo, S. S. (2013). Prevalence of tet genes mediating tetracycline resistance in *Escherichia coli* clinical isolates in Osun State, Nigeria. *European journal of*

microbiology & immunology. *3*(2):135-40.

Opintan, J. A., Newman, M. J., Arhin, R. E., Donkor, E. S., Gyansa-Lutterodt, M., & Mills-Pappoe, W. (2015). Laboratory-based nationwide surveillance of antimicrobial resistance in Ghana. *Infection and drug resistance*, *8*, 379–389. https://doi.org/10.2147/IDR.S88725

Orton, R. O. N. (2011). Challenges in the microbiological diagnosis and management of hVISA infections. *Pathology. 43*(4):357–361. doi:10.1097/PAT.0b013e3283464ca3

Osman, S., La Duc, M. T., Dekas, A., Newcombe, D., & Venkateswaran, K. (2008). Microbial burden and diversity of commercial airline cabin air during short and long durations of travel. *The ISME Journal*. *2*(5):482–497. doi:10.1038/ismej.2008.11

Osundiya,O.O., Oladele, R.O. & Oduyebo. O.O. *(2013)*. Multiple Antibiotic Resistance (Mar) Indices of Pseudomonas And Klebsiella Species Isolates in Lagos University Teaching Hospital. *African Journal of Clinical and Experimental Microbiology. 14*(3):164–168.

Pagès, J., Amaral, L., & Fanning, S. (2011). An Original Deal for New Molecule: Reversal of Efflux Pump Activity, A Rational Strategy to Combat Gram-negative Resistant Bacteria. *Current Medicinal Chemistry. 18*(19):2969–2980.

Pasupuleti, M., Schmidtchen, A., & Malmsten, M. (2012). Antimicrobial peptides: key components of the innate immune system. *Critical Reviews in Biotechnology. 32*:143–171. doi:10.3109/07388551.2011.594423

Park, D., Yeom, J., Lee, W. J., & Lee, K. (2013). Assessment of the Levels of Airborne Bacteria, Gram-negative Bacteria, and Fungi in Hospital Lobbies. *International Journal of Environmentaal Research of Public Health*. 10(2):541–555. doi:10.3390/ijerph10020541

Patel, R. (2005). Biofilms and Antimicrobial Resistance. *Clinical Orthopaedics and Related Research*. (437):41–47. doi:10.1097/01.blo.0000175714.68624.74

Patel, S. J., & Saiman, L. (2010). Antibiotic resistance in neonatal intensive care unit pathogens: mechanisms, clinical impact, and prevention including antibiotic stewardship. *Clinics in perinatology*. *37*(3):547-63.

Paterson, D. L., & Bonomo, R. A. (2005). Extended-Spectrum-Lactamases: a Clinical Update. *Clinical Microbiology Reviews. 18*(4):657–686. doi:10.1128/CMR.18.4.657

Peleg, A. Y., Jara, S., Monga, D., Eliopoulos, G. M., Moellering, R. C., & Mylonakis, E. (2009). Galleria mellonella as a Model System to Study *Acinetobacter baumannii* Pathogenesis and Therapeutics. *53*(6):2605–2609. doi:10.1128/AAC.01533-08

Pendleton, J. N., Gorman, S. P., & Gilmore, B. F. (2013). Clinical relevance of the ESKAPE pathogens. *Expert Review of Anti-infective Therapy*. *11*(3):297–308. doi: 10.1586/eri.13.12

Pereira, R. H. V, & Leão, R. S. (2019). Patterns of virulence factor expression and antimicrobial resistance in Achromobacter xylosoxidans and Achromobacter ruhlandii isolates from patients with cystic fibrosis. *Epidemiology and Infection. 145*(3):600-606.doi:10.1017/S0950268816002624

Peters, B. M., Jabra-rizk, M. A., May, G. A. O., Costerton, W., & Shirtliff, M. E. (2012). Polymicrobial Interactions: Impact on Pathogenesis and Resistance. *25*(1). doi:10.1128/CMR.00013-11

Phillips, P. L., & Schultz, G. S. (2012). Molecular Mechanisms of Biofilm Infection: Biofilm Virulence Factors. *Advances in wound care*. *1*(3):109-114. doi: 10.1089/wound.2011.0301

Piddock, L. J. V. (2006). Clinically Relevant Chromosomally Encoded Multidrug Resistance Efflux Pumps in Bacteria. *Clinical Microbiology Reviews. 19*(2):382–402. doi:10.1128/CMR.19.2.382

Pier, G. B. (2007). Pseudomonas aeruginosa lipopolysaccharide: A major virulence factor, initiator

of inflammation and target for effective immunity. *International Journal of Medical Microbiology*. *297*(5):277-295. doi:10.1016/j.ijmm.2007.03.012

Pirrone, M., Pinciroli, R., & Berra, L. (2016). Microbiome, biofilms and pneumonia in the ICU. *Current Opinion in Infectious Diseases.* *29*(2):160-6. doi: 10.1097/QCO.0000000000000255.

Pitout, J. D., Nordmann, P., & Poirel, L. (2015). Carbapenemase-Producing *Klebsiella pneumoniae*, a Key Pathogen Set for Global Nosocomial Dominance. *Antimicrobial agents and chemotherapy*. *59*(10):5873-5884. doi: 10.1128/AAC.01019-15

Plipat, N., Spicknall, I. H., Koopman, J. S., & Eisenberg, J. N. S. (2013). The dynamics of methicillin-resistant Staphylococcus aureus exposure in a hospital model and the potential for environmental intervention *BMC Infectious Disease.* *17*(13):595. doi: 10.1186/1471-2334-13-595.

Poza, M., Gayoso, C., Gómez, M. J., Rumbo-Feal, S., Tomás, M., Aranda, J., Fernández, A., & Bou, G. (2012). Exploring bacterial diversity in hospital environments by GS-FLX Titanium pyrosequencing. *PloS one*, *7*(8), e44105. https://doi.org/10.1371/journal.pone.0044105

Prashanth, K., & Badrinath, S. (2006). Nosocomial infections due to Acinetobacter species: Clinical findings, risk and prognostic factors. *Indian Journal of Medical Microbiology*. *24*(1):39–44. doi:10.4103/0255-0857.19893

Prestinaci, F., Pezzotti, P., & Pantosti, A. (2016). Antimicrobial resistance: a global multifaceted phenomenon. *Pathogens and Global Health*. *109*(7):309–318. doi:10.1179/2047773215Y.0000000030

Price, L. B., Hungate, B. A., Koch, B. J., Davis, G. S., & Liu, C. M. (2017). Colonizing opportunistic pathogens (COPs): The beasts in all of us. *PLOS Pathog*. *13*(8):e1006369.

doi:10.1371/journal.ppat.1006369

Prieto, A. M. G., Schaik, W. Van, Rogers, M. R. C., Coque, T. M., Baquero, F., Corander, J., & Willems, R. J. L. (2016). Global Emergence and Dissemination of Enterococci as Nosocomial Pathogens: Attack of the Clones. *Frontiers in Microbiology*. *7*:1–15. doi:10.3389/fmicb.2016.00788

Pruden, A. (2014). Balancing Water Sustainability and Public Health Goals in the Face of Growing Concerns about Antibiotic Resistance. *Environmental Science Technology*. *48*(1):5-14doi:10.1021/es403883p

Qudiesat, K., Elkarmi, A., Hamad, M., & Abussaud, M. (2009). Assessment of airborne pathogens in healthcare settings. *Journal of Microbiology*. *3*(2), 066–076.

Ramarao, N., Nielsen-leroux, C., & Lereclus, D. (2012). The Insect *Galleria mellonella* as a Powerful Infection Model to Investigate Bacterial Pathogenesis. *Journal of Visualized Experiments.* 1–7. doi:10.3791/4392

Rampelotto, P. H. (2010). Resistance of Microorganisms to Extreme Environmental Conditions and Its Contribution to Astrobiology. *Sustainability*. *2*(6):1602-1623. doi:10.3390/su2061602

Reed, D., & Kemmerly, S. A. (2009). Infection control and prevention: a review of hospital-acquired infections and the economic implications. *The Ochsner journal*. *9*(1), 27-31.

Report, G. (2014). Antimicrobial resistance: Global Report on Surveillance. WHO Report, ISBN:9789241564748, April 2014

Report, S. (2004). Emergence of methicillin resistant Staphylococcus aureus (MRSA) bacteraemia among children in England and Wales, 1990–2001, *139*(6), 378–379. doi:10.1136/adc.2003.028712

Rezania, S., Amirmozaffari, N., Tabarraei, B., & Jeddi-tehrani, M. (2011). Extraction, Purification and Characterization of Lipopolysaccharide from *Escherichia coli* and *Salmonella typhi*. *Avicenna Journal of Medical Biotechnology*. *3*(1):3–9.

Rinder, H. (2001). Heteroresistance: An under-recognised confounder in diagnosis and therapy? *Journal of Medical Microbiology*. *50*(12):1018–1020. doi:10.1099/0022-1317-50-12-1018

Rosadini, C. V, & Kagan, J. C. (2017). ScienceDirect Early innate immune responses to bacterial LPS Charles V Rosadini and Jonathan C Kagan. *Current Opinion in Immunology*. *44*:14–19. doi:10.1016/j.coi.2016.10.005

Rosenfeld, Y., & Shai, Y. (2006). Lipopolysaccharide (Endotoxin)-host defense antibacterial peptides interactions: Role in bacterial resistance and prevention of sepsis. *1758*:1513–1522. doi:10.1016/j.bbamem.2006.05.017

Rossolini, G. M., Arena, F., Pecile, P., & Pollini, S. (2014). ScienceDirect Update on the antibiotic resistance crisis. *Current Opinion in Pharmacology*. *18*:56–60. doi:10.1016/j.coph.2014.09.006

Ruppé, É., Woerther, P. L., & Barbier, F. (2015). Mechanisms of antimicrobial resistance in Gram-negative bacilli. *Annals of intensive care*. *5*(1):61. doi: 10.1186/s13613-015-0061-0

Rusin, P., Maxwell, S., & Gerba, C. (2002). Comparative surface-to-hand and fingertip-to-mouth transfer efficiency of Gram-positive bacteria, gram-negative bacteria and phage. *Journal of Applied Microbiology*. *93*(4):585-92.

Rudkin, J. K., Mcloughlin, R. M., Preston, A., & Massey, R. C. (2017). Bacterial toxins: Offensive, defensive or something else altogether ?. *PLOS Pathog*. *13*(9):1–12.

Russotto, V., Cortegiani, A., Raineri, S. M., & Giarratano, A. (2015). Bacterial contamination of inanimate surfaces and equipment in the intensive care unit. *Journal of Intensive Care*, 1–8.

doi:10.1186/s40560-015-0120-5

Saadoun, I., Ali, I., & Tayyar, A. (2018). Airborne Gram-negative Bacilli in the Indoor Environment of King Abdullah University Hospital, Jordan and Their Antibiotic Susceptibility. *Biomed J Sci & Tech Res. 4*(4), 1–4. doi:10.26717/BJSTR.2018.04.001091

Sabnis, A., Ledger, E., Pader, V., & Edwards, A. M. (2018). Antibiotic interceptors: Creating safe spaces for bacteria. *PLOS pathog. 14*(4):e1006924. doi:10.1371/journal.ppat.1006924

Sahalan, A. Z., Abdul, A. H., Hing, H. L., & Abdul Ghani, M. K. (2013). Divalent Cations (Mg_{2+}, Ca_{2+}) protect bacterial outer membrane damage by polymyxin B. *Sains Malaysiana*. *42*(3):301-306.

Samuel, F. (2015). Original Article Microbiological Assessment of Indoor Air of Teaching Hospital Wards: A Case of Jimma University Specialized Hospital. *Ethiopian Journal of Health Science. 25*(2): 117–122.

Scheckler, W. E., Brimhall, D., Buck, A. S., Farr, B. M., Garibaldi, R. A., Gross, P. A., … Martone, J. (2014). Hospitals: Epidemiology Report. *Infection Control of Hospital Epidemiolology. 19*(2):114-24.

Schlaberg, R., Simmon, K. E., & Fisher, M. A. (2012). A Systematic Approach for Discovering Novel, Clinically Relevant Bacteria. *Emerging Infectious Disease. 18*(3):422 430. doi: 10.3201/eid1803.111481

Schnaitman, C. A., & Klena, J. D. (1993). Genetics of lipopolysaccharide biosynthesis in enteric bacteria. *Microbiological reviews*. *57*(3):655-82.

Schwab, F., Gastmeier, P., & Meyer, E. (2014). The Warmer the Weather, the More Gram-negative Bacteria - Impact of Temperature on Clinical Isolates in Intensive Care Units. *PLOS ONE*. 9(3):e91105. doi:10.1371/journal.pone.0091105

Sengupta, S., Chattopadhyay, M. K., & Grossart, H. P. (2013). The multifaceted roles of antibiotics and antibiotic resistance in nature. *Frontiers in microbiology*. *4*:47. doi:10.3389/fmicb.2013.00047

Sergent, A., Slekovec, C., Pauchot, J., Jeunet, L., Bertrand, X., Hocquet, D., & Talon, D. (2012). Bacterial contamination of the hospital environment during wound dressing change. *Orthopaedics & Traumatology: Surgery & Research*. *98*(4):441–445. doi:10.1016/j.otsr.2012.02.005

Shittu, A., Nu, U., Udo, E., & Lin, J. (2018). Characterization of meticillin-resistant *Staphylococcus aureus* isolates from hospitals in KwaZulu-Natal province, Republic of South Africa. *Journal of Medical Microbiol. 58*(9):1219–1226.doi:10.1099/jmm.0.011452-0

Sievert, D. M., Ricks, P., Edwards, J. R., Schneider, A., Patel, J., Srinivasan, A., Kallen, A., Limbago, B., Fridkin, S., & National Healthcare Safety Network (NHSN) Team and Participating NHSN Facilities (2013). Antimicrobial-resistant pathogens associated with healthcare-associated infections: summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2009-2010. *Infection control and hospital epidemiology*, *34*(1), 1–14. doi:10.1086/668770

Singh, B. R. (2018). ESKAPE Pathogens in Animals and their Antimicrobial Drug Resistance. *Dairy and Vetenary Science Journal. 7*(3):555715. doi:10.19080/JDVS.2018.07.555715

Sit, P. S., Shuan, C., Teh, J., Idris, N., Sam, I., Faridah, S., & Sulaiman, H. (2017). Prevalence of methicillin-resistant Staphylococcus aureus (MRSA) infection and the molecular characteristics of MRSA bacteraemia over a two-year period in a tertiary teaching hospital in Malaysia. *BMC Infect Dis. 17*(274):1–14. doi:10.1186/s12879-017-2384-y

Smith, D., Alverdy, J., An, G., Coleman, M., Garcia-Houchins, S., Green, J., Keegan, K., Kelley,

S. T., Kirkup, B. C., Kociolek, L., Levin, H., Landon, E., Olsiewski, P., Knight, R., Siegel, J., Weber, S., … Gilbert, J. (2013). The Hospital Microbiome Project: Meeting Report for the 1st Hospital Microbiome Project Workshop on sampling design and building science measurements, Chicago, USA, June 7th-8th 2012. *Standards in genomic sciences*. *8*(1):112-7. doi:10.4056/sigs.3717348

Sola, C., Lamberghini, R. O., Ciarlantini, M., Egea, A. L., Gonzalez, P., Diaz, E. G., & Bocco, J. L. (2011). Heterogeneous vancomycin-intermediate susceptibility in a community-associated methicillin-resistant *Staphylococcus aureus* epidemic clone, in a case of Infective Endocarditis in Argentina. *Annals of Clinical Microbiology and Antimicrobials*. *10*(1):15. doi:10.1186/1476-0711-10-15

Solomon, F. B., Wadilo, F. W., Arota, A. A., & Abraham, Y. L. (2017). Antibiotic resistant airborne bacteria and their multidrug resistance pattern at University teaching referral Hospital in South Ethiopia. *Annals of Clinical Microbiology and Antimicrobials*. *16(*1):29. doi:10.1186/S12941-017-0204-2

Spellberg, B., & Gilbert, D. N. (2014). The Future of Antibiotics and Resistance: A Tribute to a Career of Leadership by John Bartlett. *Clinical Infectious Disease.* *59*(2):71–75. doi:10.1093/cid/ciu392

Srinivas, P., & Rivard, K. (2017). Polymyxin Resistance in Gram-negative Pathogens. *Current Infectious Disease Reports.* 19(11):7–9. doi:10.1007/s11908-017-0596-3

Sserwadda, I., Lukenge, M., Mwambi, B., Mboowa, G., Walusimbi, A., & Segujja, F. (2018). Microbial contaminants isolated from items and work surfaces in the post-operative ward at Kawolo general hospital, Uganda. *BMC Infectious Diseases.* *18*(68):6. doi:10.1186/s12879-018-2980-5

Stieglmeier, M., Wirth, R., Kminek, G., & Moissl-eichinger, C. (2009). Cultivation of Anaerobic and Facultatively Anaerobic Bacteria from Spacecraft-Associated Clean Rooms. *Applied and Environmental Microbiology*. *75*(11):3484–3491. doi:10.1128/AEM.02565-08

Stokes, H. W., & Gillings, M. R. (2011). antibiotic resistance genes into Gram-negative pathogens. *FEMS Microbiology Review*. *35*:790–819. doi:10.1111/j.1574-6976.2011.00273.x

Suleyman, G., Alangaden, G., & Bardossy, A. C. (2018). The Role of Environmental Contamination in the Transmission of Nosocomial Pathogens and Healthcare-Associated Infections. *Current Infectious Disease Report*. *20*(6):12. doi: 10.1007/s11908-018-0620-2.

Sun, J., Deng, Z., & Yan, A. (2014). Bacterial multidrug efflux pumps: Mechanisms, physiology and pharmacological exploitations. *Biochemical and Biophysical Research Communications*. *453*(2):254–267. doi:10.1016/j.bbrc.2014.05.090

Sydnor, E. R., & Perl, T. M. (2011). Hospital epidemiology and infection control in acute-care settings. *Clinical microbiology reviews*. *24*(1):141-73.

Tacconelli, E., Carrara, E., Savoldi, A., Kattula, D., & Burkert, F. (2017). Global Priority List of Antibiotic-Resistant Bacteria To Guide Research, Discovery, And Development of New Antibiotics. Retrieved from http://www.cdc.gov/drugresistance/threat-report-2013/

Tadesse, G., Tessema, T. S., Beyene, G., & Aseffa, A. (2018). Molecular epidemiology of fluoroquinolone resistant *Salmonella* in Africa: A systematic review and meta-analysis. *PLOS ONE*. *13*(2):e0192575. doi:10.1371/journal.pone.0192575

Tagoe, D. N. a, Baidoo, S., Dadzie, I., Tengey, D., & Agede, C. (2011). Potential sources of transmission of hospital acquired infections in the volta regional hospital in ghana. *Ghana Medical Journal*. *45*(1):22–26. doi:10.4314/gmj.v45i1.68918

Taylor, P., & Ramachandran, G. (2014). Gram-positive and Gram-negative bacterial toxins in

sepsis A brief review. *Virulence. 5*(1):37–41. doi:10.4161/viru.27024

Toole, G. A. O. (2011). Microtiter Dish Biofilm Formation Assay. *Journal of Visualized Experiments.* 10–11. doi:10.3791/2437

Toussaint, A., & Merlin, C. (2002). Mobile Elements as a Combination of Functional Modules. *Plasmid. 47*(1):26–35. doi:10.1006/plas.2001.1552

Tran, G. M., Ho-le, T. P., Ha, D. T., Tran-nguyen, C. H., Nguyen, T. S. M., Pham, T. T. N., & Nguyen, T. V. (2017). Patterns of antimicrobial resistance in intensive care unit patients : a study in Vietnam. *BMC Infectious Disease. 17*(1):429 doi:10.1186/s12879-017-2529-z

Tsai, C. J., Mei, J., Loh, S., & Proft, T. (2016). *Galleria mellonella* infection models for the study of bacterial diseases and for antimicrobial drug testing. *Virulence*. *7*(3), 214–229. doi:10.1080/21505594.2015.1135289

Tumbarello, M., Trecarichi, E. M., Fiori, B., Losito, A. R., Inzeo, D., Campana, L., & Fadda, G. (2012). Multidrug-Resistant Proteus Mirabilis Bloodstream Infections: Risk Factors and Outcomes. *Antimicrobial Agents and Chemotherapy. 56*(6):3224-3231. doi:10.1128/AAC.05966-11

Umar, D., Basheer, B., Husain, A., Baroudi, K., Ahamed, F., & Kumar, A. (2015). Evaluation of Bacterial Contamination in a Clinical Environment, *Journal International Oral Health. 7*(1): 53–55.

Utt, E., & Wells, C. (2016). The global response to the threat of antimicrobial resistance and the important role of vaccines. *Pharmaceuticals Policy and Law. 18*:179–197. doi:10.3233/PPL-160442

Vadivoo, N. S., & Usha, B. (2018). ESKAPE pathogens: Trends in antibiotic resistance pattern. *International Journal of Microbiology. 7*(3):26-32. doi:10.26611/1008732

Vaez, H., Faghri, J., Isfahani, B. N., Moghim, S., Yadegari, S., & Fazeli, H. (2019). Efflux pump regulatory genes mutations in multidrug resistance *Pseudomonas aeruginosa* isolated from wound infections in Isfahan hospitals. *Advancement in Biomedical Research. 3*:117. doi:10.4103/2277-9175.133183

Vanhems, P., Barrat, A., Cattuto, C., Pinton, J. F., Khanafer, N., Régis, C., Kim, B. A., Comte, B., & Voirin, N. (2013). Estimating potential infection transmission routes in hospital wards using wearable proximity sensors. *PLOS ONE. 8*(9):e73970. doi:10.1371/journal.pone.0073970

Verónica Mata-Haro, Mónica Reséndiz-Sandoval & Jesús Hernández (2014). *In vitro* differential modulation of immune response by probiotics in porcine peripheral blood mononuclear cells. *Food and Agricultural Immunology.* 25:2, 209-219. doi: 10.1080/09540105.2013.768962

Ventola, C. L. (2015). The Antibiotic Resistance Crisis Part 1: Causes and Threats. *Pharmacy and Therapeutics*. *40*(4), 277–283.

Vickery, K., Deva, A., Jacombs, A., Allan, J., Valente, P., & Gosbell, I. B. (2012). Presence of biofilm containing viable multiresistant organisms despite terminal cleaning on clinical surfaces in an intensive care unit. *Journal of Hospital Infection*. *80*(1), 52–55. doi:10.1016/j.jhin.2011.07.007

Vouga, M., & Greub, G. (2016). Emerging bacterial pathogens: the past and beyond. *Clinical Microbiology and Infection*. *22*(1):12–21. doi:10.1016/j.cmi.2015.10.010

Wang, G., Mishra, B., Lau, K., Lushnikova, T., Golla, R., & Wang, X. (2015). Antimicrobial peptides in 2014. *Pharmaceuticals (Basel, Switzerland)*. *8*(1):123-50. doi:10.3390/ph8010123

Warwick, C., & Corning, S. (2013). Managing patients for zoonotic disease in hospitals. *JRSM Short Rep*ort. 1–9. doi:10.1177/2042533313490287

Weber, D. J., & Rutala, W. A. (2001). Risks and Prevention of Nosocomial Transmission of Rare Zoonotic Diseases. *Clinical Infectious Disease. 32*(3):446-56.

Wecke, T., & Mascher, T. (2011). Antibiotic research in the age of omics: from expression profiles to interspecies communication. *Journal of Antimicrobial Chemotherapy. 66*(12):2689–2704. doi:10.1093/jac/dkr373

WHO. (2004). Microbial fact sheets. *World Health Organization Guidelines for Drinking- ...*, 221–296. Retrieved from, WHO Report, page accessed in 2019 http://scholar.google.com/scholar?hl=en&btnG=Search&q=intitle:Microbial+fact+sheets#0

WHO. (2012). WHO HIV Drug Resistance Report 2012, 84. Site visited in 2019

Wielders, C. L., Fluit, A. C., Brisse, S., Verhoef, J., & Schmitz, F. J. (2002). mecA gene is widely disseminated in *Staphylococcus aureus* population. *Journal of clinical microbiology*. *40*(11):3970-3975.

Wilson, J. W., Schurr, M. J., LeBlanc, C. L., Ramamurthy, R., Buchanan, K. L., & Nickerson, C. (2002). Mechanisms of bacterial pathogenicity. *Postgraduate Medical Journal*. *78*(918): 216-224. do:10.1136/pmj.78.918.216

Wojda, I. (2016). Immunity of the greater wax moth Galleria mellonella. *Insect Science. 24*(3):1–16. doi:10.1111/1744-7917.12325

Wojgani, H., Kehsa, C., Cloutman-green, E., Gray, C., Gant, V., & Klein, N. (2012). Hospital Door Handle Design and Their Contamination with Bacteria: A Real Life Observational Study. Are We Pulling against Closed Doors ? *PLOS ONE. 7*(10):e40171. doi:10.1371/journal.pone.0040171

Wright, G. D., (2010).Antibiotic resistance in the environment: a link to the clinic?.*Current Opinion in Microbiology*. *13*(5):589-594. doi: 10.1016/j.mib.2010.08.005.

Xiao, S., Li, Y., Wong, T., & Hui, D. S. C. (2017). Role of fomites in SARS transmission during the largest hospital outbreak in Hong Kong. *PLOS ONE*. *12*(7):e0181558.

Yadav, J., Kumar, A., Mahor, P., Kumar, P., Yadav, H., & Kumar, P. (2015). Distribution of airborne microbes and antibiotic susceptibility pattern of bacteria during Gwalior trade fair, Central India. *Journal of the Formosan Medical Association*. *114*(7):639–646. doi:10.1016/j.jfma.2013.04.006

Yagoub, O. S., & El Agbash, A. (2010). Isolation of Potential Pathogenic Bacteria from the Air of Hospital-Delivery and Nursing Rooms. *Journal of Applied Sciences*. *10*(11):1011–1014. doi:10.3923/jas.2010.1011.1014

Yallew, W. W., Kumie, A., & Yehuala, F. M. (2017). Risk factors for hospital-acquired infections in teaching hospitals of Amhara regional state , Ethiopia : A matched-case control study, 1–11.

Yang, H., Chen, G., Cheng, J., Liu, Y., Hu, L., Ye, Y., & Li, J. (2014). Discovery of a fluoroquinolone-resistant *Serratia marcescens* clinical isolate without quinolone resistance-determining region mutations. *Annals of laboratory medicine*. *34*(6):487-488.

Yang, C., Hsu, P., Chang, H., Cheng, C., & Lee, M. (2013). Clinical significance and outcomes of *Clostridium perfringens* bacteremia — a 10-year experience at a tertiary care hospital. *International Journal of Infectious Diseases*, *17*(11):955–960. doi:10.1016/j.ijid.2013.03.001

Yevutsey, S. K., Buabeng, K. O., Aikins, M., Anto, B. P., Biritwum, R. B., Frimodt-Møller, N., & Gyansa-Lutterodt, M. (2017). Situational analysis of antibiotic use and resistance in Ghana: policy and regulation. *BMC public health*. *17*(1):896. doi:10.1186/s12889-017-4910-7

Yassin, M. F., & Almouqatea, S. (2010). Assessment of airborne bacteria and fungi in an indoor and outdoor environment. *International Journal of Environmental Science & Technology*. *7*(3):535–544. 10.1007/BF03326162

Yu, Z., Qin, W., Lin, J., Fang, S., & Qiu, J. (2015). Antibacterial Mechanisms of Polymyxin and Bacterial Resistance. *BioMed Research International. 2015*:11 doi: 10.1155/2015/679109

Zapka, C. A., Campbell, E. J., Maxwell, S. L., Gerba, C. P., Dolan, M. J., Arbogast, J. W., & Macinga, D. R. (2011). Bacterial Hand Contamination and Transfer after Use of Contaminated Bulk-Soap-Refillable Dispensers. *Soil, Water and Environmental Science*. *77*(9):2898–2904. doi:10.1128/AEM.02632-10

Zavascki, A. P., Goldani, L. Z., Li, J., & Nation, R. L. (2007). Polymyxin B for the treatment of multidrug-resistant pathogens: a critical review. *Journal of Antimicrobial Chemotherapy*. *60*:1206–1215. doi:10.1093/jac/dkm357

Zemouri, C., Soet, H. De, Crielaard, W., & Laheij, A. (2017). A scoping review on bio-aerosols in healthcare and the dental environment. *PLOS ONE*. *12*(5):e0178007. doi: 10.1371/journal.pone.0178007.

Zhang, L., Dhillon, P., Yan, H., Farmer, S., & Hancock, R. E. (2000). Interactions of bacterial cationic peptide antibiotics with outer and cytoplasmic membranes of *Pseudomonas aeruginosa*. *Antimicrobial agents and chemotherapy*. *44*(12):3317-21.

Zhang, T., Wang, C. G., Lv, J. C., Wang, R. S., & Zhong, X. H. (2010). Survey on tetracycline resistance and antibiotic-resistant genotype of avian *Escherichia coli* in North China. *Poultry Science*. 91(11) 2774–2777.

Zhao, J., Chen, Z., Chen, S., Deng, Y., Liu, Y., Tian, W., Huang, X., Wu, C., Sun, Y., Sun, Y., Zeng, Z., … Liu, J. H. (2010). Prevalence and dissemination of oqxAB in *Escherichia coli*

isolates from animals, farmworkers, and the environment. *Antimicrobial agents and chemotherapy*. *54*(10):4219-24. doi: 10.1128/AAC.00139-10

Zheng, J. X., Wu, Y., Lin, Z. W., Pu, Z. Y., Yao, W. M., Chen, Z., Li, D. Y., Deng, Q. W., Qu, D., & Yu, Z. J. (2017). Characteristics of and Virulence Factors Associated with Biofilm Formation in Clinical *Enterococcus faecalis* Isolates in China. *Frontiers in Microbiology*. *8*:2338. doi:10.3389/fmicb.2017.02338

GENERAL APPENDIX
Appendix I
A. Table 1: Specific Cultural and Morphological Characteristics of Identified Strains

Category	Cultural and Morphological Characteristics									Probable Genus
	Gram Reaction	Cell Shape	Size	Sporulation	Opacity	Elevation	Surface	Edge	Lactose	
Group A	Positive	Cocci	Large/large colony	Non-spore former	Opaque	Convex	Smooth	Rounded	Positive	Staphylococcus
Group B	Positive	Cocci	Small/large colony	Non-spore former	Opaque/ Translucent	Convex	Flat/Smooth	Rounded/ Pointed	Negative	Streptococcus
Group C	Positive	Rod	Large colony	Spore former	Translucent	Slightly convex	Rough/dry	Irregular	Negative	Bacillus
Group D	Positive	Cocci	Small/large colony	Non-spore former	Opaque/ Translucent	Slightly convex	Smooth	Rounded	Positive	Enterococcus
Group E	Positive		Large colony	Spore former	Translucent	No elevation (flat colonies)	Flat/Filamentous	Rounded	Positive	Clostridium
Group F	Negative	Rod	Large	Non-spore former	Opaque	Slightly convex	Flat/mucoid	Entire	Negative	Acinteobacter
Group G	Negative	Rod	Large	Non-spore former	Opaque	Convex	Curve/filamentous	Rounded/ Pointed	Negative	Acetobacter
Group H	Negative	Rod	Large	Non-spore former	Opaque	Varies	Curved	undefined	Negative	Campylobacter
Group I	Negative	Rod	Large	Non-spore former	Opaque	Convex	Smooth/shiny	Rounded/ Entire	Positive	Citrobacter
Group J	Negative	Rod	Large	Non-spore former	Opaque	Convex	Smooth/shiny	Rounded/ Entire	Positive	Enterobacter
Group K	Negative	Rod	Small	Non-spore former	Translucent	Raised	Mucoid/smooth	Entire	Positive	*Escherichia coli*
Group L	Negative	Rod	Large	Non-spore former	Opaque	Rounded convex	Shiny/mucoid	Undulate	Positive	Klebsiella
Group M	Negative	Rod	Small	Non-spore former	Transparent	Convex	Flat/filamentous	Irregular	Positive	Proteus
Group N	Negative	Rod	Large	Non-spore former	Transparent	Umbonate	Shiny/mucoid	Wavy	Negative	Pseudomonas
Group X	Negative	Rod	Small	Non-spore former	Translucent	Convex	Smooth	Rounded/ Entire	Negative	Salmonella
Group Y	Negative	Rod	Small	Non-spore former	Opaque	Convex	Umbonate	Entire	Positive	Serratia

B. Table 2: Biochemical Profiles of Isolated and Identified Strains

Gram Positive	Urease/ Catalase	NO_3 Red.	Citrate	Motility	Coagulase	Starch hydrolysis	Methyl Red	Voges-Proskeur	Oxidase Test	Probable genus
Group A	-/+	+	+	-	+	+	+	+	-	**Staphylococcus**
Group B	+/-	+	V	-	V	+	v	-	v	**Streptococcus**
Group C	-/+	+	+	+	V	+	+	+	v	**Bacillus**
Group D	-/-	+	+	-	-	+	+	+	-	**Enterococcus**
Group E	v/-	+	V	-	-	+	-	-	-	**Clostridium**
Gram Negative	**Urease/ Catalase**	**NO_3 Red.**	**Citrate**	**Motility**	**Indole**	**H_2S**	**Methyl Red**	**Voges-Proskeur**	**Oxidase Test**	**Probable genus**
Group F	v/+	-	+	**v**	-	-	-	-	v	**Acinetobacter**
Group G	+/+	+	-	**v**	-	-	+	-	-	**Acetobacter**
Group H	+/+	+	-	+	+	-	+	-	+	**Campylobacter**
Group I	-/+	+	+	+	+	+	+	-	-	**Citrobacter**
Group J	+/+	+	+	+	+	-	-	+	-	**Enterobacter**
Group K	-/+	+	-	+	V	-	+	-	-	***Escherichia coli***
Group L	+/+	+	+	**v**	-	-	v	-	-	**Klebsiella**
Group M	+/+	+	V	+	-	+	-	-	-	**Proteus**
Group N	-/+	-	+	+	V	V	-	-	+	**Pseudomonas**
Group X	-/+	+	+	+	-	+	-	-	-	**Salmonella**
Group Y	-/+	V	+	+	**V**	-	v	+	v	**Serratia**

C. Table 3: Carbohydrate Fermentation Profiles of Isolated and Identified Strains

Category	Glucose		Mannose		Fructose		Maltose		Galactose		Arabinose		Xylose		Sucrose		Sorbitol		Probable genus
	C	G	C	G	C	G	C	G	C	G	C	G	C	G	C	G	C	G	
Group A	+	A	+	A	+	A	+	A	+/-	A	+	A	+	A	+	A	V	A	**Staphylococcus**
Group B	+	A	+	A	+	-	-	-	-	-	+	A	+	-	+	A	+	+	**Streptococcus**
Group C	+	A	+	A	+	A	+	A	V	v	+	A	-	A	+	A	+	A	**Bacillus**
Group D	+	A	-	-	+	+	+	+	-	-	-	-	-	-	+	V	+	A	**Enterococcus**
Group E	+	A	-	-	+	+	+	+	-	-	-	-	-	-	v	V	-	-	**Clostridium**
Group F	+	A	+	A	-	-	v	V	+	A	V	V	+	A	-	-	+	v	**Acinetobacter**
Group G	+	A	-	-	+	A	+	A	V	v	-	-	+	A	+	A	-	-	**Acetobacter**
Group H	+	A	-	-	+	+	+	+	+	A	-	-	-	-	+	A	-	-	**Campylobacter**
Group I	+	A	+	V	+	A	+	A	-	-	+	A	-	-	+	A-	V	v	**Citrobacter**
Group J	+	A	+	A	+	A	+	A	+	v	+	A	+	A	+	A	-	-	**Enterobacter**
Group K	+	A	+	A	+	A	+	A	+	A	+	A	+	A	+	A	-	-	***Escherichia coli***
Group L	+	A	V	V	+	A	v	A	+	A	V	A	+	A	v	A	V	v	**Klebsiella**
Group M	-	-	V	V	+	A	+	A	-	-	+	A	+	A	+	A	+	v	**Proteus**
Group N	+	A	+	A	-	-	+	A	+	A			+	A	-	-	+	A	**Pseudomonas**
Group X	+	A	+	A	+	A	+	A	+	A	+	A	-	-	-	-	V	v	**Salmonella**
Group Y	+	A	+	A	+	A	+	A	+	A	+	A	+	A	+	A	+	A	**Serratia**

D. Table 4: Diversity of bacterial strains from Indoor and Outdoor air of hospital environment

Sample location	Indoor Air				Outdoor Air				Temp ^{0}C (Indoor/Outdoor)
	Nº/samples collected	Nº/strains identified	CR(CFUm^{-3})	Strains identified	Nº/samples collected	Nº/strains identified	CR(CFUm^{-3})	Strains identified	
NICU	**15**	**32**			**7**	**13**			
Location A	7	12	3.9x10^2	$^{\sigma}$*Bacillus* spp., *Streptococcus pneumoniae*, *Enterococcus faceium*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *E. coli*	4	8	2.6x10^2	$^{\sigma}$*Bacillus* spp., *Escherichia coli* *Streptococcus pneumoniae* *Enterococcus faecium* *Staphylococcus aureus*, *Pseudomonas. aeruginosa*, *Acetobacter aceti*	25^{Indoor}/37^{outdoor}
Location B	5	11	2.1x10^2		3	5	2.1x10^2		27 ^{Indoor}/32^{outdoor}
Location C	3	9	1.8x10^2		-	-	-		25-32^{Indoor}
ICU	**22**	**53**			**11**	**16**			
Location A	11	24	4.3x10^2	$^{\sigma}$*Bacillus* spp., *Streptococcus pneumoniae*, *S. durans*, *Enterococcus faecium*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Citrobacter freundii*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Escherichia coli*, *Clostridium perferinges*	2	4	1.3x10^2	$^{\sigma}$*Bacillus* spp., *Escherichia coli* *Streptococcus pneumoniae* *Enterococcus faecium*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*,	25 ^{outdoor}/41^{outdoor}
Location B	5	16	2.2x10^2		3	6	2.7x10^2		27 ^{Indoor}/35^{outdoor}
Location C	6	13	3.0x10^2		6	6	2.9x10^2		28^{Indoor}/30^{outdoor}
Surgical Room	**4**	**9**	1.8x10^2	*B.* cereus, *B. subtilis*, *E. faecalis*, *E. coli*, *S. aureus*, *P. aeruginosa*	-	-	-		18-20^{Indoor}
Waiting Room	**7**	**10**	2.8x10^2	*B. subtilis*, *E. coli*, *S. aureus*, *P. aeruginosa*,	-	-	-		25-27^{Indoor}
Maternity Department	**5**	**11**	2.5x10^2	*B. subtilis*, *E. faecalis*, *E. coli*, *S. aureus*, *P. aeruginosa S. pneumoniae*	-	-	-		25-28^{Indoor}

$^{\sigma}$*Bacillus* spp: *cereus, subtilis, thuringiensis, atrophaeus, manliponensis, abyssalis;* $p < 0.05$ (Samples relative to the strains identified)

E. Table 5: Diversity of bacterial strains from hospital fomites

Location	Fomites	N°/samples collected	N°/strains identified	CR(CFUm^{-3})	Strains Identified
NICU		**47***	**93**		
Location A		**24***	**52**		
	Faucet	2	9	3.2x10^2	*Bacillus* cereus, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Citrobacter freundii*, *Enterobacter* cloacae, *Klebsiella oxytoca*, *E. coli*
	Tablets	1	4	1.8x10^2	*B. subtilis*, *B. cereus*, *E. coli*, *S. aureus*, *P. aeruginosa*
	Room Handles	2	11	3.9x10^2	*Bacillus* cereus, *Streptococcus pyogenes*, *S. entericus*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Citrobacter freundii*, *Enterobacter* cowanii, *E. cloacae*, *E. taylore*, *Klebsiella oxytoca*, *Proteus mirabilis*, *E. coli*, *Salmonella enterica*
	Toilets i.) Seats ii.) Sinks iii.) Handles	9	12	4.1x10^2	
	Sinks	2	4	2.1x10^2	*B.* cereus, *B. subtilis*, *E. faecalis*, *E. coli*, *S. aureus*, *P. aeruginosa*, *Citrobacter freundii*,
	Beddings	2	7	1.3x10^2	*B. subtilis*, *E. faecalis*, *E. coli*, *S. aureus*, *P. aeruginosa*, *Citrobacter freundii*
	Waste-Bin	2	5	1.1x10^2	*B. cereus*, *E. coli*, *S. aureus*, *P. aeruginosa*, *Acinetobacter baumanni*
Location B		**23***	**44**		
	Faucet	2	10	3.2x10^2	*Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Citrobacter freundii*, *Enterobacter* cloacae, *Klebsiella pneumoniae*, *E. coli*
	Tablets	1	4	1.3x10^2	*B. cereus*, *E. coli*, *S. aureus*, *P. aeruginosa*
	Room Handles	2	12	4.7x10^2	*Bacillus* cereus, *B. subtilis*, *Streptococcus entericus*, *Enterococcus faecalis*., *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Citrobacter freundii*, *Enterobacter* spp., *Klebsiella* pneumoniae, *K. oxytoca*, *Proteus mirabilis*, *E. coli*
	Toilets i.) Seats ii.) Sinks iii.) Handles	8	7	3.1x10^2	
	Sinks	2	3	1.0x10^2	*Enterococcus faecium*, *E. coli*, *S. aureus*, *P. aeruginosa*, *Klebsiella pneumoniae*, *Citrobacter freundii*, *Salmonella* spp.

	Beddings	2	5	$1.7x10^2$	*B. subtilis, E. faecalis, S. aureus, S. pneumoniae, Citrobacter freundii, E. coli, P. aeruginosa, Acinetobacter baumanni*
	Waste-Bin	2	3	$1.0x10^2$	
ICU		**72***	**137**		
Location A		**25***	**48**		
	Faucet	3	9	$3.0x10^2$	*E. coli, Staphylococcus aureus, Pseudomonas aeruginosa, Citrobacter freundii, Enterobacter cloacae, Klebsiella pneumoniae*
	Tablets	2	5	$1.1x10^2$	*Escherichia coli, Bacillus cereus, Staphylococcus aureus, Pseudomonas aeruginosa,*
	Room Handles	3	11	$4.5x10^2$	*Enterobacter cloacae, E. aerogenes, E. cowanii, E. cloacae complex, Klebsiella pneumoniae, K. oxytoca K. variicola, E. coli, Pseudomonas aeruginosa, P. alcaligen, Citrobacter freundii, Serratia marcescens, S. marcescens subsp., Proteus mirabilis, P. vulgaris, Acinetobacter baumanni, Campylobacter entiritis, Salmonella enterica, Acetobacter aceti, #Bacillus* spp., *E. faecalis, S. aureus*
	Toilets i.) Seats ii.) Sinks iii.) Handles	10	9	$3.6x10^2$	
	Sinks	2	6	$1.4x10^2$	*P. aeruginosa, Enterobacter cloacae, Klebsiella oxytoca, Citrobacter freundii, Enterococcus faecium, E. coli, S. aureus, Salmonella enterica*
	Beddings	3	5	$1.7x10^2$	*Acinetobacter baumanni, Enterobacter cloacae B. subtilis, E. faecalis, S. aureus, S. pneumoniae, Citrobacter freundii, E. coli, P. aeruginosa,*
	Waste-Bin	2	3	$1.3x10^2$	
Location B		**22***	**36**		
	Faucet	2	6	$2.4x10^2$	*Citrobacter freundii, Enterobacter* cloacae, *Klebsiella pneumoniae, E. coli*
	Tablets	2	4	$1.0x10^2$	*Staphylococcus aureus, Bacillus subtilis, Escherichia coli*
	Room Handles	3	9	$4.0x10^2$	*Enterobacter cloacae, E. aerogenes, E. cloacae complex, Klebsiella pneumoniae, K. oxytoca E. coli, Pseudomonas aeruginosa, P. alcaligens, Citrobacter freundii, Serratia marcescens, S. marcescens subsp., Proteus mirabilis, Acinetobacter baumanni, Campylobacter enteritis, Salmonella enterica, Bacillus cereus, E. faecalis, S. aureus*
	Toilets i.) Seats ii.) Sinks iii.) Handles	9	7	$3.8x10^2$	

	Sinks	2	3	$1.1x10^2$	*Salmonella enterica, P. aeruginosa, Enterobacter cloacae, Citrobacter freundii, Enterococcus faecium, E. coli, S. aureus,*
	Beddings	2	3	$1.1x10^2$	*Acinetobacter baumanni, Enterobacter cloacae, B. subtilis, E. faecalis,*
	Waste-Bin	2	4	$0.9x10^2$	*Staphylococcus aureus, S. pneumoniae, Citrobacter freundii, E. coli, S. aureus, P. aeruginosa*
Location C		**27***	**53**		
	Faucet	3	10	$3.4x10^2$	*Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Citrobacter freundii, Enterobacter* cloacae *Klebsiella pneumoniae*
	Tablets	2	5	$1.0x10^2$	*Escherichia coli, Bacillus cereus, Staphylococcus aureus, Pseudomonas aeruginosa,*
	Room Handles	3	12	$4.2x10^2$	*Enterobacter cloacae, E. aerogenes, E. cowanii, E. cloacae complex, Klebsiella pneumoniae, K. oxytoca, K. variicola, E. coli, Pseudomonas aeruginosa, P. alcaligens, Citrobacter freundii, Serratia marcescens, S. marscenses subsp., Proteus mirabilis, P. vulgaris, Acinetobacter baumanni, Campylobacter enteritis, Salmonella enterica, Acetobacter aceti, Bacillus cereus, B. subtilis, E. faecalis, S. aureus*
	Toilets i.) Seats ii.) Sinks iii.) Handles	12	13	$4.8x10^2$	
	Sinks	3	6	$1.0x10^2$	*P. aeruginosa, Enterobacter cloacae, Klebsiella* oxytoca, *Citrobacter freundii Enterococcus faecium, E. coli, S. aureus, Salmonella enterica*
	Beddings	2	4	$1.3x10^2$	*Citrobacter freundii, E. coli, S. aureus, P. aeruginosa, Acinetobacter baumanni, Enterobacter cloacae B. subtilis, E. faecalis, S. pneumoniae, Bacillus cereus*
	Waste-Bin	2	3	$1.0x10^2$	
Maternity Department		**9***	**14**		
	Faucet	2	4	$1.7x10^2$	*Escherichia coli, Bacillus cereus, Staphylococcus aureus, Pseudomonas aeruginosa*
	Tablets	1	2	$1.0x10^2$	*Bacillus cereus, Staphylococcus aureus, Escherichia coli,*
	Room Handles	2	5	$2.1x10^2$	*Acinetobacter baumanni, Enterobacter cloacae B. subtilis, E. faecalis, S. aureus, S. pneumoniae, Bacillus cereus, Citrobacter freundii, E. coli, P. aeruginosa,*
	Sinks	2	2	$0.7x10^2$	*Staphylococcus aureus, P. aeruginosa, Klebsiella pneumoniae, Citrobacter freundii,*
	Waste-Bin	2	1	$0.9x10^2$	Streptococcus *pneumoniae, Bacillus cereus, Staphylococcus aureus, E. coli*
Waiting Room	Tablets/chairs	**5***	**8**	$2.7x10^2$	*Citrobacter freundii, E. coli, S. aureus, P. aeruginosa, B. subtilis, E. faecalis, S. aureus, S. pneumoniae*

***** - Total samples from the same location pooled together; $p < 0.05$ (Samples relative to the strains identified)

F. Table 6: Bacteria Growth at Different Salinity

Strains	Growth at different salt concentrations (%)			
	25	30	35	40
ENAB1	1.152	0.960	0.990	1.050
ENAB2	0.780	0.479	0.516	0.530
PGAB1	0.853	0.661	0.729	0.712
PGAB2	0.943	0.645	0.716	0.683
CTAB1	0.296	0.445	0.315	0.229
CTAB2	0.836	0.584	0.670	0.565
KBAB1	0.939	0.583	0.670	0.573
KBAB2	0.963	0.500	0.219	0.547
SRAB1	0.739	0.673	0.450	0.333
SRAB2	0.679	0.713	0.598	0.463
ACNAB1	1.048	0.797	0.474	0.798
ACNAB2	0.633	0.523	0.470	0.5227
ECAB01	0.836	0.584	0.670	0.565
ECAB02	0.956	0.684	0.670	0.505
PRMAB1	0.268	0.492	0.651	0.368
PRMAB2	0.441	0.397	0.234	0.212
ENTAB1	1.036	0.684	0.470	0.365
ENTAB2	0.753	0.583	0.882	0.578
STAB1	0.895	0.524	0.486	0.580
STAB2	0.268	0.438	0.651	0.213

G. SWARM MOTILITY

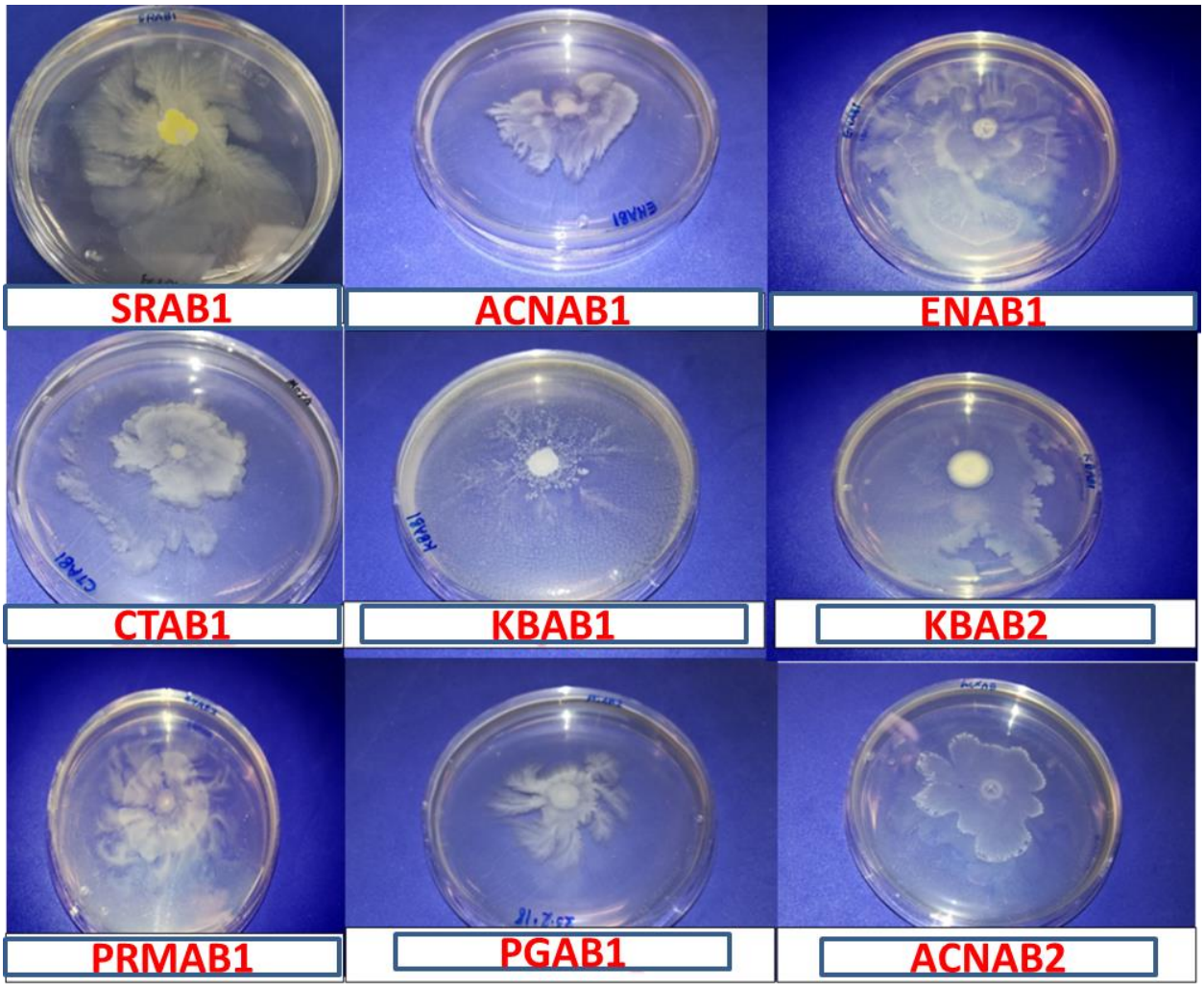


Figure 1: Advanced swarm motility bacterial phenotypes after 96 hours at 37^0C

H. QUALITATIVE BIOFILM

Figure 2: Crystal violet biofilm forming assay A – weak biofilm producer strains, B – moderate producer, C – strong producer

Appendix II

A. Table 1: Antimicrobial profiles of Gram-negative bacterial strains indicating the zone of inhibitions

R – Resistance = +, growth (≤5 mm), S – Susceptible = -, growth inhibition (10-25 mm), H^R – unclear zone of inhibition, *E. coli*

Antibiotics (ug)	Resistance Profiles (Zone of inhibition, mm)															
	ENAB1	ENAB2	KBAB1	KBAB2	PGAB2	PGAB2	CTAB1	CTAB2	SRAB1	SRAB2	PRMAB1	PRMAB2	ACNAB1	ACNAB2	ECAB01	ECAB02
Flucloxacillin (5)	+	+	+	+	+	+	H^R	+	+	+	+	+	+	H^R	+	+
Erythromycin (5)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cloxacillin (5)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ceftriaxone (30)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cotrimoxazole (25)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nitrofurantoin (200)	+	+	+	+	+	+	+	+	+	+	H^R	+	+	+	+	+
Chloramphenl (30)	+	+	+	+	H^R	+	+	+	+	+	+	+	+	+	+	+
Tetracycline (10)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cefotaxime (10)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cefuroxime (30)	+	+	+	+	+	+	+	+	H^R	+	+	+	+	+	+	+
Penicillin (15)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ampicillin (10)	+	H^R	-	+	+	H^R	+	+	+	-	+	H^R	+	+	+	+
Nalidixic Acid (30)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gentamicin (10)	+	+	+	-	H^R	+	H^R	+	+	+	+	+	+	+	+	+
Ceftazidime (30)	+	+	+	+	+	+	+	+	+	+	+	+	H^R	H^R	+	+

ATCC 3047 (was inhibited by 90% of all the antibiotics)

Figure 1: Population Analysis Profiling of Heteroresistance positive strains indicating the difference between highest inhibitory concentrations and highest non-inhibitory concentrations as 8-fold or greater

B. POPULATION ANALYSIS PROFILING OF HETERORESISTANCE POSITIVE STRAINS

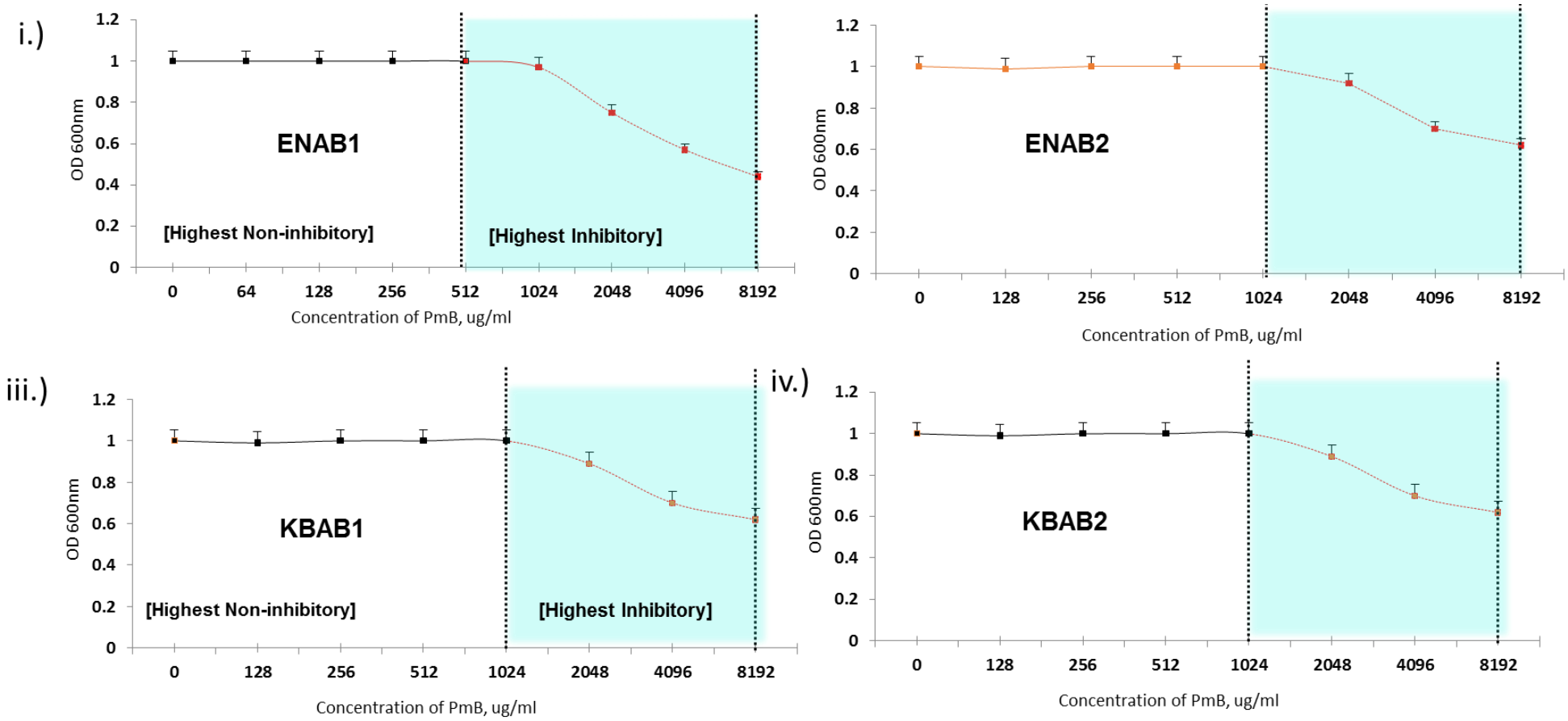


Figure 1: Population Analysis Profiling of Heteroresistance positive strains indicating the difference between highest inhibitory concentrations and highest non-inhibitory concentrations as 8-fold or greater

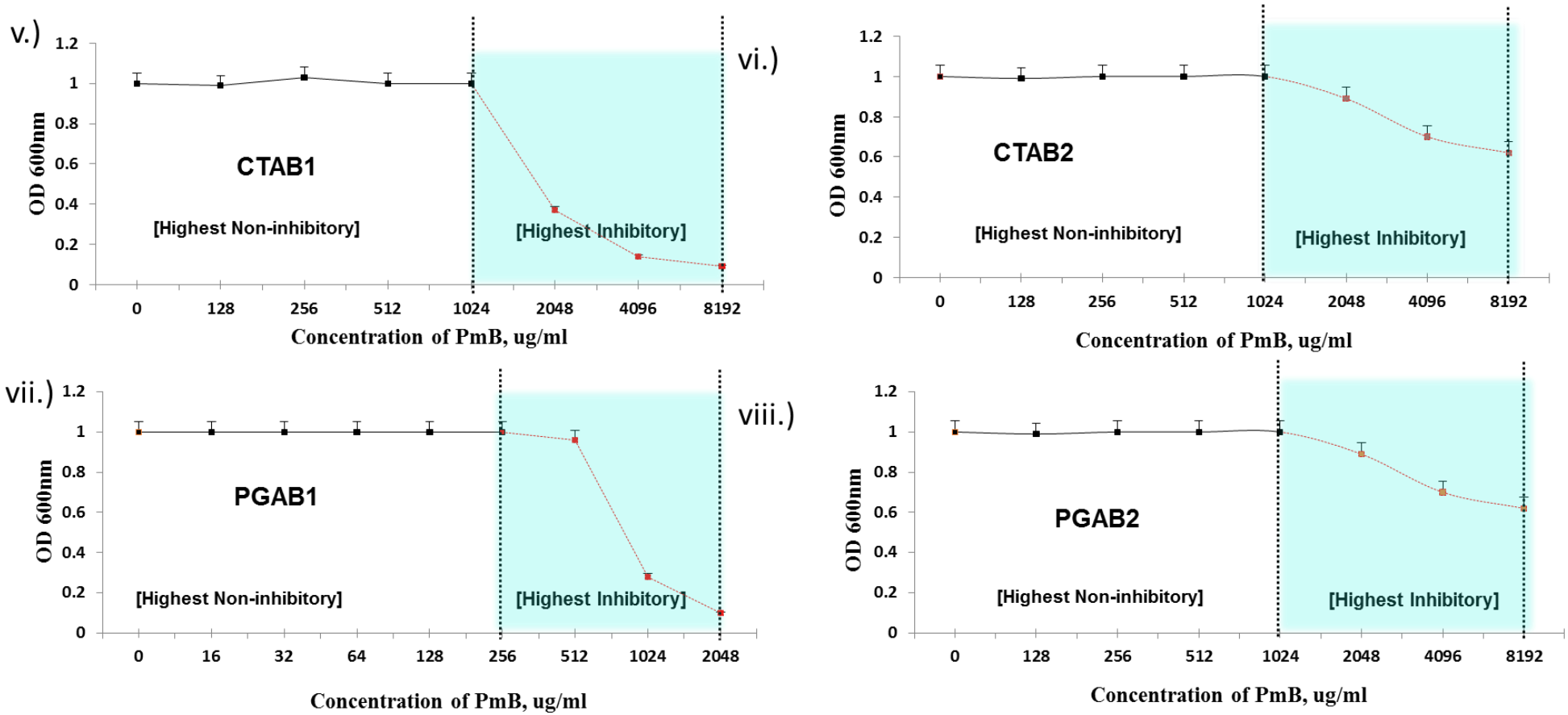


Figure 1: Population Analysis Profiling of Heteroresistance positive strains indicating the difference between highest inhibitory concentrations and highest non-inhibitory concentrations as 8-fold or greater

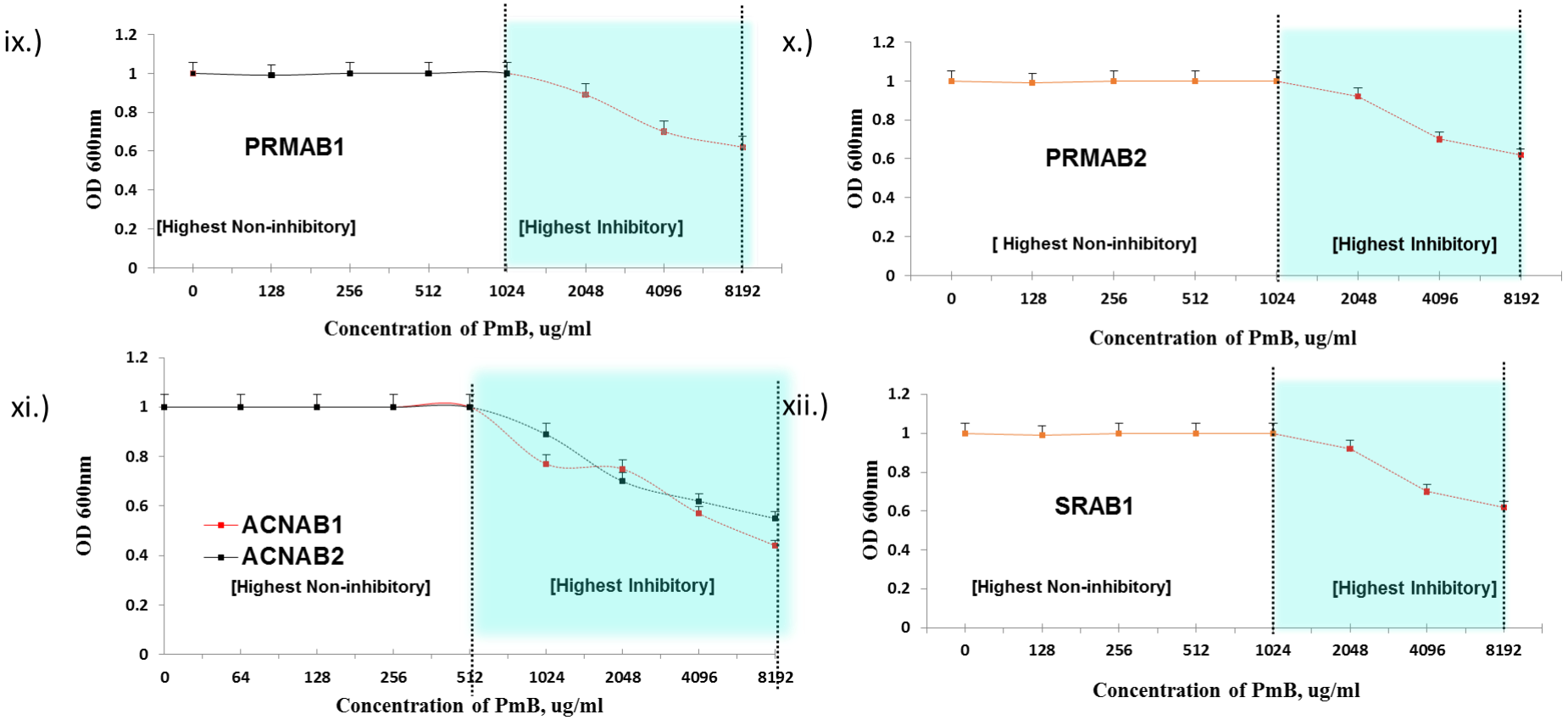


Figure 1: Population Analysis Profiling of Heteroresistance positive strains indicating the difference between highest inhibitory concentrations and highest non-inhibitory concentrations as 8-fold or greater

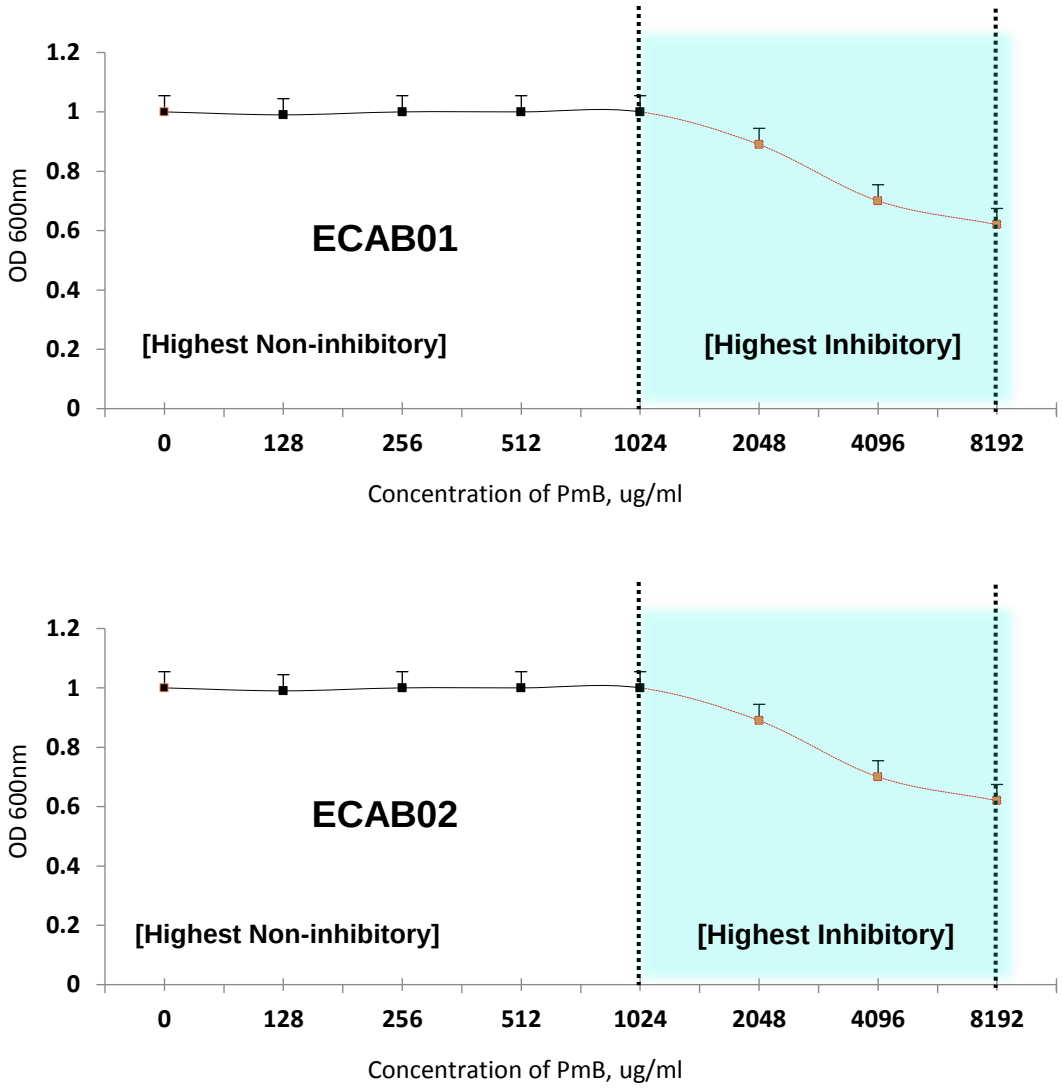


Figure 1: Population Analysis Profiling of Heteroresistance positive ECAB01/02 strains indicating the difference between highest inhibitory concentrations and highest non-inhibitory concentrations as 8-fold or greater

Appendix III

A. Galleria Mellonella research relevance

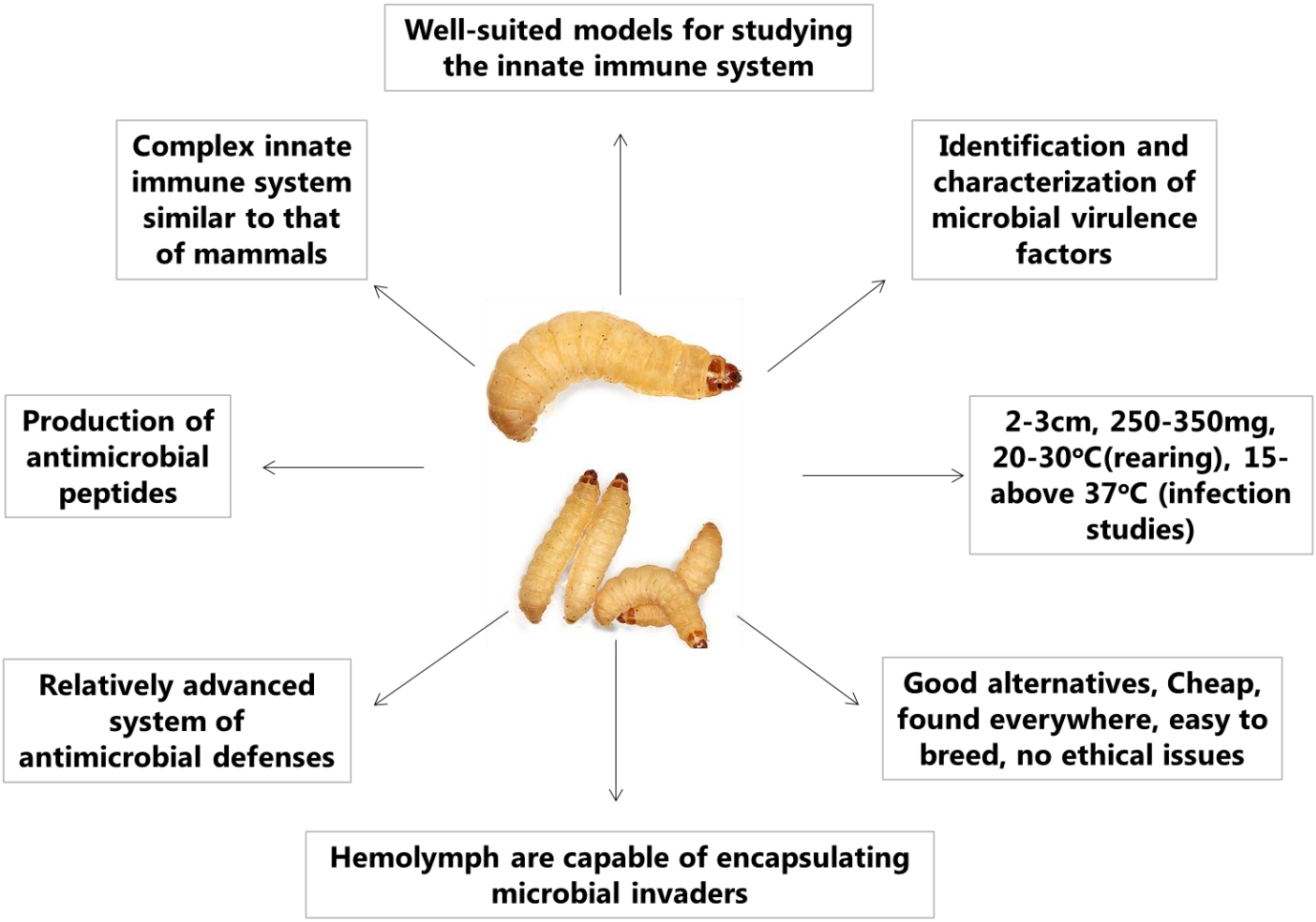


Figure 1a: Non-mammalian *Galleria mellonella* as good alternatives to animal models

B. *GALLERIA MELLONELLA* EXPERIMENTAL SET UP

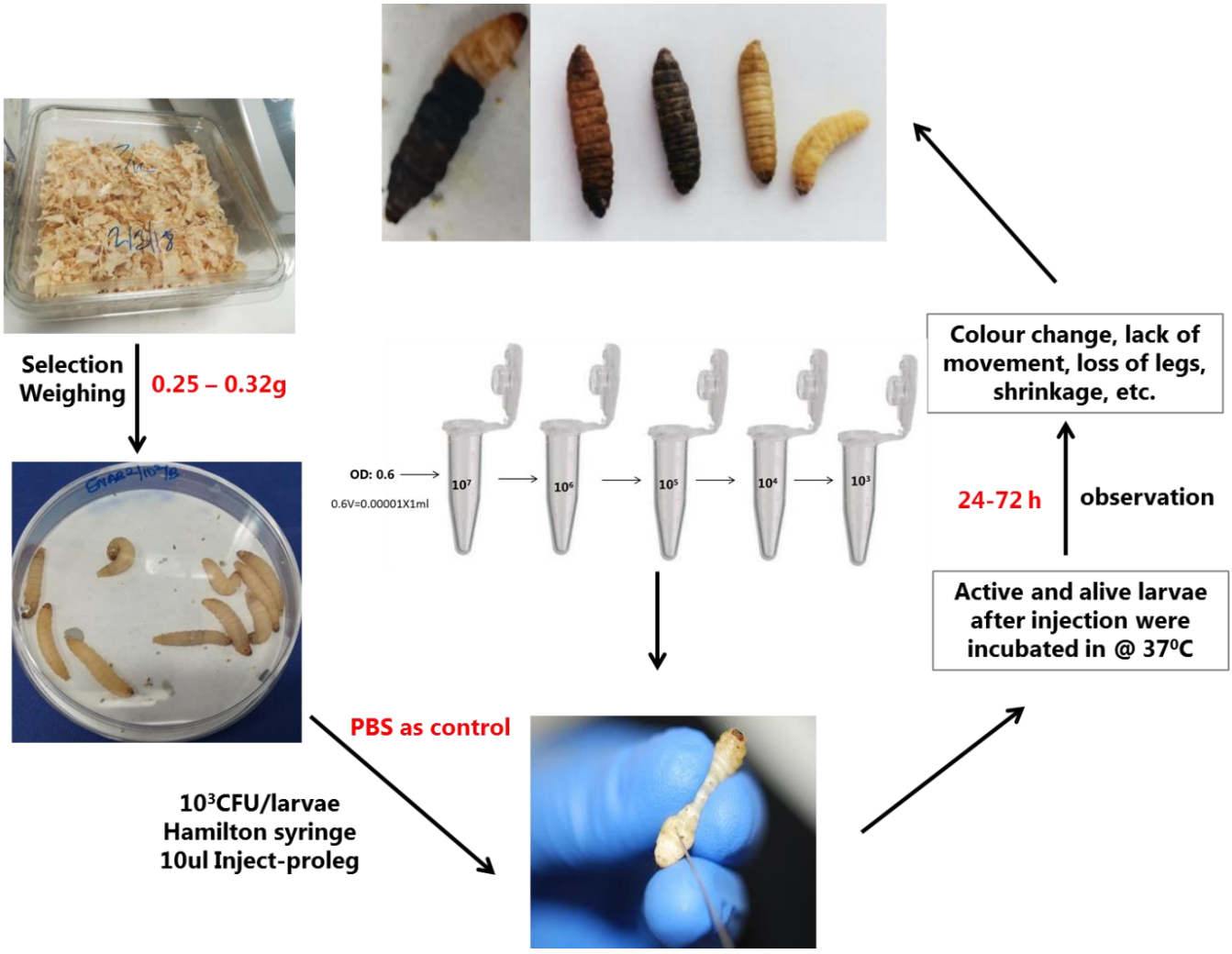


Figure 1b: *Galleria mellonella* experimental set up

C. *EXTREME VIRULENCE OF STRAINS IN GALLERIA MELLONELLA*

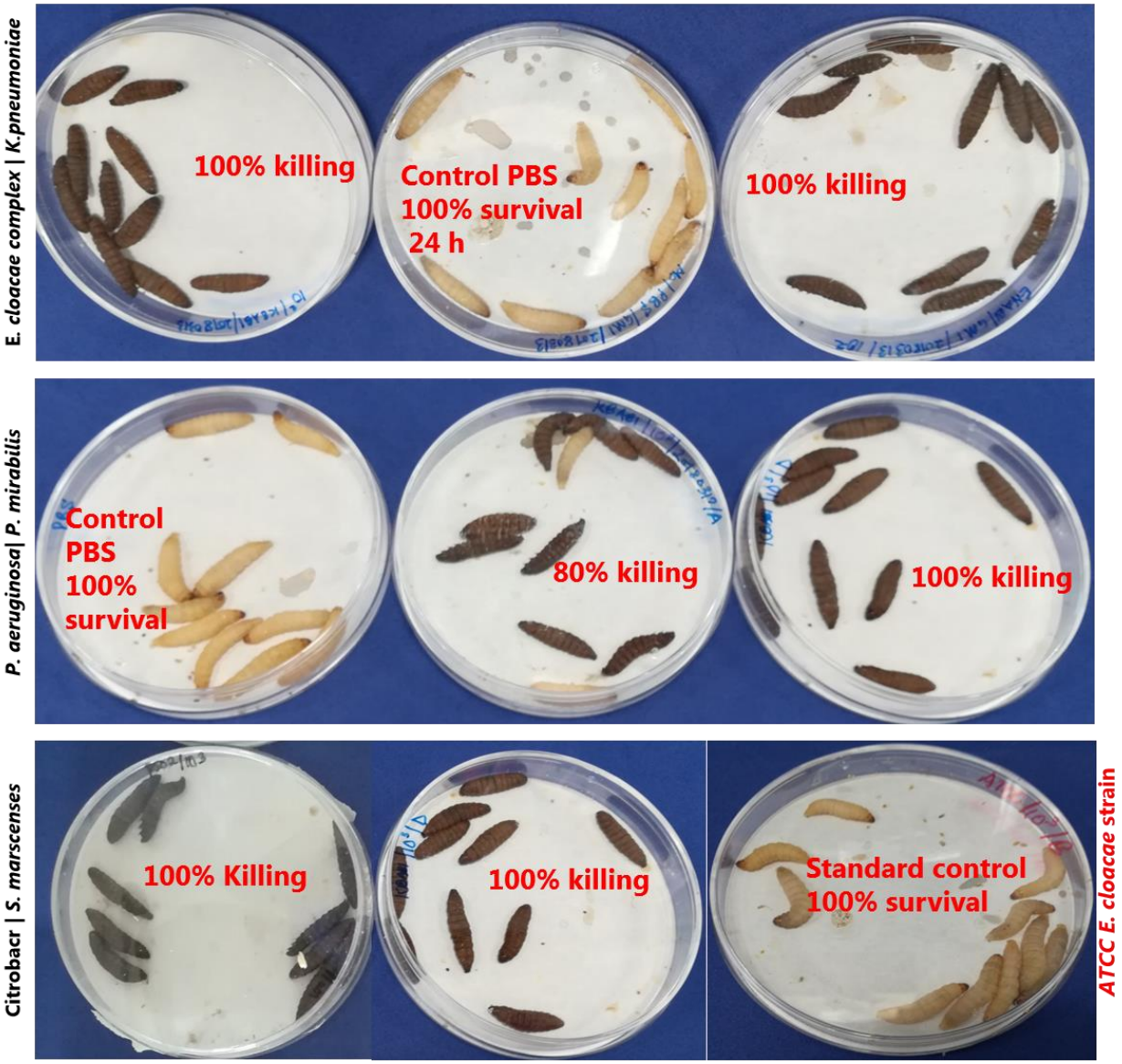


Figure 2: Extreme virulence (10 cells/larvae) of selected strains in GMI model system relative to PBS and ATCC positive controls

APPENDIX IV

A. REPRESENTATIVE GELS SHOWING SOME OF THE DETECTED RESISTANT MARKERS

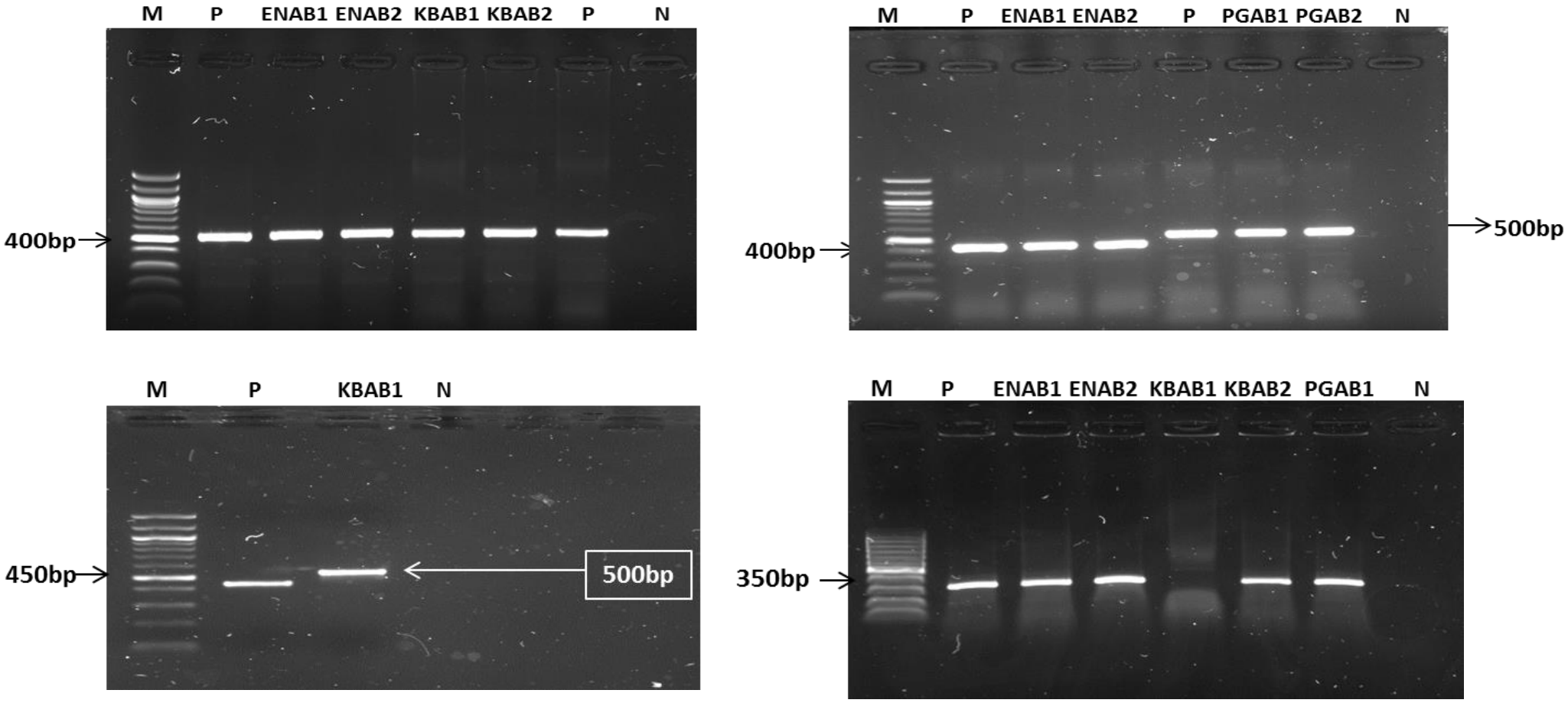


Figure 1: PCR detection of resistance markers, M (Molecular marker), P/N (Positive/Negative controls); (a.) *blactx-M* (400bp); (b) *gyrA* (400bp, *ENAB1* and *ENAB2*, (500, *PGAB1* and *PGAB2*); (c.) *Klebsiella pneumoniae Carbapenemase (blaKPC)* (500bp); (d.) *tetA* (350bp)

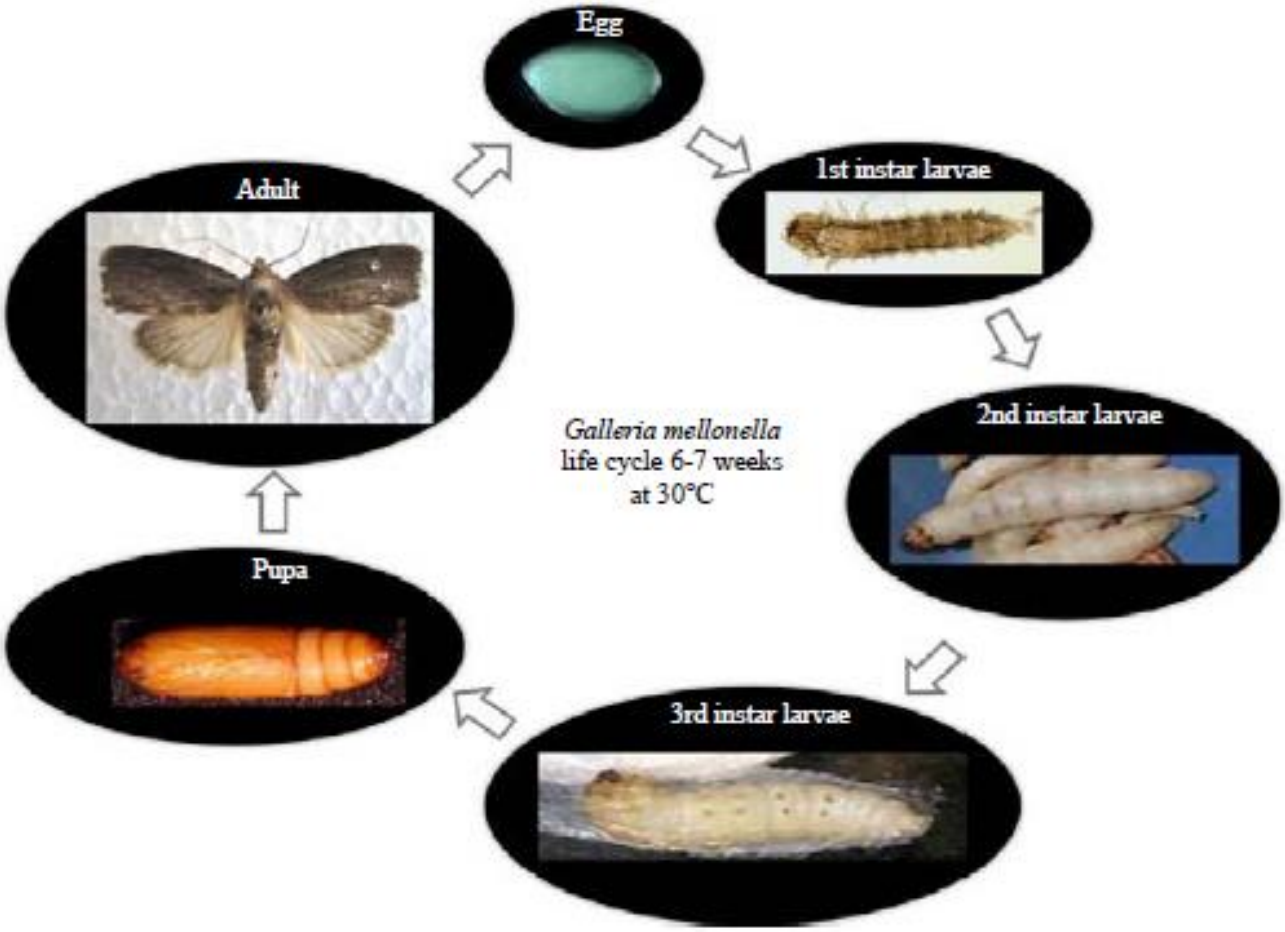
B. Table 1: LIST OF PRIMERS USED IN THIS STUDY

Target genes	Primer sequences (5'-3')		References
	Forward	**Reverse**	
GyrA	AAATCTGCCCGTGTCGTTGGT	GCCATACCTACGGCGATACC	Kim *et al.*, 2009
GyrB	GAAATGACC CGCCGTAAA	ACGACCGATACCACAGCC	Kim *et al.*, 2009
MefA	GGGAGATGAAAAGAAGGAGT	TAAAATGGCACCGAAAG	Daly *et al.*, 2004
MefE	GCTAGTGGATCGTCATGATAGG	TTCCCGAAACGGCTAAACTGGT	Daly *et al.*, 2004
ermB	GAAAAGGTACTCAACCAAATA	ATGAACGGTACTTAAATTGTTTAC	Okamoto *et al.*, 2002
mecA	GTGAAGATATACCAAGTGATT	ATGCGCTATAGATTGAAAGGAT	Ebadi and Ashrafi, 2018
qnrA	TCAGCAAGAGGATTTCTCA	GGCAGCACTATTACTCCCA	Wang *et al.*, 2003
qnrB	CCTGAGCGGCACTGAATTTAT	GTTTGCTGCTCGCCAGTCGA	Wang *et al.*, 2012
ampC	AACACACTGATTGCGTCTGAC	CTGGGCCTCATCGTCAGTTA	Moghaddam *et al.*, 2014
blaKPC	TGTTGCTGAAGGAGTTGGGC	ACGACGGCATAGTCATTTGC	Mlynarcik *et al.*, 2016
blaCTX	GCTGTTGTTAGGAAGTGTGC	CCATTGCCCGAGGTGAAG	Shibata *et al.*, 2006
tetA	GGCCTCAATTTCCTGACG	AAGCAGGATGTAGCCTGTGC	Guillaume *et al.*, 2000
prmA	CATTTCCGCGCACTGTCTGC	CAGGTTTCAGTTGCAAACAG	Jayol *et al.*, 2015
prmB	ACCTACGCGAAAAGATTGC	GATGAGGATAGCGCCCATGC	Jayol *et al.*, 2015
phoP	GAGCTTCAGACTACTATCGA	GGGAAGATATGCCCAACAG	Jayol *et al.*, 2015
phoQ	ATACCCACAGGAGGTCATCA	CAGGTGTCTGACAGGGATTA	Jayol *et al.*, 2015

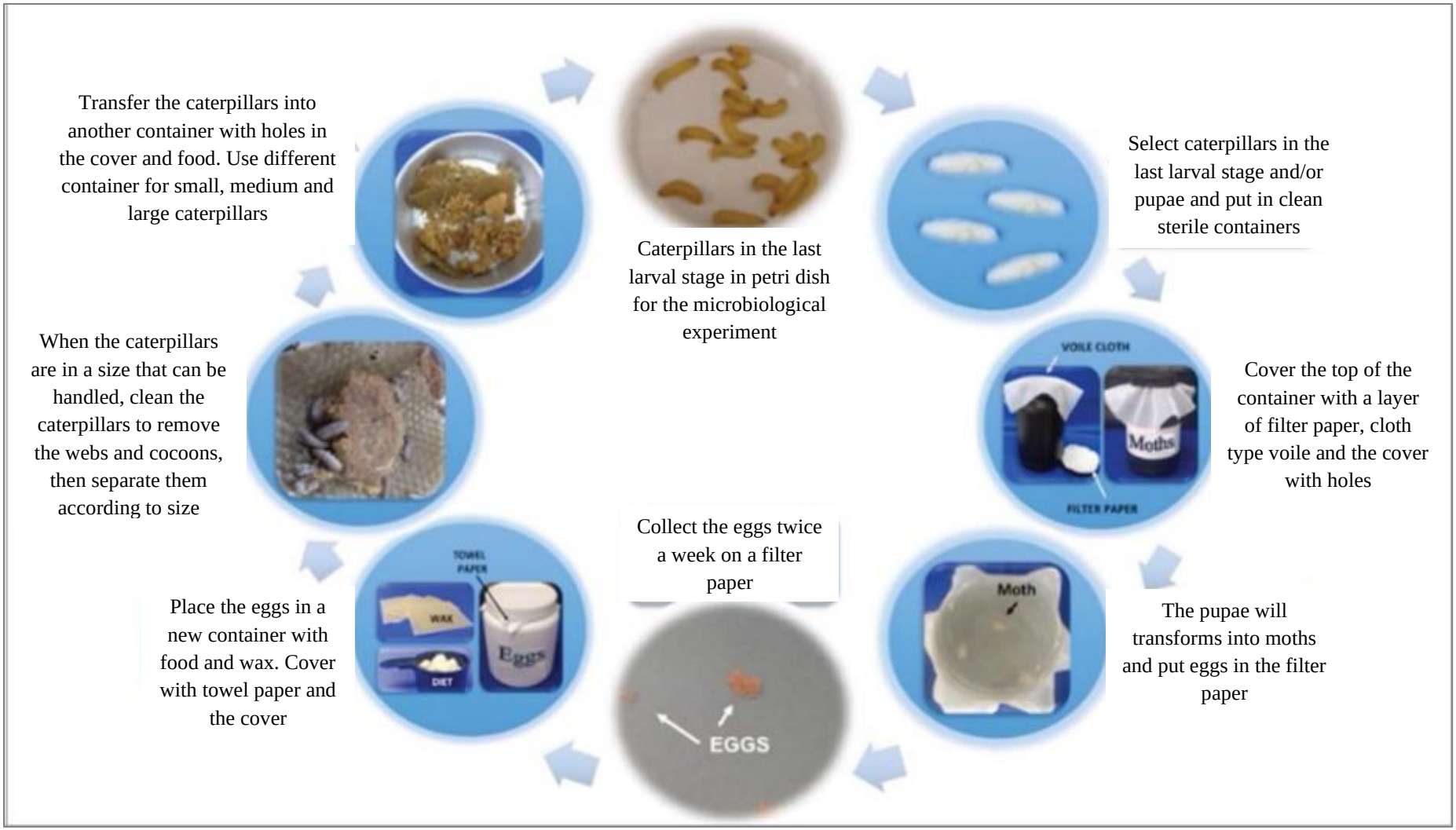
GENERAL INFORMATION ON THE STUDY

Galleria mellonella husbandry

Galleria mellonella is a wax moth of the family Pyralidae and it is found throughout the world. *G. mellonella* laid eggs in the spring with four life stages. Males generate ultrasonic sound pulses, alongside pheromones in mating. The larvae of *G. mellonella* are also often used as a model organism in research.



The stages of *Galleria mellonella* development takes 6-7 weeks and starts from eggs to caterpillar, three larvae stages, pupa and finally adult. 2nd instar larvae is preferred model for research purpose as the immune system is better developed here and produce antimicrobial peptides similar to human innate immune system. The larvae are also well-suited models for studying the innate immune system.



Process for *Galleria mellonella* rearing

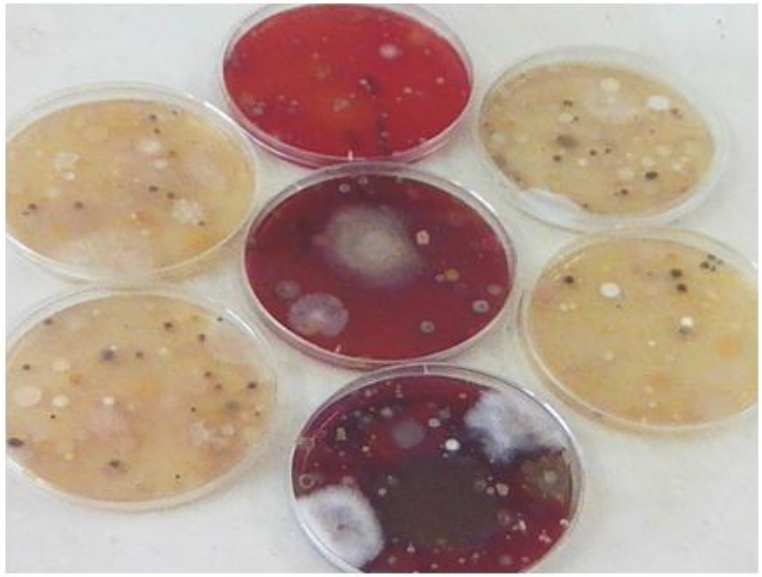
Clinical Laboratory Standard Institute and EUCAST Guidelines for resistance

Antimicrobial Agent (ug)	CLSI breakpoint µg/mL		EUCAST breakpoint µg/mL		Strains
Conventional	Susceptible ≥ (mm)	Resistance ≤ (mm)	Susceptible	Resistance	
Flucloxacillin (5)	20	5	20	5	Enterobacteriaceae
Erythromycin (5)	20	5	20	5	
Cloxacillin (5)	20	5	20	5	
Ceftriaxone (30)	20	5	20	5	
Cotrimoxazole (25)	20	5	20	5	
Nitrofuratoin (200)	20	5	20	5	
Chloramphenl (30)	20	5	20	5	
Tetracycline (10)	20	5	20	5	
Cefotaxime (10)	20	5	20	5	
Cefuroxime (30)	20	5	20	5	
Penicillin (15)	20	5	20	5	
Ampicillin (10)	20	5	20	5	
Nalidixic Acid (30)	20	5	20	5	
Gentamicin (10)	20	5	20	5	
Ceftazidime (30)	20	5	20	5	
Imipenem (10)	24	<21	24	<21	
Meropenem (10)	21	<15	21	<15	

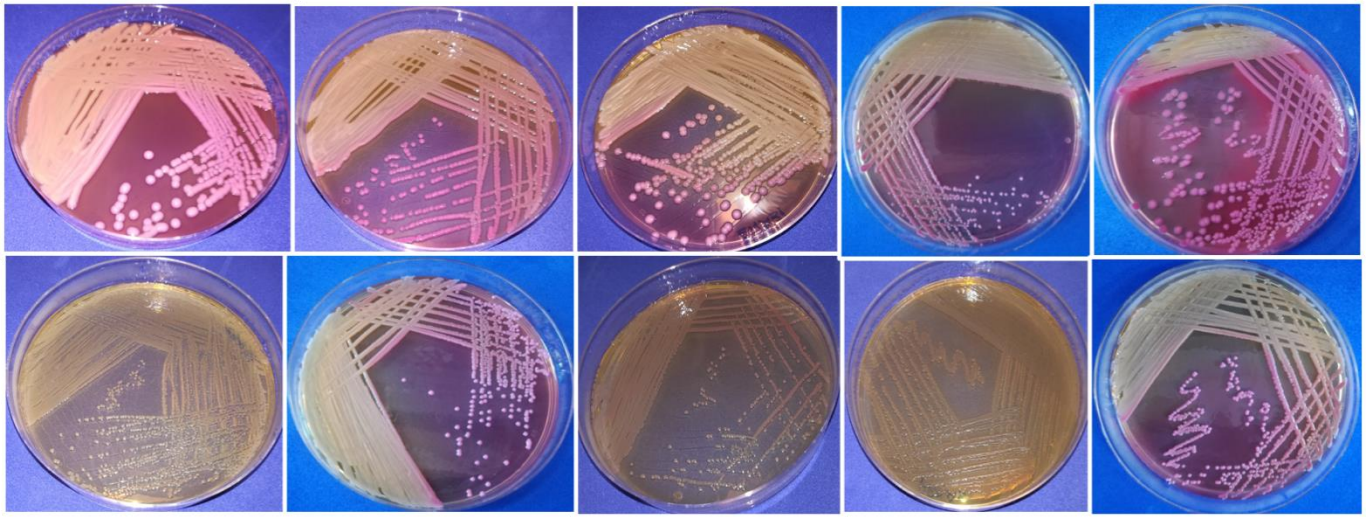
Breakpoint	Polymyxin B and Colistin E		Imipenem		Meropenem	
	Susceptible (µg/ml)	Resistance (µg/ml)	Susceptible (ug/ml)	Resistance (ug/ml)	Susceptible (ug/ml)	Resistance (ug/ml)
	≤ 2	≥ 4	≤ 2	≥ 4	≤ 2	≥ 8

CLSI standard guidelines (12th edition)

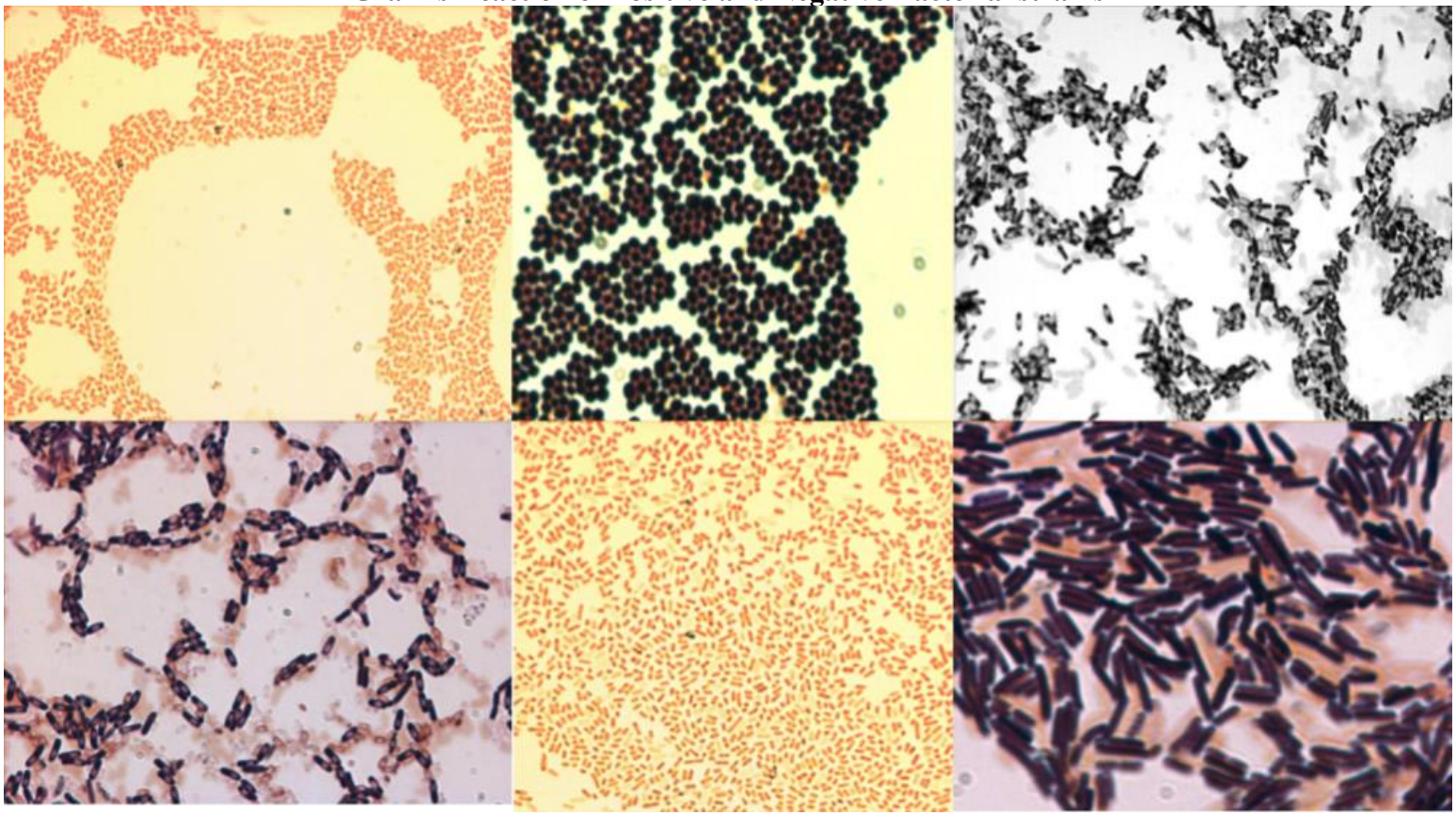
Different bacterial colonies from the exposed plates (37^0C, 24-48 hours)



Random agar plates showing bacterial colonies (37^0C, 18-24 hours)



Gram's Reaction of Positive and Negative Bacterial strains



***One Sample t-test**

Samples Collected	Strains	Identified
Theoretical mean	0.000	0.000
Actual mean	17.71	36.00
Number of values	7	7
One sample t test		
t, df t=3.266, df=6	t=5.937, df=6	
P value (two tailed)	0.0171	0.0010
P value summary	*	**
Significant (alpha=0.05)?	Yes	Yes

How big is the discrepancy?		
Discrepancy	17.71	36.00
SD of discrepancy	14.35	16.04
SEM of discrepancy	5.424	6.063
95% confidence interval	4.443 to 30.99	21.16 to 50.84
R squared	0.6400	0.8546

***Wilcoxon Signed Rank Test**

Samples Collected	Strains	Identified
Theoretical median	0.000	0.000
Actual median	13.00	32.00
Number of values	7	7
Wilcoxon Signed Rank Test		
Sum of signed ranks (W)	28.00	28.00
Sum of positive ranks28.00	28.00	
Sum of negative ranks	0.000	0.000
P value (two tailed)	0.0156	0.0156
Exact or estimate?	Exact	Exact
P value summary	*	*
Significant (alpha=0.05)?	Yes	Yes

*One-sample t-test followed by Wilcoxon Signed Rank test was performed using GraphPad Prism version 6.0.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com