Antibiotic Resistance in Gram-negative Bacteria in Brunei Darussalam: Molecular Characterisation, Epidemiology, Surveillance and Virulence

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A thesis submitted in partial fulfillment of the requirements of the Degree of Doctor of Philosophy by the University of London

Statement of Originality

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Abstract

Antimicrobial resistance (AMR) presents a global threat to human and animal health. Southeast Asia (SEA) is seen as a potential hotspot for the emergence and dissemination of new resistance mechanisms. The World Health Organization highlights research on multi-drug resistant Gram-negative bacteria (Enterobacteriaceae, *Acinetobacter* and *Pseudomonas* spp) as a critical priority.

This study reports findings from Brunei Darussalam. A molecular epidemiological surveillance study conducted using contemporary MDR *Escherichia coli, Klebsiella pneumoniae, A. baumannii* and *P. aeruginosa* of human and avian (farmed poultry) origin. Resistance to carbapenems and polymyxins was investigated using phenotypic (susceptibility, serotyping) and genotypic (PCR, sequencing, typing) methods. A selective bacterial culture media was developed and evaluated as a screening media for polymyxin-resistant (PR) strains. Immunochromatographic assays were used in the rapid identification of novel resistance determinants (OXA-48-like, MCR-1). Pathogenicity, fitness and virulence was correlated with host strain background and the carriage of AMR plasmids by resistant isolates.

Carbapenem-resistant (CR) *A. baumannii* were prevalent amongst hospital isolates and produced OXA-23 carbapenemases, similar to those reported worldwide. CR *K. pneumoniae* (CRKP) with plasmid encoded OXA-232 carbapenemase were found as part of a hospital outbreak. All identified as members of sequence type 231, a hi-risk epidemic clone in SEA. In separate point

prevalence studies, polymyxin resistant *E. coli* were found in 58% of poultry and 41.7% of human faecal samples. All produced phosphoethanolamine transferases encoded by *mcr* genes supported by diverse plasmid backbones (IncHI2, IncI2, IncX4) and host strain sequence types (n= 40). Additionally, a novel variant, *mcr-1.8,* localised on a 63,056 bp IncI2 conjugative plasmid was identified and characterized in *E. coli* O88:H31 ST101. PR strains were co-resistant to quinolones, aminoglycosides and phenicols. CTX-M β-lactamases (CTX-M-3 and CTX-M-65) were found in 2 strains but none co-produced a carbapenemase.

Comparison of human and avian MCR producing *E. coli* revealed only 6 sequence types were common to both humans and poultry. Polymyxin resistant strains harboured multiple virulence factors (VF) but no Avian Pathogenic (APEC) or Shiga Toxin (STEC) producing strains were found. Virulence of human and poultry isolates assessed in a *Galleria mellonella* infection model showed differences in survival rates that did not correlate with virulence score. When virulence of polymyxin resistant transconjugants (TC) was assessed only one which encoded MCR-1.8 exhibited heightened virulence. Growth kinetics showed no obvious differences despite plasmid acquisition.

This study demonstrates the high rate of resistance to carbapenems and polymyxins in critical Gram-negative bacteria in Brunei Darussalam. Plasmid-mediated polymyxin resistance in *E. coli* was found to be endemic in poultry and highly prevalent in the human samples studied. Unlike carbapenem resistance, polymyxin resistance was not associated with any predominant clone and did not

readily correlate with virulence properties of the strain. However, given the high background rates, enhanced surveillance for MCR-1 within more virulent Enterobacterial backgrounds is warranted, particularly in Southeast Asia countries.

Acknowledgments

First and foremost, I would like to thank my supervisor Dr David W Wareham for his knowledgeable experience, expertise in this area and endless support as well as being a friend throughout my PhD journey. My dearest family (Rosinah, Khairel, Liah) for their love, support and endless prayers. I love you all.

I am grateful to Dr Hj Osmali for his confidence in me to pursue my PhD. And my esteemed colleagues who believed in me.

I would like to thank our AMR group (Dr Jonathan W Betts, Dr Lynette M Phee, Sadia Miah, Maria Nieto) and collaborators (Dr Paul G Higgins from University of Cologne, Germany, Dr Apostolos Liakopoulos from Lieden University, Netherlands and Dr David C Bean from Federation University, Australia) for their work and support.

Microbiology Laboratory Services, Department of Laboratory Services, RIPAS Hospital, Veterinary Laboratory Services, Department of Agriculture and Agrifood, and poultry farms in Brunei Darussalam for allowing me to collect and analyze human faecal and chicken caecal samples.

To my best friends for always being there. To Allah, without his direction I would not be where I am today. Finally, I would like to dedicate this thesis to my late dad (Haji Abdul Momin), an inspirational human being, who pushes me to challenge myself every day. This is from me to you. I love you.

Publications, Presentations and Collaborations

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Muhd Haziq F Abdul Momin, Sadia S Miah, David W Wareham. Antimicrobial Resistance Surveillance of Human Faecal Samples Reveals a High Carriage Rate of *E. coli* MCR-1 in Brunei Darussalam. *Abstracts of the 28th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), Madrid, Spain* (2018) P0415.

Sadia Miah and Maria Nieto (Masters Student) – working on polymyxin resistance project which includes Antibiotic Susceptibility Testing (AST), broth microdilution for polymyxin MIC, conjugation and PCR.

Dr Jonothan W Betts – working on Coris OXA-48 K-SeT.

Dr Lynette M Phee – working on *G. mellonella* virulence statistics and survival curve analysis in colistin-resistant *E. coli* strains.

Dr Apostolos Liakopolous – working on the molecular analysis of the OXA-232 producing *K. pneumoniae* and novel *mcr-1-like* variant.

Dr David C Bean – working on CHROMagar COL-*APSE* media and molecular analysis

on the novel *mcr-1-like* variant.

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Abbreviations

AB	*Acinetobacter baumannii*
AME	Aminoglycoside modifying enzyme
AMK	Amikacin
AMP	Ampicillin
AMR	Antimicrobial resistance
APEC	Avian-pathogenic *E. coli*
ASEAN	Association of Southeast Asian Nation
ATCC	American Type Culture Collection
AUG	Augmentin (amoxicillin-clavulanate)
AZT	Aztreonam
bla	beta(β)-lactamase
BLAST	Basic local alignment tool
bp	base pair
BSI	Bloodstream infection
C	Chloramphenicol
CAZ	Ceftazidime
CDC	Centers for Disease Control and Prevention
CFU	Colony forming units
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
COL	Colistin
CPD	Cefpodoxime
CR	Carbapenem-resistant
CRE	Carbapenem-resistance Enterobacteriaceae
CTX	Cefotaxime
DNA	Deoxyribonucleic acid
EC	*E. coli*
EDTA	Ethylenediaminetetraacetic acid
EPEC	Enteropathogenic *E. coli*
ETP	Ertapenem
ESBL	Extended spectrum beta-lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
ExPEC	Extra-intestinal pathogenic *E. coli*
FAO	Food and Agriculture Organization of the United Nation
FOX	Cefoxitin
FQ	Fluoroquinolone
G3CREC	Gram-negative third generation cephalosporin-resistant *E. coli*
HAI	Hospital-acquired infection
I	Intermediate
IMI	Imipenem
KP	*Klebsiella pneumoniae*
LPS	Lipopolysaccharide

MALDI-Tof	Matrix assisted laser desorption/ionisation – time of flight
MDR	Multidrug-resistant
MEM	Meropenem
MIC	Minimum inhibitory concentration
MIN	Minocycline
MLST	Multi locus sequence typing
NCTC	The National Collection of Type Cultures
NDM	New Delhi Metallo β-Lactamase
NT	Non typeable
OD	Optical density
OIE	World Organisation for Animal Health
ORF	Open Reading Frame
OXA	Oxacillin hydrolyzing capabilities
PA	*Pseudomonas aeruginosa*
PCR	Polymerase chain reaction
PDR	Pandrug resistant
PFGE	Pulsed-field gel electrophoresis
PHE	Public Health England
PMPR	Plasmid-mediated polymxin resistance
PMQR	Plasmid-mediated quinolone resistance
PNPG	p-nitro-phenyl glycerol
PR	Polymyxin-resistant
RIPAS	Raja Isteri Pengiran Anak Hajah Saleha Hospital (Brunei)
R	Resistance
RLH	Royal London Hospital
S	Sensitive
SEA	Southeast Asia
ST	Sequence type
STEC	Shiga toxin-producing *Escherichia coli*
SXT	Co-trimoxazole (trimethoprim-sulfamethoxazole)
TAE	Tris-acetate-ethylenediaminetetraacetic acid
TEM	Temocillin
TIG	Tigecycline
TOB	Tobramycin
TRIM	Trimethoprim
UK	United Kingdom
VF	Virulence factor
WGS	Whole Genome Sequencing
WHO	World Health Organization

Chapter 1

Introduction

1.1 Antimicrobial Resistance (AMR)

1.1.1 Antimicrobial resistance (AMR): an end of the antibiotic era?

Antimicrobial resistance (AMR) poses a major threat to public health in the 21st century (Woolhouse and Farrar, 2014). According to a Lord Jim O'Neill, a leading UK economist, tasked with analyzing the global problem of rising drug resistance, whereby in 2050 the attributable number of deaths caused by AMR every year is predicted to rise to 10 million people (O'Neill, 2014). AMR deaths will exceed diseases caused by cancer, cholera, diabetes, diarrheal disease, measles, tetanus and road traffic accidents (O'Neill, 2014). The World Health Organization (WHO) further emphasized that AMR threatens the ability to treat common infectious diseases, resulting in prolonged illness, disability and death (WHO, 2018).

A highly diverse collection of genes encoding resistance to antibiotics (β-lactam, tetracycline and glycopeptide) have been found from an ancient DNA of 30,000-year-old Beringian permafrost sediments samples, this shows that antibiotic use and misuse favor resistance through selection pressure (D'Costa et al., 2011). The first synthetic antibiotic used systemically and paved the way for the antibiotic revolution in medicine was sulfonamide drugs (Skold, 2000). However, in the late 1930s, sulfonamide resistance was reported (Skold, 2000). In the late 1940s, the naturally occurring antibiotic benzylpenicillin (Penicillin G) discovered by Sir Alexander Fleming in 1928, recovered from the mold *Penicillium notatum* was also short lived due to resistance (Piddock, 2012; Sengupta et al., 2013). Since then,

resistance has developed in every major class of antibiotics (polymyxins, tetracyclines, carbapenems, cephalosporins, tigecyclines) (Figure 1).

The AMR crisis is further magnified with the movement of resistance genes through plasmid-mediated horizontal gene transfer (HGT) between different bacterial species which increases the variety of bacterial populations possessing multidrug-resistant (MDR) potential (Holmes et al., 2016). The various mechanism of HGT for which exchange of genetic information takes place includes transformation (extracellular donor DNA taken up recipient bacterium), transduction (donor DNA packaged in bacteriophage infects the recipient bacterium) and conjugation (through mating the donor bacterium transfer DNA to the recipient) (Carroll et al., 2016). In addition, the spread of bacteria carrying resistance genes through vertical transfer (from mother to daughter cells) are also known as clones (Woodford et al., 2011). More importantly are the high-risk clones which are able to disseminate resistance gene globally (Woodford et al., 2011).

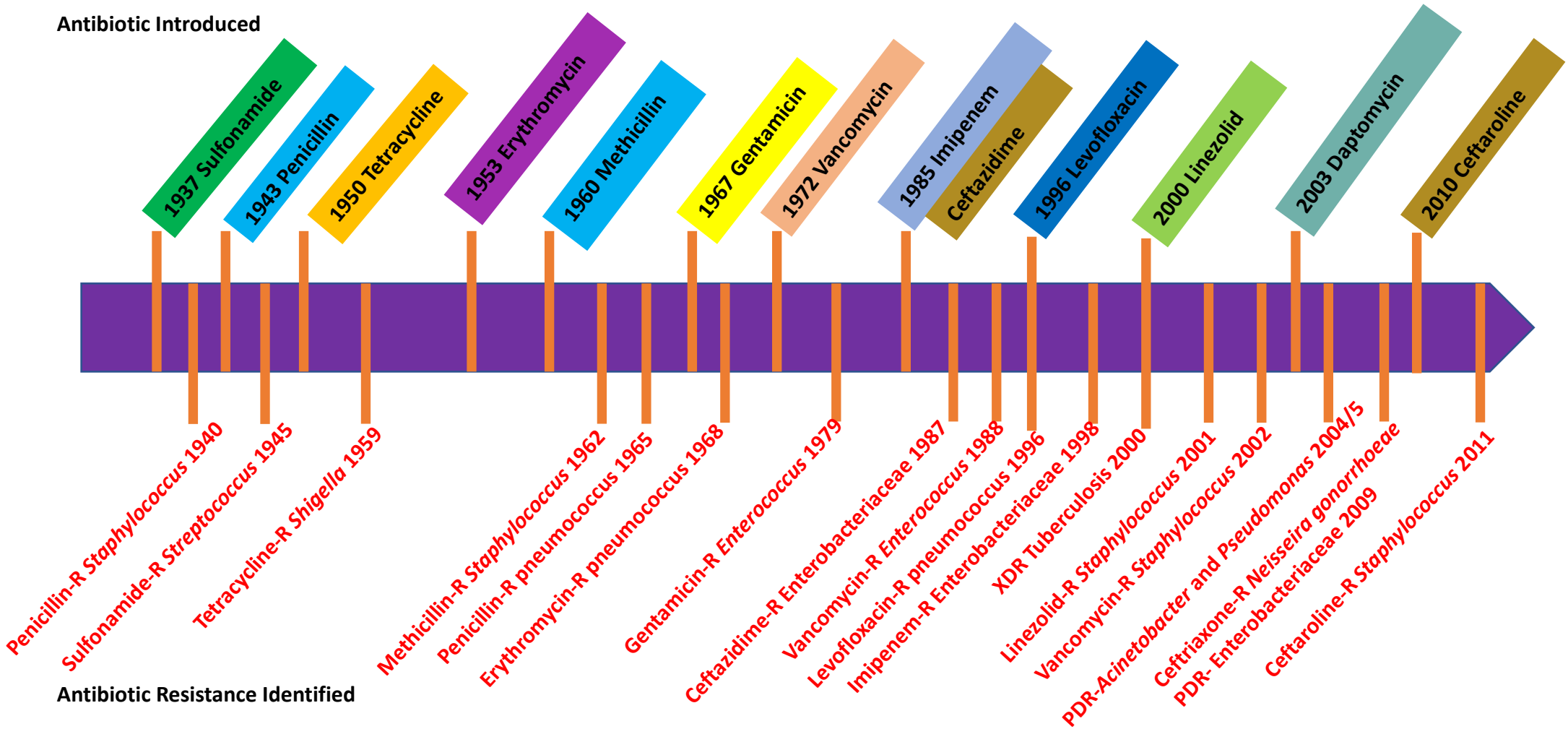


Figure 1: Timeline of antibiotic introduced and the development of resistance. R- resistance. Adapted from (CDC, 2013).

1.1.2 AMR a global problem

With increasing numbers of carbapenem-resistant pathogen, in 2017 the coordinating group of the Member States (Israel, Switzerland, Sweden, Netherlands, South Africa, France, USA and Austria) were tasked by the WHO after their request to develop a global priority pathogen list (Table 1) of antibiotic-resistant bacteria to help in prioritizing the research and development (R&D) of new and effective antibiotic treatments (WHO, 2017a). The criteria for prioritization was as follows; all-cause mortality, healthcare and community burden, prevalence of resistance, 10-year trend of resistance, transmissibility, preventability in hospital and community settings, treatability and current pipeline (WHO, 2017a).

1. Critical priority

i. *Acinetobacter baumannii*, carbapenem-resistant

ii. *Pseudomonas aeruginosa*, carbapenem-resistant

iii. *Enterobacteriaceae*, carbapenem-resistant, 3rd generation cephalosporin-resistant

2. High priority

i. *Enterococcus faecium*, vancomycin-resistant

ii. *Staphylococcus aureus*, methicillin-resistant, vancomycin intermediate and resistant

iii. *Helicobacter pylori*, clarithromycin-resistant

iv. *Campylobacter*, fluoroquinolone-resistant

v. *Salmonella spp.*, fluoroquinolone-resistant

vi. *Neisseria gonorrhoeae*, 3rd generation cephalosporin-resistant, fluoroquinolone-resistant

3. Medium priority

i. *Streptococcus pneumoniae*, penicillin-non-susceptible

ii. *Haemophilus influenza*, ampicillin-resistant

iii. *Shigella spp.*, fluoroquinolone-resistant

Table 1. Global priority pathogen lists of antibiotic-resistant bacteria for Research & Development of new antibiotics. Adapted from (WHO, 2017a)

1.1.3 AMR in Southeast Asia (SEA)

There are 10-member states of ASEAN (Association of Southeast Asian Nation) Brunei Darussalam, Cambodia, Indonesia, Laos, Malaysia, Myanmar, Philippines, Singapore, Thailand and Vietnam (Figure 2). Most of these states are still under-developed and surveillance for antimicrobial resistance can be challenging due to limited microbiology tools.

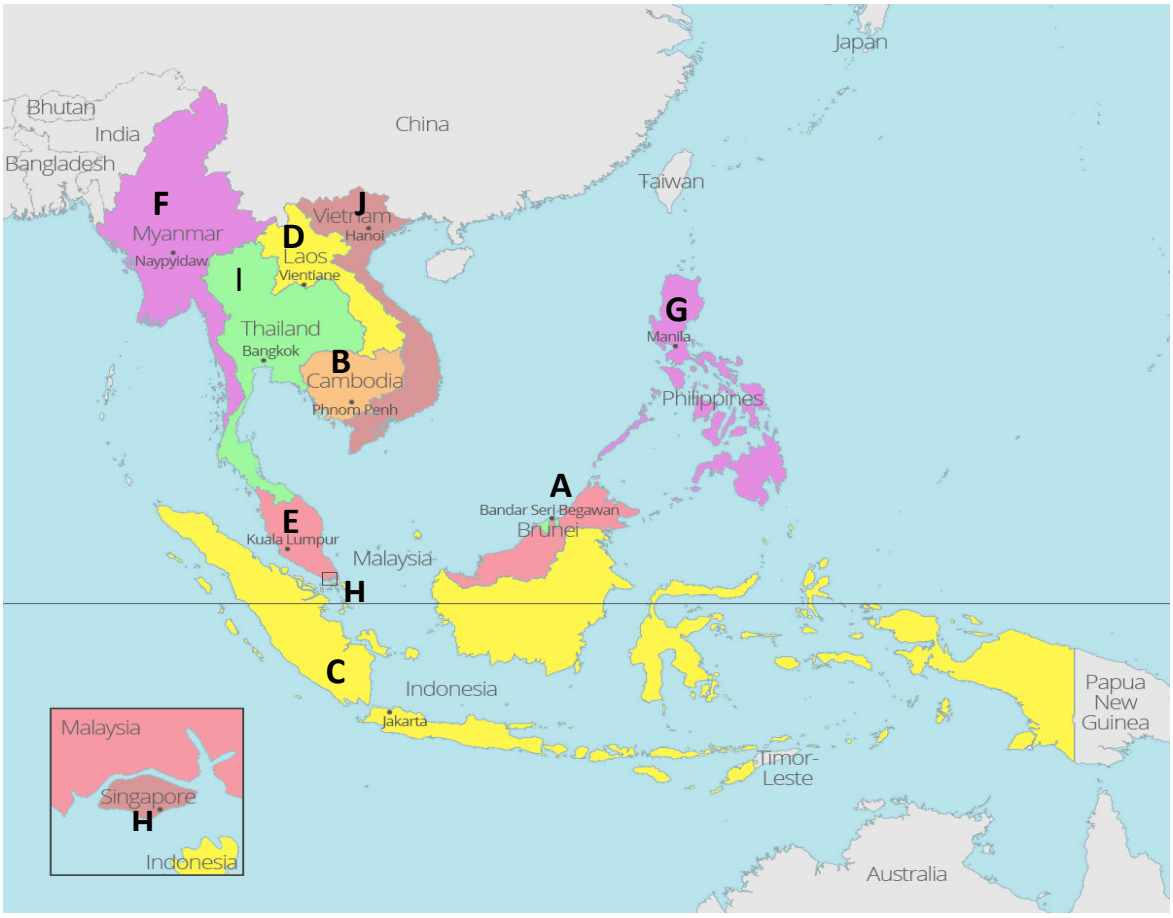


Figure 2. Map of Southeast Asia (SEA) and the ASEAN member states; A) Brunei Darussalam, B) Cambodia, C) Indonesia, D) Laos, E) Malaysia, F) Myanmar, G) Philippines, H) Singapore, I) Thailand and J) Vietnam. Map taken from ASEAN *UP*, www.aseanup.com

As of May 2016, there are only four countries with established National Antimicrobial Surveillance Programmes; Singapore, Thailand, Malaysia and the Philippines (Hsu et al., 2017). The highest rates of carbapenem resistance in Gram-negative bacteria in Southeast Asia are reported in *Acinetobacter baumannii* followed by Enterobacteriaceae (Hsu et al., 2017). However, AMR data for the East Malaysian states (Sabah and Sarawak) and Indonesian state (Kalimantan) in the Borneo island are unknown.

1.1.4 Brunei Darussalam at a glance

Brunei Darussalam (capital, Bandar Seri Begawan) is situated on the island of Borneo flanked between Sabah and Sarawak (Malaysian states) (Figure 3). As of 2018, Brunei's population is approximately 433, 517, with a total area of 5,765 square kilometers (2,226 sq mi). Brunei Darussalam comprises of four districts namely; Brunei-Muara, Tutong, Kuala Belait and Temburong. All districts have their own hospitals and laboratory services (PMO). However, due to limited capacity in terms of services provided by the district hospitals, Raja Isteri Pengiran Anak Hajah Saleha (RIPAS) Hospital (main hospital, located in Brunei-Muara district) is the main center for most of the medical and laboratory services offered to patients.

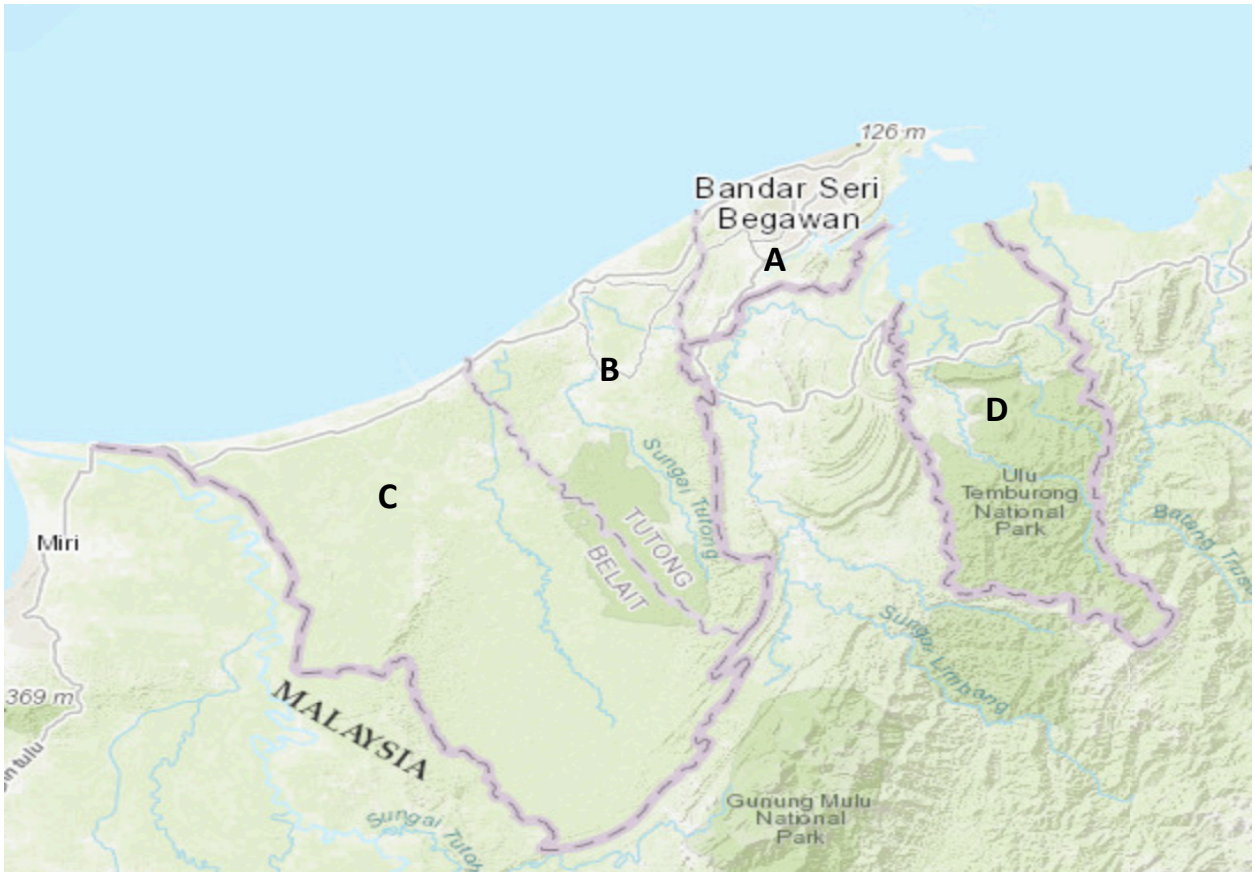


Figure 3. Map of Brunei Darussalam consisting of four districts; A) Brunei-Muara, B) Tutong, C) Belait and D) Temburong. Map available at ArcGIS, www.arcgis.com.

1.1.4.1 AMR in Brunei Darussalam

Little is known about AMR in Brunei Darussalam. However, like many other countries, Hospitals in Brunei are threatened with infections caused by pathogens in the WHO critical lists, causing a public health threat. Despite limited information on AMR in Brunei, monitoring and reporting of these resistant pathogens are routinely done by the Microbiology Laboratory Services in RIPAS Hospital using the WHONET 5.4 system (a Microsoft Windows-based database software for the

entry, reporting, and analysis of microbiology laboratory test results (O'Brien et al., 2001). Although, the WHONET program is excellent for entry, analysis and reporting of resistant data, the software is not intended to function as a complete patient management system (O'Brien et al., 2001).

1.2 Gram-negative pathogens

Gram-negative pathogens of interest in this study includes the Non-fermenters *(Acinetobacter baumannii, Pseudomonas aeruginosa)* and Enterobacteriaceae (*Escherichia coli, Klebsiella pneumoniae*). These pathogens can cause a multitude of infections such as urinary tract infection, intra-abdominal infection, nosocomial pneumonia, septicemia and meningitis (CDC, 2011).

The importance of Gram-negative pathogens was highlighted in a study done by de Kraker and colleagues where it was predicted that there would be an increase in Gram-negative third generation cephalosporin-resistant *E. coli* (G3CREC) surpassing Gram-positive methicillin-resistant *Staphylococcus aureus* (MRSA) bacteremias (de Kraker et al., 2011). Data were analysed from the European Antibiotic Resistance Surveillance System (EARSS) from 2005-2009 (de Kraker et al., 2011) .

1.2.1 Structure of Gram-Negative Bacteria

Gram-negative bacteria (GNB) have a unique structure. They have multiple layers of membranes that protects them from foreign attack (immune system, antibiotics) before reaching the cytoplasm. The first outer layer is the capsule, followed by the outer membrane, a thin layer of peptidoglycan layer and an inner cytoplasmic cell membrane. Other features of GNB structure includes flagella, pili, nucleoid, ribosomes and plasmids (Figure 4). Magnified structure of GNB cell wall is shown in Figure 5.

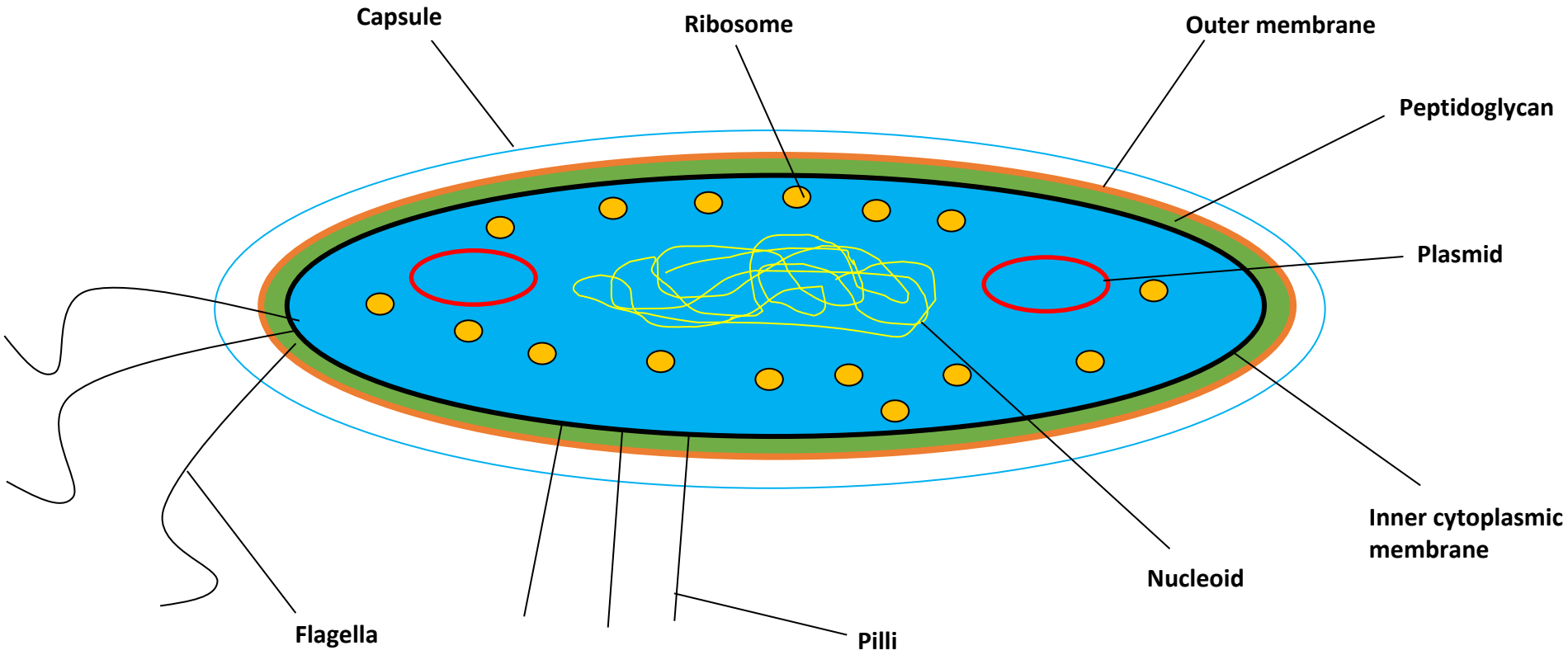


Figure 4. Overview structure of Gram-negative bacteria. Adapted from (Carroll et al., 2016)

1.2.2 Mobile Genetic Elements (MGE) in bacteria

Mobile Genetic Elements are important for the exchange of genetic information between bacteria, between species, on a chromosome or between chromosome (Carroll et al., 2016). This includes plasmid, transposon, insertion element, integron and gene cassette.

1.2.2.1 Plasmid

Plasmids play an important role in the dissemination of antimicrobial resistance and present one of the most difficult challenges (Carattoli, 2013). Plasmids are small circular DNA that is able to self-replicate in a host cell (Carattoli et al., 2005). Plasmids vary in sizes from a few to more than several hundred kilobases (kb) found in nearly all bacterial species (Waters, 1999). Plasmids contain genes essential for initiation and control of replication and accessory genes that may be useful to their bacterial hosts such as antimicrobial resistance or virulence genes (Amabile-Cuevas and Chicurel, 1992; Bergstrom et al., 2000; Thomas, 2000). In addition, plasmids are classified based on incompatibility (Inc) groups (Novick, 1987). Incompatibility grouping is based on the procedure by conjugation or transformation, of a plasmid of an “unknown” Inc group into a strain carrying a plasmid of a known Inc group (Carattoli et al., 2005).

If the plasmids, currently present in the bacteria are eliminated, the incoming plasmid is assigned to its same Inc group (Datta and Hedges, 1971). “Incompatible” is when the plasmids have the same replication control, whereas plasmids with different replication controls are “compatible” (Carattoli et al., 2005). Hence, on this basis two plasmids cannot be propagated in the same cell line belonging to the same Inc group (Datta and Hughes, 1983; Couturier et al., 1988). Examples of these Inc groups includes Inc HI1, HI2, I2, I, X, Y, FIA, FIB, P and N (Carattoli et al., 2005).

1.2.2.2 Transposons

Transposons are DNA sequences containing several genes, including those necessary for their migration from one genetic locus to another (Carroll et al., 2016). And during their migration, they create insertion mutations (Carroll et al., 2016).

1.2.2.3 Insertion elements (IS)

Insertion elements are short transposons (0.75-2.0 kbp long) producing insertion mutations (Carroll et al., 2016). In addition, they carry enzymes needed to promote their own transposition to another genetic locus but cannot replicate on their own (Carroll et al., 2016).

1.2.2.4 Integron

An integron is a genetic element that possesses a site, *attI*, at which additional DNA (in the form of gene cassettes) is able to integrate by site-specific recombination (Bennett, 1999). It also encodes an enzyme, integrase, that mediates these site-specific recombination events (Bennett, 1999).

1.2.2.5 Gene cassette

Gene cassettes are discrete mobile genetic elements that may exist as free, circular, non-replicating DNA molecules when moving from one genetic site to another, (Collis and Hall, 1992) but which are normally found as linear sequences that constitute part of a larger DNA molecule, such as a plasmid or bacterial chromosome (Bennett, 1999). In addition, gene cassettes normally contain only a single gene and an additional short sequence (called a 59-base element), that functions as a specific recombination site (Hall et al., 1991). The size of the cassettes are small, normally in the order of 500–1000bp (Bennett, 1999). Furthermore, genes that are carried on gene cassettes usually lack promoters (region of DNA where transcription is initiated and encoded proteins to be expressed at high levels) (Carroll et al., 2016) and are expressed from a promoter on the integron (Wiedemann et al., 1986; Collis and Hall, 1995).

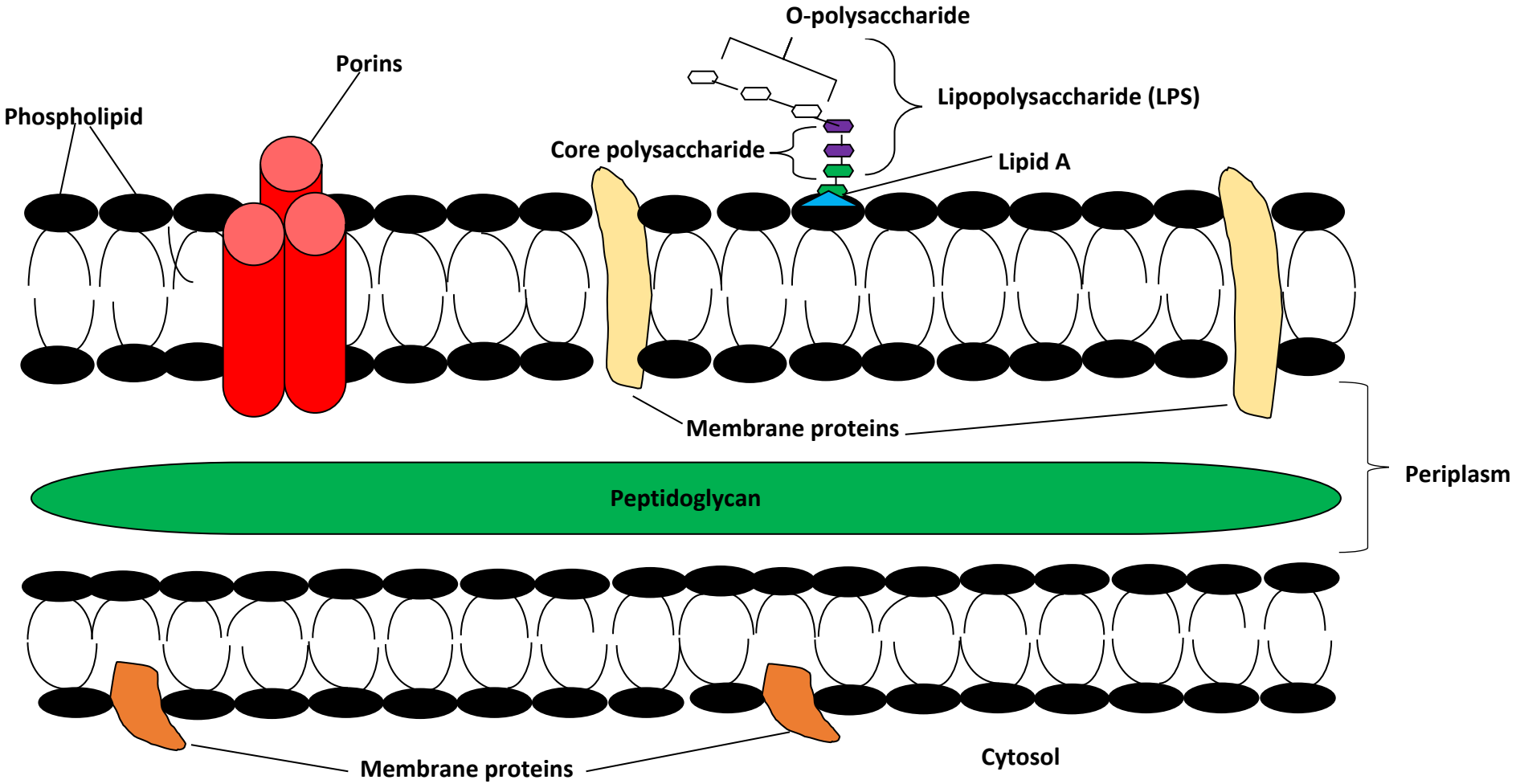


Figure 5. Gram-negative bacteria cell wall structure. Adapted from (Carroll et al., 2016)

1.3 Important WHO Gram-negative critical pathogen lists

1.3.1 *Acinetobacter baumannii*

The genus *Acinetobacter* was first described in 1911 by a Dutch microbiologist, Beijerinck isolated from a soil sample, which was named *Micrococcus calcoaceticus* (Henriksen, 1973). *Acinetobacter baumannii* (*A. baumannii*) are Gram-negative, strictly aerobic, non-fastidious, non-motile and non-fermenting coccobacilli bacteria where the genus is a member of the order *Gammaproteobacteria* and belongs to the family Moraxellaceae (Peleg et al., 2008; Evans et al., 2013). More recently, twitching motility has been reported in *A. baumannii* mediated by type IV pili by the action of extension and retraction of the pili (Vijayakumar et al., 2016). *A. baumannii* has been classified as one of the ESKAPE organism (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and Enterobacter species) causing nosocomial infections and effectively "escaping" the effects of antibacterial drugs (Boucher et al., 2009).

A. baumannii can cause a diverse range of infections (respiratory tract, urinary tract, bloodstream, skin and soft tissue, device-related) and are frequently isolated from immunocompromised patients hospitalised in the intensive care unit (ICU) (Potron et al., 2015). In the United States (1986-2003) between 5% and 10% of all ICU-acquired pneumonia and 1.6% of ICU-urinary tract infection (UTI) was caused by *A. baumannii* (Gaynes et al., 2005). In another large study done on nosocomial bloodstream infection (BSI) in the US hospitals (1995-2002), *A.*

baumannii was found to be the 10th most common etiologic agent accountable for 1.3% of all monomicrobial nocosomial BSI (Wisplinghoff et al., 2004). The overall crude mortality from *A. baumannii* BSI was 34% to 43.4% in the ICU, compared to 16.3% outside the ICU (Peleg et al., 2008). Of greater concern, higher mortality in post-neurosurgical *A. baumannii* meningitis with mortality as high as 70%, (Peleg et al., 2008) usually seen in patients with an external ventricular drainage setup after neurosurgery (Metan et al., 2007). However, the direct effects of *A. baumannii* on mortality appear less well defined (Peleg et al., 2008).

1.3.2 *Pseudomonas aeruginosa*

In the 1850s, Sédillot observed a blue-green discharge frequently present and associated with infection in surgical wound dressings (Lyczak et al., 2000). The non-fluorescence bluish pigment is pyocanin, where only *Pseudomonas aeruginosa* (*P. aeruginosa*) produces this pigment (Carroll et al., 2016). *P. aeruginosa* is a motile, non-fermenting, opportunistic Gram-negative bacteria belonging to the family Pseudomonadaceae, (Alhazmi, 2015) which also belongs to the ESKAPE pathogen group (Boucher et al., 2009). In the 1960s, *P. aeruginosa* has emerged as an important human pathogen (Doggett, 1979). By 1961, this organism can cause both severe acute and chronic infections (Freeman, 1916).

In 2006-2007, the National Healthcare Safety Network (NHSN) in the United States reported that 8% of all hospital-associated infections were due to *P. aeruginosa*, 3% causing central line-associated bloodstream infections, 6% of surgical site

infections, 10% of catheter-associated urinary tract infection and 16% of ventilator-associated pneumonia (VAP) infections (Hidron et al., 2008). The major predisposing factors to the acquisition of *P. aeruginosa* in hospitals are mechanical ventilation, antibiotic therapy, surgery, and chemotherapy (Thuong et al., 2003).

Nosocomial bacteremia leading to sepsis associated with *P. aeruginosa* accounts for mortality greater than 50% (Whitecar et al., 1970; Fishman and Armstrong, 1972) and as high as 70% in febrile neutropenic patients (Bodey et al., 1985). *P. aeruginosa* gains access to the blood stream from the local site of infection by breaking down epithelial and endothelial tissue barriers (Kurahashi et al., 1999). In order to evade bactericidal activity of the serum complement, *P. aeruginosa* produces smooth LPS; full-length O side-chain of the bacteria. (Hancock et al., 1983; Pier and Ames, 1984)

In Cystic fibrosis (CF), 90% of individuals suffering become infected with *P. aeruginosa* during their lifetime (Alhazmi, 2015). This pathogen is the leading cause of morbidity and mortality among those patients and a dominant pathogen present in chronic lung infection in CF (Alhazmi, 2015). CF is an autosomal recessive genetic disorder caused by a mutation in a gene on chromosome 7 known as CFTR (cystic fibrosis transmembrane conductance regulator) (Alhazmi, 2015). The most common mutation is in the ΔF508 (or F508del), which is a three-nucleotide deletion of a phenylalanine residue and subsequent defective

intracellular processing of the CFTR protein that is an important chloride channel (Bobadilla et al., 2002). CF affects 1:2,500 in the Caucasian population (Ratjen and Doring, 2003) and mortality are due to lung disease (Bobadilla et al., 2002).

1.3.3 *Klebseilla pneumoniae*

Klebsiella pneumoniae was first described by Carl Friedlander in 1882 as a bacterium isolated from the lungs of patients who had died from pneumonia (Freidlander, 1882). *Klebsiella pneumoniae* (*K. pneumoniae*) is a Gram-negative, lactose-fermenting, non-motile, aerobic, rod-shaped bacterium with a prominent capsule and belongs to the family Enterobacteriaceae (Carroll et al., 2016) also part of the ESKAPE pathogen group (Boucher et al., 2009). They have large polysaccharide capsule and lacks motility (Carroll et al., 2016). *Klebsiella* species are found ubiquitously in nature, including in plants, animals, and humans (Carroll et al., 2016).

These pathogens are an opportunistic hospital-acquired infection (HAI) causing several types of infections in humans, including respiratory tract infections, urinary tract infections (UTIs), and bloodstream infections, (Podschun and Ullmann, 1998) infecting the critically ill and immunocompromised patients. Furthermore, *K. pneumoniae* are found in the respiratory tract and faeces of about 5% of normal individuals (Carroll et al., 2016). In the 1980s and 1990s community-acquired hypervirulent *K. pneumoniae* has emerged from the Asian Pacific Rim

causing pyogenic liver abscess affecting Asian males and this was mostly associated with the K1 encapsulated strains (Martin and Bachman, 2018).

Klebsiella species have been identified as the third leading cause of HAIs in the United States (9.9%) behind *Clostridium difficile* and *Staphylococcus aureus* (Magill et al., 2014) and the second leading cause of bloodstream infections (BSI) caused by Gram-negative bacteria, behind only *E. coli* (Podschun and Ullmann, 1998; Magill et al., 2014). Hospital-acquired *K. pneumoniae* BSI is associated with the primary underlying cancer disease, while liver abscess and diabetes mellitus are highly associated among community-acquired (CA) *K. pneumoniae* BSI (Kang et al., 2006).

1.3.4 *Escherichia coli*

Escherichia coli (E. coli) was first isolated by Theodor Escherich from an infant stool sample in 1886 and named Bacterium *coli commune* (the common colon bacillus) (Shulman et al., 2007). *E. coli* is fascinatingly diverse bacteria adapted to diverse environmental conditions and lifestyles (Hufnagel et al., 2015). They are facultatively anaerobic Gram-negative rods belonging to the family Enterobacteriaceae and member of the normal intestinal flora (Hufnagel et al., 2015). There are a total of over 15,000 genes that make up the *E. coli* pangenome (Touchon et al., 2009; Kaas et al., 2012). Hence, due to the genomic plasticity of various *E. coli* isolates allows this bacterium the ability to proliferate and survive

in an array of environments (Kaper et al., 2004; Tenaillon et al., 2010). A major niche of *E. coli* is found in the lower intestinal tract of mammals, birds and reptiles (Smith, 1965a).

E. coli becomes pathogenic when it reaches tissue outside of the normal intestinal such as urinary tract, biliary tract and other sites in the abdominal cavity (Carroll et al., 2016). Approximately, 90% of first UTI in young women are caused by *E. coli* (Carroll et al., 2016). Most of UTI involving the bladder or kidney are caused by a small number of O antigen types with virulence factors facilitating colonization and subsequent clinical infections (Carroll et al., 2016). Other associated disease caused by *E. coli* are diarrheal diseases that are common worldwide (Carroll et al., 2016). There are seven groups of *E. coli* pathotypes classified by their pathogenicity profiles (clinical disease, virulence factors and phylogenetic profiles); Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E. coli* (ETEC), Shiga toxin-producing *E. coli* (STEC), Enteroinvasive *E. coli* (EIEC), Enteroaggregative *E. coli* (EAEC), Diffusely Adherent *E. coli* (DAEC) and Adherent Invasive *E. coli* (AIEC) (Clements et al., 2012). In developing countries ETEC, EPEC and EAEC contributes to infantile diarrhoea with potentially fatal consequences when untreated (Clements et al., 2012). In contrast, in developed countries these infections are mild and self-limiting (Clements et al., 2012). EHEC and more recently EAEC and STEC are associated with food poisoning outbreaks in developed countries (Clements et al., 2012). While AIEC is associated with Crohn's disease but does not cause diarrhoeal disease (Croxen and Finlay, 2010).

Pathogenic *E. coli* strains are identified in the environment, food or clinical samples, and for understanding epidemiology, serotyping based on antigens is one of the requirements (Wang et al., 2010). Serotyping method is based on its O (somatic), H (flagellar) and K (capsular) surface antigen profiles (Kauffman, 1944) which exist in many combinations, and their combinations make specific serotypes (Nataro and Kaper, 1998). The O-antigen is a component of the thermostable LPS found in the cell wall of *E. coli* (Orskov et al., 1977). An important virulence factors which play a major role in the pathogen-host interactions (DebRoy et al., 2011). The H-antigen is the flagellar filament of *E. coli* and it is made up of a single protein, flagellin (Wang et al., 2010). The importance of H-antigen in *E. coli* is in the typing because strains causing epidemic diarrheal diseases can be differentiated from the normal stool flora by its unique O:H antigenic combinations (Evans and Evans, 1996). As with the capsular (K) antigens in *E. coli*, they have virulence capabilities of resisting phagocytosis by the host leucocytes (Glynn and Howard, 1970).

Another important determinant for pathogenic *E. coli* is by grouping them according to their phylotypes. *E. coli* are composed of four main phylogenetic groups (A, B1, B2 and D2) (Clermont et al., 2000). Phylo-groups B2 or D strains are responsible for extra-intestinal infection than members of A or B1 (Picard et al., 1999; Johnson and Stell, 2000). Assigning *E. coli* isolates to these groups are determined by PCR assay to detect the genes *chuA* and *yjaA* and a DNA fragment

TspE4.C2, later characterized as a putative lipase esterase gene (Gordon et al., 2008).

1.4 Definitions of multidrug-resistant (MDR), extensively drug-resistant (XDR) and pandrug-resistant (PDR)

Resistant bacteria are categorized based on their antibiotic resistance profiles. MDR is when bacteria are non-susceptible to at least one agent in three or more antimicrobial categories (Magiorakos et al., 2012). While XDR bacteria are non-susceptible to at least one agent in all but two or fewer antimicrobial categories (Magiorakos et al., 2012). PDR bacteria are non-susceptible to all agents in all antimicrobial categories (Magiorakos et al., 2012). These definitions were proposed based on a joint initiative by the European Centre for Disease Prevention and Control (ECDC) and Centers for Disease Control and Prevention (CDC) (Magiorakos et al., 2012).

1.5 Antimicrobial agents

1.5.1 Definition

Antimicrobial agents are a natural or synthetic compound that kill or inhibit the growth of microorganisms (fungi, virus, bacteria) (Carroll et al., 2016). In the context of an antibiotic, it is a chemical substance that prevents bacterial growth by inhibiting the cell from dividing (bacteriostatic) or by killing them (bactericidal) (Carroll et al., 2016).

1.5.2 Overview of the mode of actions of antibiotics

Summary of the mode of action of antibiotics is shown in Figure 6.

1.5.2.1 Inhibitors of cell wall synthesis

The cell wall of bacteria is made of peptidoglycan (long sugar polymers) (Kahne et al., 2005). The peptidoglycan undergoes cross-linking of the glycan strands by the action of transglycosidase while the peptide chains extend from the sugars in the polymers to form cross-links from one peptide to another (Kahne et al., 2005). In the presence of PBPs (penicillin binding proteins), the D-alanly-alanine portion of peptide chain is cross linked by glycine residues (Reynolds, 1989). The cross-linking aids in strengthening the cell wall (Kapoor et al., 2017).

The primary target of β-lactam antibiotics are the PBPs where during the interaction of the PBP with the β-lactam ring, prevents the peptidoglycan being synthesised (Kapoor et al., 2017). This leads to the disruption of the peptidoglycan layer and lysis of bacterium (Džidic et al., 2008).

Gylcopeptide antibiotics binds to the D-alanly D-alanine portion of the peptide side chain of the precursor peptidoglycan subunit (Kapoor et al., 2017). For

example, vancomycin (large drug molecule) will prevent the binding of D-alanyl subunit with the PBP, hence inhibiting cell wall synthesis (Grundmann et al., 2006; Džidic et al., 2008).

1.5.2.2 Inhibitors of cell membrane function

Cell membranes are important barriers that segregate and regulate the intra- and extracellular flow of substances (Kapoor et al., 2017). A disruption or damage to this structure could result in leakage of important solutes essential for the cell's survival (Kapoor et al., 2017). Because this structure is found in both eukaryotic and prokaryotic cells, the action of this class of antibiotics is often poorly selective and can often be toxic for systemic use in the mammalian host (Kapoor et al., 2017). Most clinical usage is therefore limited to topical applications, for examples; polymyxin B and colistin (Kapoor et al., 2017).

1.5.2.3 Inhibitors of protein synthesis

The process of utilising information in bacterial DNA to synthesise an RNA molecule referred to mRNA (messenger RNA) is known as transcription (Kapoor et al., 2017). And in translation, the ribosome synthesis proteins present in mRNA (Kapoor et al., 2017). The bacterial 70S ribosome is composed of two ribonucleoprotein subunits, which are the 30S and 50S subunits (Yoneyama and

Katsumata, 2006). Hence, these subunits are targeted by antibiotics inhibiting protein synthesis (Vannuffel and Cocito, 1996; Johnston et al., 2002).

For example, the action of tetracyclines (doxycycline, minocycline or chlortetracycline) on the 30S subunit of the ribosome (Kapoor et al., 2017). These antibiotics act upon the conserved sequences of the 16S rRNA of the 30S ribosomal subunit to prevent binding to tRNA to the A site (Wise, 1999; Yoneyama and Katsumata, 2006).

With the 50S subunit, chloramphenicol interacts with the conserved sequences of the peptidyl transferase cavity of the 23S rRNA of the 50S subunit, thus inhibiting protein synthesis by preventing binding of tRNA to the A site of the ribosome (Vannuffel and Cocito, 1996; Yoneyama and Katsumata, 2006).

1.5.2.4 Inhibitors of nucleic acid synthesis

Fluoroquinolones (FQ) antibiotics inhibit the bacterial enzyme DNA gyrase (Kapoor et al., 2017). The function of this enzyme to nick the double-stranded DNA, introduces supercoiling and then reseals the nicked ends (Kapoor et al., 2017). This process is necessary to prevent excessive positive supercoiling of the strands when they separate to allow replication or transcription which are carried

out by two A subunits and two B subunits of the DNA gyrase (Kapoor et al., 2017). A subunit carries out nicking of DNA, B subunit introduces negative supercoils and then the strand is resealed back by A subunit (Kapoor et al., 2017). The FQ will bind to the A subunit with high infinity and interfere with its strand cutting and resealing function (Kapoor et al., 2017).

1.5.2.5 Inhibitors of other metabolic processes

Both sulfonamides and trimethoprim inhibit distinct steps in the folic acid pathway (Kapoor et al., 2017). However, the combination of both drugs acts as a distinct step on the same biosynthetic pathways that shows synergy and a reduced mutation rate for resistance (Yoneyama and Katsumata, 2006). Sulfonamides will competitively inhibit dihydropteroate synthase with higher affinity for the enzyme than the natural substrate, p-amino benzoic acid (PABA) (Kapoor et al., 2017). While trimethoprim act at a later stage of folic acid synthesis and inhibits enzyme dihydrofolate reductase (Kapoor et al., 2017).

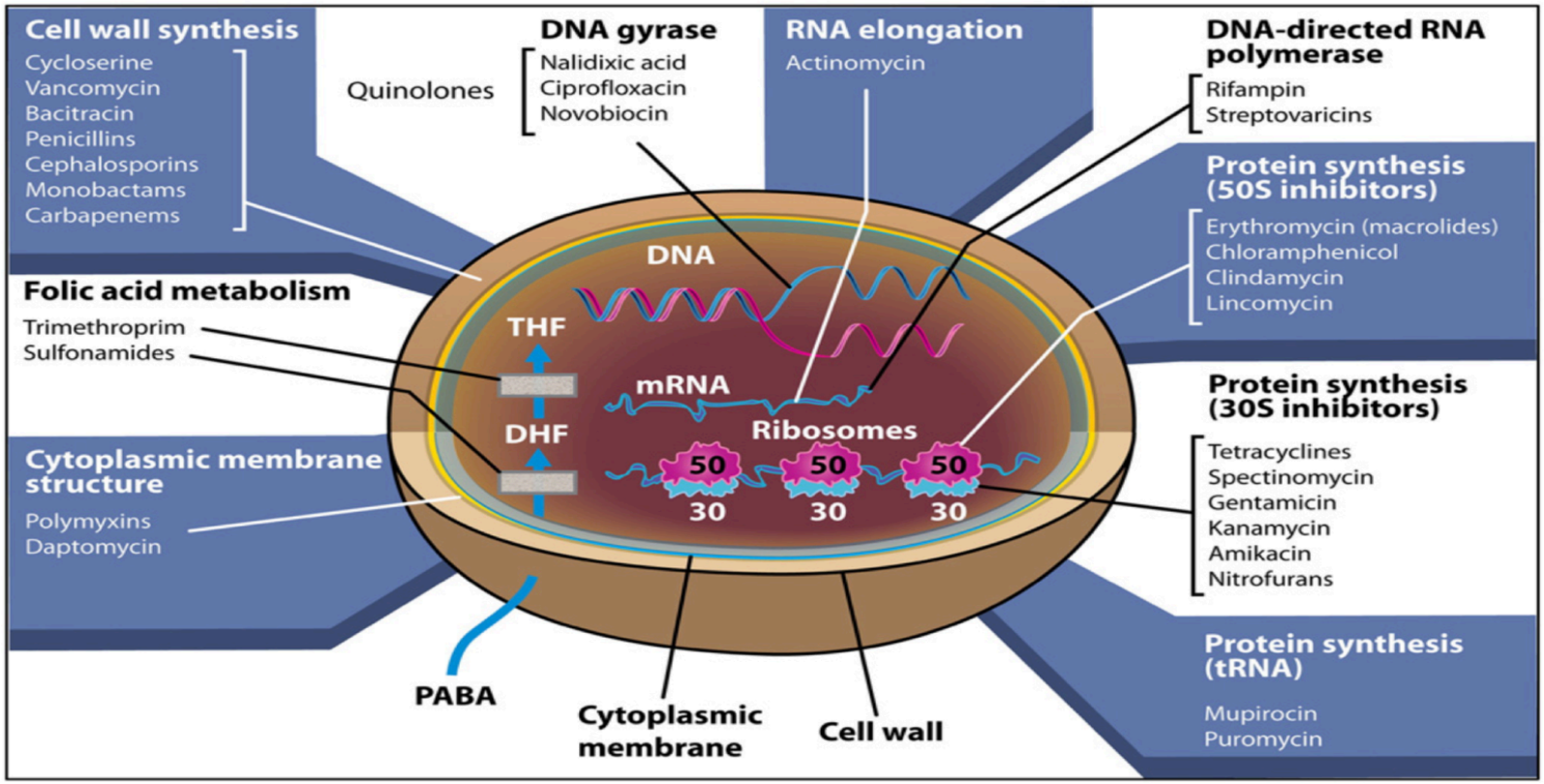


Figure 6. Mechanism of actions of antibiotics on bacteria. Taken from (Brooks and Brooks, 2014)

1.5.3 Overview of antibiotic resistance mechanisms

Summary of antibiotic resistance mechanisms is shown in Figure 7.

1.5.3.1 Enzymatic inactivation of antibiotic

The most common mechanism of antibiotic resistance (Courvalin et al., 2010). The production of enzymes can either be intrinsic (encoded by the chromosomal gene carried by the bacteria) or acquired through horizontal gene transfer (plasmid or transposons) (Courvalin et al., 2010). For example ß-lactamase enzymes which hydrolyse ß-lactam ring of ß-lactam antibiotics. (Courvalin et al., 2010)

1.5.3.2 Target modification

1.5.3.2.1 Enzymatic

Polymyxin resistance is an example of target modification by the overproduction of phosphoethanolamine transferase enzymes (Courvalin et al., 2010). The enzyme causes modification of the LPS (lipopolysaccharide) by attaching to L-Ara4N (4-amino-4-deoxy-L-arabinose) or 2-aminoethanol residue of the phosphate group on the LPS, hence preventing polymyxin penetration and binding (Nummila et al., 1995).

1.5.3.2.2 Mutational

Mutations can be spontaneous causing amino acid changes or nucleotide substitution (Courvalin et al., 2010). With quinolone resistance, type II topoisomerase is targeted, which is composed of DNA gyrase (DNA replication) and topoisomerase IV subunits (chromosomal segregation during cell division) (Courvalin et al., 2010). Mutations in the genes encoding these subunits have been reported; *gyrA* and *gyrB* in DNA gyrase subunit while *parC* and *parE* in topoisomerase IV subunit (Courvalin et al., 2010).

1.5.3.2.3 Antibiotic sequestration and target protection

Sequestration of antibiotic by bacteria neutralizes the effect of the drug (Courvalin et al., 2010). This mechanism of resistance has been observed with third generation cephalosporin (C3G) in "derepressed" mutants of Gram-negative bacilli overproducing chromosomal cephalosporinase AmpC, due to the relative stability of beta-lactams to the enzyme (Sanders and Sanders, 1985).

With regards to target protection, bacteria produce a protein that can protect the target from interacting with antibiotics (Courvalin et al., 2010). For example, with tetracycline, the Tet(O) and Tet(M) proteins can displace tetracycline from the ribosomal A site (aminoacyl-tRNA), allowing protein synthesis to continue normally (Connell et al., 2003).

1.5.3.2.4 Loss of membrane permeability and active efflux

Loss of membrane permeability has been seen in fosfomycin resistance due to a deficiency in glycerol-3-phosphate active transport system which this antibiotic uses to enter the cytoplasm, leading to impermeability (Arca et al., 1997). While in *P. aeruginosa*, defect and synthesis in the porin, OprD (facilitating diffusion of basic amino acids and gluconate) due to mutations can prevent the entry of imipenem causing resistance (Dib et al., 1995; Pai et al., 2001).

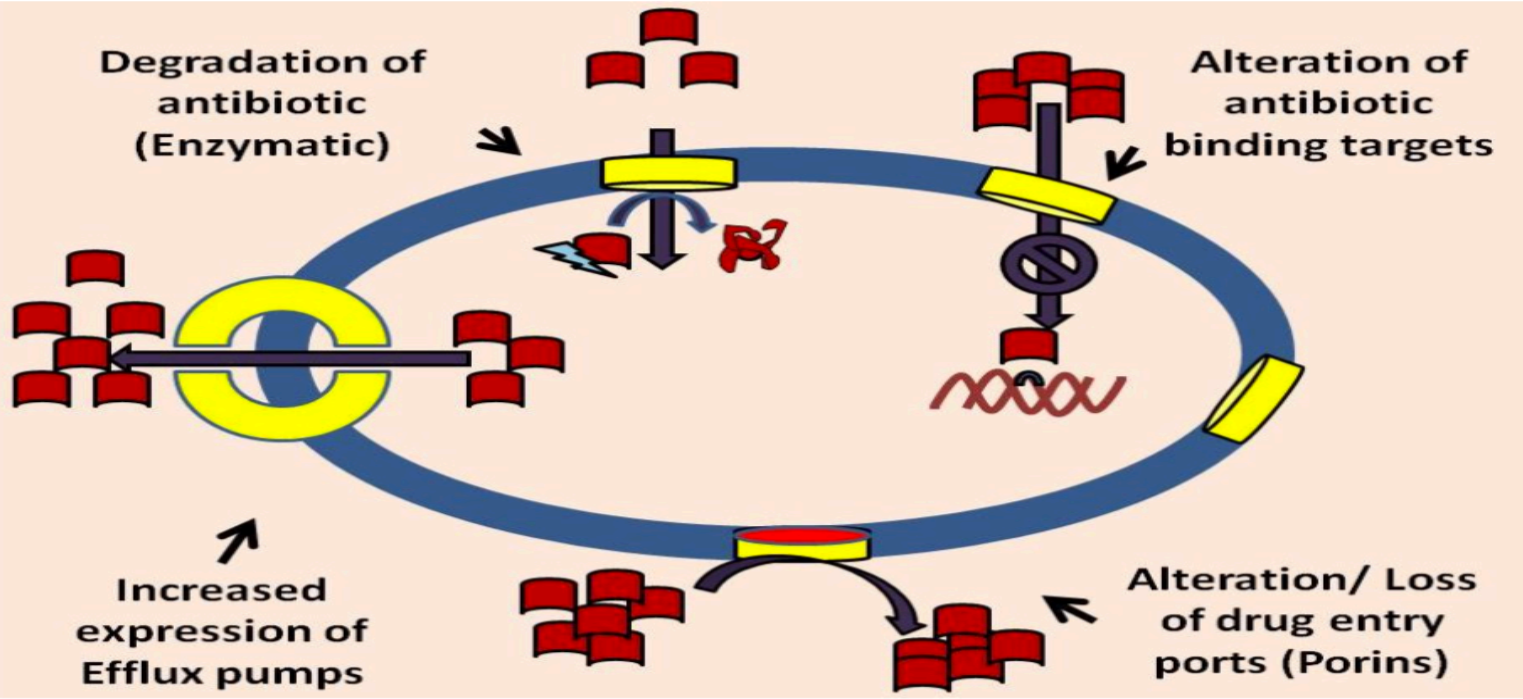


Figure 7. Summary of the mechanism of antibiotic resistance. Red colour represents antibiotics. Yellow colour channels are drug entry ports/porins. Taken from (Andersen et al., 2015).

1.6 Antibiotics and mechanisms of resistance

1.6.1 Carbapenem

Carbapenems (doripenem, ertapenem, imipenem and meropenem) possess the broadest spectrum of activity and greatest potency against Gram-positive and Gram-negative bacteria (Papp-Wallace et al., 2011). They work by inactivating the PBP (penicillin binding proteins), enzymes with an essential role in cell wall synthesis, leading to cell death (Vollmer et al., 2008; Yao et al., 2012). Carbapenem are often used as antibiotic of last-resort when patients with infections become gravely ill or are suspected of harbouring resistant bacteria (Bradley et al., 1999; Paterson, 2000; Paterson, 2002; Paterson and Bonomo, 2005; Torres et al., 2007).

1.6.1.1 Carbapenem and other β-lactam antibiotic resistance mechanisms

The most important mechanism of carbapenem resistance is the production of β-lactamases (carbapenemases) (Munita and Arias, 2016). β-lactamases are classified according to their ambler classification based on their amino acid homology which includes Class A, B, C and D β-lactamases (Ambler, 1980; Jaurin and Grundström, 1981). Most important are the class A, B and D carbapenemases (Munita and Arias, 2016). These enzymes can be found on the bacterial chromosome or plasmids (Palzkill, 2013).

There are more than 1000 different β-lactamases described (www.lahey.org/studies) and from the new current website http://www.laced.uni-stuttgart.de/ (TEM, SHV and class B enzymes) and National Centre for Biotechnology Information, NCBI https://www.ncbi.nlm.nih.gov/pathogens/isolates#/refgene/

1.6.1.1.1 Class A β-lactamases

An important property of Class A β-lactamases is the presence of a serine residue in the catalytic site, which is also shared with class C and D enzymes (Munita and Arias, 2016). Most class A enzymes are inhibited by clavulanic acid and they can hydrolyse monobactams but not cefoxitin and cefotetan (Munita and Arias, 2016).

There is a wide range of proteins having different catalytic activities with Class A enzymes, extending from penicillinases (TEM-1, SHV-1), Extended-spectrum β-lactamases, ESBLs (CTX-M) to carbapanemases (KPC) prevalent in several Gram-negative species carried on plasmids (Munita and Arias, 2016). However, three are typically chromosomally encoded which includes IMI [imipenem-hydrolyzing enzyme], SME [*Serratia marcescens* enzyme] and NMC [not-metallo-enzyme carbapenemase] (Queenan and Bush, 2007).

KPC, a clinically important class A enzyme was first reported in 1996 from *K. penumoniae* isolate, recovered from a patient in North Carolina, USA (Yigit et al., 2001). Although KPC carbapenemases are predominantly found in *Klebsiella* spp, and they have also been reported in other gram-negatives, including *Enterobacter* spp., *E. coli*, *Proteus mirabilis*, *Salmonella* spp (Munita and Arias, 2016) and in non-lactose fermenters such as *P. aeruginosa* (Poirel et al., 2010b). There are a total of 37 KPC-like variants to date https://www.ncbi.nlm.nih.gov/pathogens/isolates#/refgene/, most of them are located in plasmids harboring transposable elements (e.g. Tn*4401*) or in association with insertion sequences like IS*Kpn6* and IS*Kpn7* (Nordmann et al., 2009). Hence, causing dissemination of variants. Interestingly, most of the endemic outbreaks of KPC and international outbreaks belongs to clonal expansion of strains of *K. pneumoniae* belonging to clonal complex 258 (CC258) and more specifically to sequence type, ST258 (Cuzon et al., 2010; Logan and Weinstein, 2017). These strains harbours bla_{KPC-2} or bla_{KPC-3} gene located on a Tn3-based transposon, Tn4401 (Kitchel et al., 2009; Cuzon et al., 2011). In terms of the propagation of bla_{KPC}, it is a complex process (Logan and Weinstein, 2017). There are two distinct genetic clades (I and II) found in a circulating ST258 *K. pneumoniae* (Logan and Weinstein, 2017). In addition, several sequence types have been found to carry bla_{KPC} associated with a variety of plasmids (Patel and Bonomo, 2013; Carattoli, 2009; Chen et al., 2014).

Another clinically important class A enzyme is the CTX-M (cefotaxime-Munich) ESBLs (Extended Spectrum β-lactamases) that have been reported worldwide, typically found in *K. pneumoniae*, *E. coli* and other Enterobacteriaceae (Munita and Arias, 2016). CTX-M-1 was the first enzyme of clinical origin that was described in enterobacterial strains isolated in Europe in the late 1980s (Bonnet, 2004). The sources of CTX-M determinants are chromosomal genes resident in members of the genus *Kluyvera* which is part of an environmental species with little or no pathogenic activity against humans (Rossolini et al., 2008). The precursor of genes encoding CTX-M-1 and CTX-M-2 group have been found in strains of *Kluyvera ascorbata* (Bonnet, 2004; Rodriguez et al., 2004). As for the precursor of genes encoding CTX-M-8 and CTX-M-9 group, it has been detected in strains of *Kluyvera georgina* (Bonnet, 2004; Olson et al., 2005). However, the original sources of genes encoding members of the CTX-M-25 and CTX-M-49 subgroups are currently unidentifiable, but most likely comprise other members of the genus *Kluyvera* (Rossolini et al., 2008).

In contrast to other Amber class A ESBLs such as TEM-3, this enzyme did not derived from TEM or SHV, it was likely acquired from *Kluyvera* spp. (an environmental bacterium and non-pathogenic) through horizontal gene transfer (Bonnet, 2004).

CTX-M enzymes are mostly associated with the insertional sequence, IS*Ecp1* and transposon, Tn*402-like* which mobilise the enzymes (Poirel et al., 2005). This is captured on broad-range conjugative plasmids or phage-like sequences that serves a vehicle for dissemination (Poirel et al., 2005). Other different IS that have been identified upstream of the *bla*$_{\text{CTX-Ms}}$ genes includes IS*CR*1, IS*10*, and IS*26* (Canton et al., 2012).

1.6.1.1.2 Class B β-lactamases

Class B metallo-β-lactamases (MBLs) have a broad substrate spectrum and have the ability to catalyze the hydrolysis of virtually all β-lactam antibiotics except for monobactams (Palzkill, 2013). They are known as metallo-β-lactamases because they utilize a metal ion (most usually Zinc) as a cofactor (instead of a serine residue) for the nucleophilic attack of the β-lactam ring (Munita and Arias, 2016). Class B MBLs are not readily inhibited by mechanism-based inhibitors such as clavulanate, sulbactam, or tazobactam that are effective against serine-based, class A β-lactamases (Perez-Llarena and Bou, 2009; Drawz and Bonomo, 2010). Apart from serine-based ezymes, MBLs are inactivated by metal chelators such as EDTA (Ethylene Diamine Tetra Acetic acid) (Drawz and Bonomo, 2010).

MBLs discovered forty years ago did not cause any serious problem for antibiotic therapy as they were located in the chromosome of non-pathogenic bacteria (Lim et al., 1988; Walsh et al., 1994). However, during the 1990's the situation changed

with the spread of IMP- and VIM-type MBL in Gram-negative pathogens, including Enterobacteriaceae, *Pseudomonas aeruginosa,* and *Acinetobacter baumannii* (Laraki et al., 1999; Lauretti et al., 1999).

The IMP- and VIM-type enzymes are encoded as gene cassettes and reside with other resistance genes within integron structures that are associated with transposons and can insert into bacterial chromosome or within plasmids (Laraki et al., 1999; Cornaglia et al., 2011).

IMP, VIM, SPM and NDM are the most of the clinically important MBLs (Munita and Arias, 2016). In the early 1990s, IMP-type enzymes were first reported in Japan in *S. marcescens* (Munita and Arias, 2016). Since then, there are currently 79 IMP-like variants (https://www.ncbi.nlm.nih.gov/pathogens/isolates#/refgene/). IMP-type enzymes have been described worldwide in Enterobacteriaceae *Pseudomonas* spp., and *Acinetobacter* spp, among other organisms (Munita and Arias, 2016). In addition, bla_{IMP} genes have been identified on large-size plasmids and forming part of Class 1 integron (Poirel et al., 2007). As with VIM-type enzymes, it was first described in the late 1990s in Verona, Italy and have since disseminated throughout the globe (Munita and Arias, 2016). VIM-type enzymes were initially found in *Pseudomonas aeruginosa*; however, its association with Class 1 integron, along with reports in locating them in different types of Mobile Genetic Element

(MGE) has led to their dissemination to many different bacterial species and major concern worldwide (Munita and Arias, 2016). Among the VIM variants described to date, VIM-2 is the most widely distributed enzyme, with reports from Europe, Asia, Africa, and the Americas (Cornaglia et al., 2011).

More recent of the MBLs was the NDM-type enzyme reported in 2008 (Munita and Arias, 2016). NDM-type enzyme was identified in *Klebsiella pneumoniae* isolate recovered from a Swedish patient who had been previously admitted to a hospital in New Delhi, India (Munita and Arias, 2016). This enzyme was designated NDM-1, in reference to its origin (Kumarasamy et al., 2010). NDM-1 shares little amino acid identity, for example 32% with VIM-1, however, its hydrolytic profile is very similar to other members of Ambler class B enzymes (Munita and Arias, 2016). Since the first description of NDM-1, eight variants of this enzyme have been identified (NDM-1 to -8) (Dortet et al., 2014c).

The bla_{NDM} gene has been found in several types of plasmids and it is readily transferable among different species of Gram-negative (Munita and Arias, 2016). This gene is also associated with the presence of insertion sequences such as the IS*Aba125*, but not usually related to integron-like structures (Nordmann et al., 2011b). In addition, NDM-type MBL genes have been present in several epidemic clones, this includes *K. pneumoniae* ST11 and ST147 and *Escherichia coli* ST131 and ST101, which are known to harbor other β-lactamase genes and antibiotic

resistance determinants (Walsh, 2010; Patel and Bonomo, 2013; Dortet et al., 2014c).

Hence it is a public health concern, as the *bla*$_{NDM}$ gene has shown to be readily transmissible among different types of Gram-negative organisms, spreading to many countries in a short period and becoming one of the most feared resistance determinants in several parts of the world (Cornaglia et al., 2011).

Interestingly, in the Indian subcontinent (India and Pakistan), the *bla*$_{NDM}$ genes are not only found disseminated among nosocomial pathogens but in community-associated isolates (Munita and Arias, 2016). Several reports have found this gene in the soil and drinking water for human consumption, suggesting that its ability to disseminated through the human microbiota (Walsh et al., 2011).

1.6.1.1.3 Class C β-lactamases

The most clinically relevant class C enzyme is AmpC, which is a cephalosporinase that is generally chromosomally-mediated (Munita and Arias, 2016). AmpC β-lactamases will hydrolyze narrow-, broad-, and expanded-spectrum cephalosporins and cephamycins and resist inhibition by clavulanate, sulbactam, and tazobactam (Thomson, 2010). When this enzyme is hyperproduced, it may

cause resistance to penicillins, aztreonam, cephamycins, and narrow-, broad-, and expanded-spectrum cephalosporins (Thomson, 2010).

Plasmid-mediated AmpC β-lactamases have been detected in some isolates of *Klebsiella* spp., *Salmonella* spp., *C. freundii*, *E. aerogenes*, *P. mirabilis*, and *E. coli* (Jacoby, 2009) and are typically associated with multidrug resistance (Thomson, 2010). The most commonly encountered plasmid-mediated AmpC β-lactamases belong to the ACT, ACC, CFE, CMY, DHA, FOX, LAT, MIR and MOX families (Jacoby, 2009; Thomson, 2010). While chromosomal AmpC has been identified in *E. cloacae*, *E. aerogenes*, *C. freundii*, *S. marcescens*, *Providencia* sp., *Morganella morganii*, and *P. aeruginosa*, among others (Munita and Arias, 2016).

The expression of *ampC* is generally inducible, controlled by complex regulatory mechanisms (Munita and Arias, 2016). For example, in *Enterobacter* spp, AmpR (transcriptional regulator of the LysR family) will act as a repressor of the transcription of bla_{AmpC} (Munita and Arias, 2016). Usually, under non-inducing conditions (absence of β-lactams), AmpR will bind to peptidoglycan precursors (UDP-MurNAc pentapeptides) and the interaction of AmpR with its cognate promoter does not occur hence, resulting in the absence of bla_{AmpC} transcription (Munita and Arias, 2016).

On the contrary, the presence of β-lactams will alter cell wall homeostasis resulting in accumulation of peptidoglycan byproducts such as anhydro-muropeptides that compete for the same AmpR binding site with the UDP-MurNAc pentapeptides (Munita and Arias, 2016). Due to this competition for the same binding site, AmpR is released and is able to interact with the bla_{AmpC} promoter, activating transcription of the gene (Jacobs et al., 1997; Johnson et al., 2013).

AmpD is another mechanism by which *ampC* is overexpressed, a cytosolic amidase that recycles muropeptides (Munita and Arias, 2016). AmpC overexpression occurs when AmpD effectively reduces the concentration of anhydro-UDP-MurNAc tri-, tetra- and pentapeptides preventing displacement of UDP-MurNAc pentapeptide from AmpR (Munita and Arias, 2016). Additionally, mutations in *ampD* are often seen in isolates that constitutively overproduce AmpC; hence, the clinical efficacy of cephalosporins is affected (Munita and Arias, 2016). Cefepime is not a good substrate for AmpC enzymes, but high-level production of AmpC may markedly increase cefepime MICs (Schmidtke and Hanson, 2006; Jacoby, 2009).

1.6.1.1.4 Class D β-lactamases

The Class D β-lactamases have emerged as a prominent resistance mechanism against β-lactam antibiotics that previously had efficacy against infections caused by pathogenic bacteria, especially by *Acinetobacter baumannii* and the Enterobacteriaceae (Antunes and Fisher, 2014).

Class D β-lactamases include a wide range of enzymes that were initially differentiated from the class A penicillinases due to their ability to hydrolyse cloxacillin and oxacillin faster than benzylpenicillin (Bush et al., 1995). Class D β-lactamases are usually not inhibited by clavulanic acid, tazobactam and sulbactam, whereas their activities may be inhibited *in vitro* by sodium chloride (NaCl) (Poirel et al., 2010a) and more efficiently by avibactam (Docquier and Mangani, 2018).

There are many OXA variants with over 779 variants currently recognised (https://www.ncbi.nlm.nih.gov/pathogens/isolates#/refgene/). OXA β-lactamase genes are found on both the chromosomes as well as the plasmids of diverse bacterial species such as *Acinetobacter*, *Shewanella*, *Pseudomonas*, and *Burkholderia* (Sanschagrin et al., 1995; Poirel et al., 2010a). In addition, most of chromosomal Class D β-lactamases have now been transferred to plasmids which pose a greater clinical threat (Bush, 2013a; Bush, 2013b).

Examples of the OXA variants are their ability to degrade third generation cephalosporins (ESBLs) (OXA-11 in *P. aeruginosa*) and carbapenems (OXA-23 detected in *A. baumannii* from Scotland) (Munita and Arias, 2016).

Additionally, OXA-23 identified in *A. baumannii* was isolated from Scotland (Poirel et al., 2010a) and found to be plasmid mediated after its transfer to *Acinetobacter junii* (Scaife et al., 1995). The genetic structures which are involved in the mobilization includes transposon and insertion elements (Munita and Arias, 2016). For example, the $bla_{\text{OXA-23}}$ gene originating from the chromosome of *A. radioresistens* may be mobilized onto plasmids diffusing into *A. baumannii* by the involvement of a composite transposon Tn*2006*, formed by two IS*Aba1* elements (Corvec et al., 2007; Mugnier et al., 2009).

Since then, OXA-23-positive *A. baumannii* strains have spread globally, to locations including France (Corvec et al., 2007), Bulgaria (Stoeva et al., 2008), Iran (Feizabadi et al., 2008), the United Arab Emirates (Mugnier et al., 2008), Tunisia (Mansour et al., 2008), Brazil (Dalla-Costa et al., 2003), and Australia (Valenzuela et al., 2007).

Another example is OXA-48, a widely disseminated class D carbapenemases which were originally described in 2001 in Turkey from a multidrug resistant isolate of *K. pneumoniae* (Munita and Arias, 2016). The $bla_{\text{OXA-48}}$ gene has been associated with insertion sequence IS*1999* in *K. pneumoniae* (Poirel et al., 2004). In addition, the

bla$_{OXA-48}$ gene was part of composite transposon Tn*1999*, made of two copies of IS*1999*, with the copy located upstream of *bla*$_{OXA-48}$ providing promoter sequences for *bla*$_{OXA-48}$ expression (Aubert et al., 2006).

Many other types of OXA enzymes have been described that are often found in a wide range of MGEs (Munita and Arias, 2016). In other instances, the OXA-containing MGE inserts in the chromosome, resulting in core-genome genes encoding OXA enzymes (Munita and Arias, 2016). For example, OXA-51 enzyme is found intrinsic to *Acinetobacter baumannii* isolates (Evans and Amyes, 2014). This enzyme is naturally found in the chromosome of this isolates (Evans and Amyes, 2014).

More importantly, successful intra- and interspecies transmission of these genes have been described with enzymes such as OXA-23 and OXA-58, currently disseminated globally (Munita and Arias, 2016).

Summary of all the ß-lactamases mentioned above are shown in Table 2.

Molecular classification of ß-lactamases	Example	Remarks
A	KPC-like	Carbapenemase
	CTX-M-like	ESBL
	SHV-1-like	Penicillinases
	TEM-1-like	
	IMI	Chromosomally encoded
	NMC	
	SME	
B	IMP-like	Carbapenemases
	NDM-like	
	SPM-like	
	VIM-like	
C	AmpC	Chromosomally encoded
	ACT-like	Plasmid encoded
	ACC-like	
	CFE-like	
	CMY-like	
	DHA-like	
	FOX-like	
	LAT-like	
	MIR-like	
	MOX-like	
D	OXA-11-like	ESBL

	OXA-23-like	Carbapenemase
	OXA-48-like	Carbapenemase

Table 2. Molecular classification of ß-lactamases.

1.6.2 Polymyxin: an old antibiotic repurposed

Polymyxins are a family of nonribosomal lipopeptide nonapeptides, isolated from *Bacillus polymyxa* in 1947 (Walsh and Wencewicz, 2016). Polymyxin B and E (colistin) were used against Gram-negative bacterial infections for decades, however, due to neurotoxicity and nephrotoxicity, this led to their replacement with subsequent generations of less toxic antibiotics (Walsh and Wencewicz, 2016).

The recent emergence of multidrug-resistant Gram-negative organisms including the rise of carbapenem resistance had led to the repurposing of polymyxins antibiotic (Walsh and Wencewicz, 2016).

Colistin disrupts membrane integrity through displacement of cations like Mg^{2+} and Ca^{2+} in the outer membrane (Falagas and Kasiakou, 2005). The lipopolysaccharide (LPS) is therefore destabilized, consequently increasing the permeability of the bacterial membrane, leading to leakage of the cytoplasmic content and ultimately causing cell death (Falagas and Kasiakou, 2005; Li et al., 2006a)

1.6.2.1 Polymyxin Resistance mechanisms

1.6.2.1.1 Chromosomal-mediated

Most mechanisms conferring resistance to colistin are directed against modifications of the lipid A moiety of lipopolysaccharide (LPS), which is the primary target of colistin (Baron et al., 2016). Mechanisms that are associated with colistin resistance across different bacterial genera may be different, but more often than not the majority always follow the common pathway of lipid A modifications with 4-amino-4-deoxy-L-arabinose (L-Ara4N) and/or phosphoethanolamine (PEtN) (Baron et al., 2016).

Resistance mechanisms described to date involve lipopolysaccharide (LPS) modification, particularly through derivatization of lipid A phosphate moieties with a sugar or ethanolamine (Wright et al., 2015). These modifications reduce the electrostatic affinity between the cationic colistin and anionic LPS (Wright et al., 2015). Mutations in the transcriptional regulatory systems controlling these LPS modifications are a common genetic mechanism leading to colistin resistance (Wright et al., 2015). For example, the PhoPQ and PmrAB two-component regulatory systems (TCRS) regulate expression of the gene (*pmrC*) that codes for the addition of phosphoethanolamine (pETN) and genes encoding biosynthesis and lipid A transfer of 4-amino-4-deoxy-L-arabinose (L-ara4N) (*pmrHFIJKLM*) (Wright et al., 2015). Other regulatory components in this pathway include PmrD

and MgrB, two connector proteins that convey feedback between the PmrAB and PhoPQ TCRS (Lippa and Goulian, 2009; Cannatelli et al., 2013; Luo et al., 2013; Lopez-Camacho et al., 2014) (**Error! Reference source not found.**). Mutations in *p mrAB*, *phoPQ*, and *mgrB* have been identified as mechanisms conferring colistin resistance in several Gram-negative pathogens, including *K. pneumoniae* (Adams et al., 2009; Beceiro et al., 2011; Lesho et al., 2013; Cannatelli et al., 2014; Jayol et al., 2014; Kim et al., 2014).

Recently, mutations in the *crrB* gene, belonging to a third two-component system (named CrrAB for Colistin Resistance Regulation) (Figure 8) are involved in LPS modifications, have also been associated with colistin resistance (Wright et al., 2015; Cheng et al., 2016). Mutations in the *crrB* gene is responsible for the increase of the *crrC* gene transcription, which in turns regulates the expression of the *pmrC* gene and the *pmrHFIJKLM* operon, through the PmrAB two-component system (Cheng et al., 2016). The expression of these genes leads to the addition of cationic groups on the LPS and consequently to colistin resistance (Jayol et al., 2017).

1.6.2.1.2 Plasmid-mediated

The encoded MCR-1 protein is a member of the phosphoethanolamine transferase enzyme family, *mcr-1* gene acquisition results in the addition of

phosphoethanolamine to lipid A (Figure 8), and consequently results in a more cationic LPS and reduction in polymyxin affinity (Poirel et al., 2017). Detailed plasmid-mediated colistin resistance mechanism would be further discussed in Chapter 4.

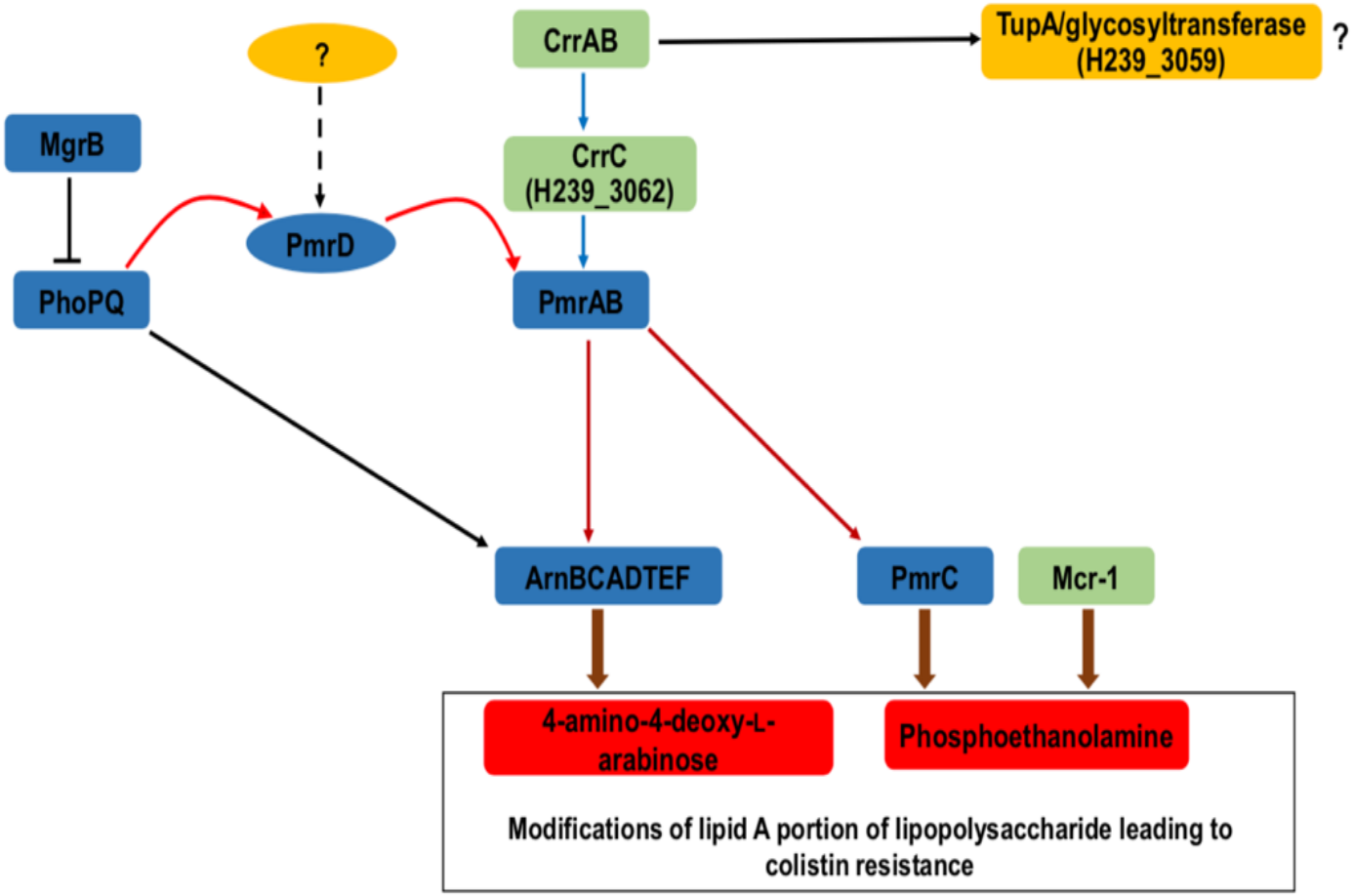

?
CrrAB
TupA/glycosyltransferase (H239_3059)
?
MgrB
CrrC (H239_3062)
PmrD
PhoPQ
PmrAB
ArnBCADTEF
PmrC
Mcr-1
4-amino-4-deoxy-L-arabinose
Phosphoethanolamine
Modifications of lipid A portion of lipopolysaccharide leading to colistin resistance

Figure 8. Two-component systems (TCSs) for colistin resistance in bacteria. Activation of the TCSs, stimulates the transcription of lipopolysaccharide (LPS) modification loci *arnBCADTEF* (*pmrHFIJKLM*) and *pmrC*, which leads to the synthesis of 4-amino-4-deoxyl-arabinose and phosphoethanolamine (PEtN), respectively.TCS CrrAB (recenltly discovered) activates PmrAB and not the PhoPQ via H239_3062 (now referred to as CrrC) with subsequent upregulation of *arnBCADTEF* and *pmrC*. In addition, the recently identified, novel PEtN-encoding gene *mcr-1*, which modifies by adding phosphoethanolamine to the lipid A of LPS. Green colour - novel protein-encoding genes. Yellow and dashed lines - missing links (genes) or pathways not yet fully understood, respectively. Taken from (Baron et al., 2016)

1.6.3 Aminoglycosides

Resistance to aminoglycosides due to target modification involves G1405 methylation of 16S rRNA (Garneau-Tsodikova and Labby, 2016). The first report of 16s rRNA methyltransferase gene was *armA* in multidrug-resistant *Klebsiella pneumoniae* isolated in France (Galimand et al., 2003). Seven other enzymes associated with 16S rRNA methylation were reported which includes *rmtA* (Yokoyama et al., 2003), *rmtB* (Doi et al., 2004), *rmtC* (Wachino et al., 2006), *rmtD* (now renamed as rmtD1) (Doi and Arakawa, 2007; Doi et al., 2007b), *rmtD2* (shares 96.4% amino acid identity with rmtD1) (Tijet et al., 2011), *rmtE* (Davis et al., 2010) and *npmA* (Wachino et al., 2007).

Aminoglycoside-modifying enzymes (AME) are the most common aminoglycoside resistance mechanism (Garneau-Tsodikova and Labby, 2016). AME catalyze the modification at −OH or $-NH_2$ groups of the 2-deoxystreptamine nucleus or the sugar moieties leading to *in vivo* resistance phenotype (Ramirez and Tolmasky, 2010). AME are divided into acetylation of an amino group [*N*-acetyltransferases (AAC)], adenylation [*O*-nucleotidyltransferases (ANT)] and phosphorylation [*O*-phosphotransferases (APH)] of a hydroxyl group (Ramirez and Tolmasky, 2010). The AME enzymes and its encoding gene are summarized in Table 3.

Enzymes	Genes	Resistance phenotype
***N*-acetyltransferases (AAC)**		
AAC(3)-I	*aac(3)-Ia*	Gentamicin
	aac(3)-Ib	
AAC(3)-II	*aac(3)-Iia*	Gentamicin, Tobramycin
	aac(3)-Iib	
	aac(3)-Iic	
AAC(3)-III	*aac(3)-IIIa*	Gentamicin, Tobramycin, Kanamycin, Neomycin, Paromycin
	aac(3)-IIIb	
	aac(3)-IIIc	
AAC(3)-IV	*aac(3)-Iva*	Gentamicin, Tobramycin
AAC(3)-VI	*aac(3)-Via*	Gentamicin
AAC(6')-I	*aac(6')-Ia*	Tobramycin, Amikacin
	aac(6')-Ib	
	aac(6')-Ic	
	aac(6')-Id	
	aac(6')-Ie	
	aac(6')-If	
	aac(6')-Ig	
	aac(6')-Ih	
	aac(6')-Ii	
AAC(6')-II	*aac(6')-Iia*	Gentamicin, Tobramycin
	aac(6')-Iib	
AAC(6')-APH(2")	*aac(6')-aph(2")*	Gentamicin, Tobramycin, Amikacin
AAC(2'-I)	*aac(2')-Ia*	Gentamicin, Tobramycin

***O*-nucleotidyltransferases (ANT)**		
ANT(2”)-I	*ant(2”)-Ia*	Gentamicin, Tobramycin, Kanamycin
	ant(2”)-Ib	
	ant(2”)-Ic	
ANT(3”)-I	*ant(3”)-Ia*	Streptomycin, Spectinomycin
ANT(4’)-I	*ant(4’)-Ia*	Tobramycin, Amikacin
ANT(4’)-II	*ant(4’)-IIa*	Tobramycin, Amikacin
ANT(6)-I	*ant(6)-Ia*	Streptomycin
***O*-phosphotransferases (APH)**		
APH(2”)-I	*aph(2”)-Ia*	Gentamicin, Tobramycin, Amikacin
APH(3”)-I	*aph(3’)-Ia*	Kanamycin, Neomycin, Paromycin
	aph(3’)-Ib	
	aph(3’)-Ic	
APH(3’)-II	*aph(3’)-IIa*	Kanamycin, Neomycin, Paromycin, Gentamicin B
APH(3’)-III	*aph(3’)-IIIa*	Kanamycin, Neomycin, Paromycin, Amikacin, Gentamicin B
APH(3’)-IV	*aph(3’)-IVa*	Kanamycin, Neomycin, Paromycin
APH(3’)-V	*aph(3’)-Va*	Neomycin, Paromycin
	aph(3’)-Vb	
	aph(3’)-Vc	
APH(3’)-VI	*aph(3’)-VIa*	Kanamycin, Neomycin, Paromycin, Amikacin, Gentamicin B
	aph(3’)-VIb	
APH(3’)-VII	*aph(3’)VIIa*	Kanamycin, Neomycin
APH(3”)-I	*aph(3”)-Ia*	Streptomycin
	aph(3”)-Ib	
APH(6)-I	*aph(6)-Ia*	Streptomycin
	aph(6)-Ib	

	aph(6)-Ic
	aph(6)-Id

Table 3. Summary of the classification of aminoglycoside modifying enzymes (AME), AME genes and its resistance phenotypes. Adapted from (Shaw et al., 1993).

Another member of an aminoglycoside antibiotic is streptomycin and resistance is mediated by the linked *strA-strB* gene pair, inactivating two phosphotransferase enzymes (aminoglycoside-3″-phosphotransferase and aminoglycoside-6″-phosphotransferase) (Shaw et al., 1993). These gene pair are widely disseminated among diverse Gram-negative bacteria and they have been detected in bacteria colonizing plants, animals, humans and farmed fish (Sundin and Bender, 1996; Sunde et al., 1998; L'Abee-Lund and Sorum, 2000).

1.6.4 Quinolones

Quinolone resistance arises due to point mutations in targeting both the gyrase and topoisomerase IV or to active efflux (Drlica and Zhao, 1997). The subunits of DNA gyrase are GyrA, a 97-kDa protein encoded by the *gyrA* gene, and GyrB, a 90-kDa protein encoded by the *gyrB* gene (Jacoby, 2005). The corresponding subunits of topoisomerase IV are ParC (75 kDa) and ParE (70 kDa) (Jacoby, 2005). DNA gyrase can introduce negative supercoils into DNA, can remove both positive and negative supercoils, and can catenate and decatenate closed circular molecules (Jacoby, 2005). DNA topoisomerase IV can also remove positive and negative

supercoils and is even better at decatenation than is gyrase (Jacoby, 2005). The two enzymes work together in the replication, transcription, recombination, and repair of DNA (Jacoby, 2005).

Resistance involves amino acid substitutions in a region of the GyrA or ParC subunit termed the "quinolone-resistance–determining region" (QRDR) (Jacoby, 2005). This region occurs on the DNA-binding surface of the enzyme, (Morais Cabral et al., 1997) and for *E. coli*, DNA gyrase includes amino acids between positions 51 and 106 (Friedman et al., 2001), with "hot spots" for mutation at amino acid positions 83 and 87 (Jacoby, 2005). The QRDR in DNA gyrase is near tyrosine 122, which is covalently bound to phosphate groups on DNA in the initial strand-breaking reaction (Jacoby, 2005).

AcrAB-TolC efflux pump in *E. coli* plays a major role in quinolone efflux and has multiple controls (Jacoby, 2005). Mutations in *acrR* (a repressor of *acrAB*) causes an increase in pump activity (Morais Cabral et al., 1997). Conversely, inactivation in *marR* (a repressor of *marA*) due to mutation allow MarA to activate *acrAB*, *tolC*, and a gene that decreases translation of *ompF*, hence collectively decreasing influx and increased efflux of quinolones (Cohen et al., 1989; Alekshun and Levy, 1997).

The plasmid-mediated quinolone resistance gene was named "*qnr*" (Jacoby, 2005). Qnr proteins were shown to be members of the pentapeptide repeat protein (PRP) family, and to be related to MfpA, a protein cloned from

Mycobacterium smegmatis that also causes (low-level) resistance to fluoroquinolones (Jacoby, 2005).

Six major Qnr families have been identified: QnrA, QnrB, QnrS, QnrC, QnrD and QnrVC (Rodriguez-Martinez et al., 2016). For example, QnrA can reverse the ability of fluoroquinolones to inhibit DNA gyrase, (Tran and Jacoby, 2002) since it can bind directly to DNA, reduce the binding of gyrase to DNA and protecting topoisomerase IV from the quinolone (Jacoby, 2005). QnrA can bind to the DNA gyrase holoenzyme and its respective subunits, GyrA and GyrB (Jacoby, 2005). This binding of Qnr to gyrase does not require the presence of an enzyme-DNA-quinolone complex since it takes place in the absence of relaxed DNA, ciprofloxacin and ATP (Tran et al., 2005a; Tran et al., 2005b)**.**

Two other mechanisms unrelated to Qnr, but also encoded by plasmid genes, have been discovered; one of these corresponds to an acetyltransferase [AAC(6)-Ib-cr] (Robicsek et al., 2006) which is a variant of an enzyme involved in aminoglycoside modification (and resistance), the other includes active efflux pumps such as QepA and OqxAB (Hansen et al., 2004; Perichon et al., 2007; Yamane et al., 2007). QepA is member of the major facilitator superfamily (MFS) transporters able to eliminate hydrophilic fluoroquinolones from the cell (ciprofloxacin and norfloxacin especially) by an active efflux mechanism, leading to a moderate (2- to 64-fold) increase in the MIC (Perichon et al., 2007; Yamane et al., 2007; Cattoir et al., 2008a; Cattoir et al., 2008b). OqxAB is an efflux system in the resistance-nodulation-cell division (RND) family of transporters, commonly found in *K.*

pneumoniae (Jacoby, 2005). OqxAB was initially recognized on transmissible plasmids responsible for resistance to olaquindox, used for growth enhancement in pigs (Sorensen et al., 2003; Hansen et al., 2004). OqxAB has a wide substrate specificity, including not only quinolones such as ciprofloxacin, flumequine, norfloxacin and nalidixic acid but also chloramphenicol and trimethoprim (Hansen et al., 2007).

1.6.5 Chlorampenicol and florfenicol

Both chloramphenicol (Cm) and its fluorinated derivative florfenicol (Ff) are both highly potent inhibitors of bacterial protein synthesis (50S subunit) (Schwarz et al., 2004). Florfenicol are mostly used in veterinary medicine and not approved for human use (White et al., 2000).

The most common mechanism in chloramphenicol resistance is enzymatic inactivation by acetylation of the drug via different types of chloramphenicol acetyltransferases (CATs) (Murray and Shaw, 1997). Other resistance mechanisms that have been reported includes efflux systems, inactivation by phosphotransferases, mutations of the target site and permeability barriers (Shaw, 1983; Murray and Shaw, 1997). CATs are composed of two defined types which distinctly differ in their structure; type A and type B CATs (Schwarz et al., 2004).

The Type A CATs have been detected in a wide variety of bacteria (Shaw, 1983) (Murray and Shaw, 1997) with at least 16 distinct groups, A-1–A-16, of *catA* genes (Schwarz et al., 2004). For example in group A-1, *catI* gene was originally identified as part of transposon Tn*9* (Alton and Vapnek, 1979) in *Escherichia coli* and has been detected on a variety of resistance plasmids of Gram-negative bacteria, such as *Acinetobacter* spp. (Elisha and Steyn, 1991), *Photobacterium damselae* subsp. *piscicida*, formerly known as *P. piscicida* (Kim and Aoki, 1993), and *Pseudomonas putida*(Nomoto and Yamagishi, 1991). Recently, *cat* genes of this group have also been identified in *S.* Typhi, (Parkhill et al., 2001) *Serratia marcescens* (Gilmour et al., 2003) and *S. flexneri* (Luck et al., 2001).

Other than chloramphenicol resistance, some of the type A CATs have specific properties, such as the capability to mediate resistance also to fusidic acid (Volker et al., 1982; Shaw, 1983) or sensitivity to inhibition by thiol-reactive reagents (Murray et al., 1990).

For Type B CATs, occasionally referred to as xenobiotic acetyltransferases, they also inactivate chloramphenicol (Cm) by acetylation (Schwarz et al., 2004). Type B *cat* genes are composed of at least five different groups: B-1–B-5 (Schwarz et al., 2004). The first type B *cat* gene (*catB1*) describe was cloned from the chromosome of *A. tumefaciens* (Tennigkeit and Matzura, 1991; Goodner et al., 2001). As with

the *catB2* gene, it was initially found on the multiresistance transposon Tn*2424* from *E. coli* (Parent and Roy, 1992).

Florfenicol resistance mechanism is unknown but, it is associated with the *flo* determinant, a highly conserved gene sequence detected in *Salmonella enterica* serovar Typhimurium DT104 (Bolton et al., 1999; Briggs and Fratamico, 1999) and in the fish pathogen *Pasteurella piscicida* (*Photobacterium damsela*) (Kim and Aoki, 1996). In addition, the *flo* gene confers resistance to both chloramphenicol and florfenicol (Kim et al., 1993; Bolton et al., 1999).

1.6.6 Fosfomycin

Mechanisms of fosfomycin resistance in Gram-negative bacteria can be conferred by defects in the transporters across the cytoplasmic membrane, amino acid substitution in the MurA active site which decreases fosfomycin binding affinity, and production of the fosfomycin-inactivating enzyme FosA (Falagas et al., 2016). FosA is an Mn^{2+}- and K^{+}-dependent dimeric glutathione *S*-transferase, it works by catalyzing the nucleophilic addition of glutathione to the epoxide ring of Fosfomycin (Bernat et al., 1997) and can be found encoded on a bacterial chromosome or a plasmid (Ito et al., 2017). The *fosA* gene was first described in *Serratia marcescens*, carried on transposon Tn*2921* located on a conjugative plasmid (Mendoza et al., 1980; Garcia-Lobo and Ortiz, 1982), but it has a high identity with chromosomal *fosA* of *Enterobacter cloacae*, where it likely originated

(Ito et al., 2017). In addition, other plasmid-borne glutathione transferase (FosA type) enzymes are FosA3, FosA4, FosA5, and FosC2 (Silver, 2007).

1.6.7 Macrolide

Acquired macrolide resistance results from a variety of mechanisms of resistance, several of which have already been reported in *Enterobacteriaceae* (Leclercq, 2002; Ojo et al., 2004). This includes target site modification by methylases encoded by *erm* genes, in particular *erm*(A), *erm*(B), and *erm*(C). (Phuc Nguyen et al., 2009)

In addition, macrolides may be inactivated by modifying enzymes that was first reported in *Enterobacteriaceae* (Arthur et al., 1987; O'Hara et al., 1989) e.g., esterases encoded by *ere*(A) or *ere*(B) genes or phosphotransferases encoded by *mph*(A), *mph*(B), and *mph*(D) genes (Phuc Nguyen et al., 2009). All of these genes confer full cross-resistance between erythromycin and azithromycin (Leclercq, 2002).

1.6.8 Sulfonamide

Sulfonamide resistance is conferred by mutations either in the DHPS (dihydropteroate synthase) gene (*folP*) (Swedberg et al., 1993) or from the acquisition of an alternative DHPS gene (*sul*) (Radstrom and Swedberg, 1988; Sundstrom et al., 1988; Perreten and Boerlin, 2003). DHPS is a catalytic enzyme in the folic acid biosynthesis pathway, resulting in the inhibition of dihydrofolic acid formation (Skold, 2000).

There are three alternative DHPS genes; *sul1* is usually located on the 3' conserved region of a class 1 integron (Skold, 1976) and is frequently identified with this potentially mobile element in the slurry and soil environment (Sundstrom et al., 1988; Rosser and Young, 1999; Guerra et al., 2003). The *sul2* gene was first identified on RSF1010 plasmid in *Escherichia coli* and has also been found on small nonconjugative resistance plasmids (Radstrom and Swedberg, 1988). While, the *sul3* gene were found during a study identified in sulfonamide resistance in pathogenic *E. coli* isolates from swine from Switzerland (Perreten and Boerlin, 2003).

1.6.9 Tetracycline

The most common tetracycline resistance mechanism in Gram-negative bacteria (*E. coli*) is the extrusion of the drug from the cytoplasm via efflux (Chopra and Roberts, 2001). Tetracycline-specific efflux pumps are members of the major facilitator superfamily (MFS) of efflux pumps (Paulsen et al., 1996). The tetracycline-specific efflux pump operates by transporting tetracycline in an energy-dependent fashion, via proton exchange, thereby reducing the intracellular concentration of the drug (Chopra and Roberts, 2001). The Tet pumps are divided into six groups based on amino acid sequence; Tet(A), Tet(B), Tet(C), Tet(D), and Tet(E) placed in group 1 due to amino acid sequence similarity (Chopra and Roberts, 2001) (Roberts, 2005). In addition, there are other Tet pumps which

includes Tet(G), Tet(H), Tet(K) and Tet(L) (Roberts, 2005). These tetracycline-specific efflux pumps not only confers resistance to tetracycline but with pump encoded by *tet*(B) gene both tetracycline and minocycline can be extruded (Guay and Rothstein, 1993; Petersen et al., 1999).

1.6.10 Trimethroprim

Mechanism to trimethroprim is caused by modifications in the target enzyme dihydrofolate reductase (dfr) encoded by *dfr* genes (Brolund et al., 2010). So far 30 *dfr* genes have been described and they are usually associated with integrons (Skold, 2001). The *dfr* genes are consecutively numbered from *dfr*1 onwards since this was the first gene observed (Skold and Widh, 1974). In addition, the most prevalent trimethoprim resistance gene among Gram-negative bacteria seems to be *dfr*1, which occurs in a cassette in both class 1 and class 2 integrons (Skold, 2001). In a study done by Brolund et al., molecular characterization of trimethroprim resistance in *E. coli* showed other *dfr* genes alongside *dfrA1* were observed which includes *dfrA5*, *dfrA7*, *dfrA14* or *dfrA17* (Brolund et al., 2010).

Study objectives

AMR is a global health threat that will contribute to 10 million death by 2050. In 2017, the World Health Organisation (WHO) has released a priority list of pathogens of great importance. Top of the priority lists are those designated critical pathogens: carbapenem-resistant *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, Enterobacteriaceae and 3rd generation cephalosporin resistant *E. coli* and *Klebsiella* spp.

With these emerging pathogens compromising the ability to treat infections, a global action plan has been developed by the WHO to tackle AMR (WHO, 2015). These plans set 5 strategic objectives which includes (WHO, 2015):

- Improving awareness and understanding of antimicrobial resistance
- Strengthening knowledge through surveillance and research
- Reducing the incidence of infection
- Optimising the use of antimicrobial agents
- Developing an economic case for sustainable investment that takes account of the needs of all countries, and increases investment in new medicines, diagnostic tools, vaccines and other interventions.

A more simplistic bundled approach could focus on 4 key points:

- **Surveillance** – screening diagnostic tools, antimicrobial stewardship
- **Effective detection** – new rapid diagnostics
- **Effective treatment** – development of antimicrobial agents
- **Effective control strategies** – international collaboration

Hence, the objectives in this study are defined in relation to this approach:

Chapter 2

- Molecular analysis (acquired resistance gene and other mobile genetic elements) of carbapenem-resistant *Acinetobacter baumannii* clinical isolates from Brunei Darussalam.

- Evaluation of an acidimetric method for the detection of carbapenemase production in carbapenem-resistant *Pseudomonas aeruginosa* clinical isolates from Brunei Darussalam.

Chapter 3

- Investigation of the mechanisms of resistance amongst carbapenem-resistant *K. pneumoniae* (CRKP) clinical isolates from Brunei Darussalam.

- Characterisation by multiple phenotypic (RAPID CARB Blue kit, Micronaut-S Beta Lactamase VII, OXA-48 K-SeT) and molecular methods of CRKP clinical isolates

from Brunei Darussalam. In addition, evaluation of an OXA-48 K-SeT lateral flow assay for the detection of OXA-48 carbapenemase in CRE clinical isolates.

Chapter 4

Part 1:

- Development and evaluation of a novel media for the detection of colistin-resistant Gram-negative pathogens.

Part 2:

- To perform antimicrobial surveillance and determine the prevalence of colistin-resistant *E. coli* isolated from chicken cloacal and human clinical faecal samples in Brunei Darussalam.

Part 3:

- Molecular analysis of novel *mcr-1* variants found during surveillance.

Chapter 5

- Investigating the impact of acquiring plasmid-mediated polymyxin resistance (PMPR) in *E. coli* isolated from chickens and humans on the survival, virulence and fitness in a *G. mellonella* infection model.

Chapter 2

Carbapenem-resistant Gram-negative Non-fermenters in Brunei Darussalam

2.1 Introduction

In this chapter, phenotypic and genotypic methods will be performed for detecting carbapenemases, resistance mechanisms and associated mobile genetic elements (MGE) in carbapenem resistant Gram-negative non-fermenters collected in Brunei Darussalam. The non-fermenters include *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, an important World Health Organization (WHO) critical pathogen lists.

With regards to detecting carbapenemases, Modified Hodge Test (MHT) is the only method used in Brunei's Microbiology Laboratory. However, there are limitations to this method such as a lack of specificity and longer time to a result. In addition, molecular diagnostic techniques are not practiced, as Brunei's Microbiology Laboratory is dependent on culture and antibiotic susceptibility testing (AST). In order to progress and combat AMR, phenotypic and genotypic tools should be performed to complement one another. Hence, introducing new phenotypic methods and molecular diagnostic tools will allow earlier detection, inform on resistance mechanisms, better treatment outcomes and improve infection control management.

Recent attention has focused on non-lactose fermenting Gram-negative bacteria such as *Acinetobacter baumannii* and *Pseudomonas aeruginosa* that predominantly cause hospital-acquired infections such as urinary tract infection

(UTI), bloodstream infection (BSI), lower respiratory tract infection (LRTI) and meningitis (CDC, 2011).

Carbapenem-resistant *P. aeruginosa* (CRPA) and *A. baumannii* (CRAB) are very well documented in Southeast Asia (SEA) (Suwantarat and Carroll, 2016). Mechanisms of CR in *P. aeruginosa* are multifactorial and involve non-enzymatic mechanism such as porin loss (*OprD* gene mutation), the combination of efflux pumps (MexAB-OprM) and overexpression of AmpC ß-lactamases (Suwantarat and Carroll, 2016). Additionaly, enzymatic mechanisms such as IMP-type metallo-β-lactamase (bla_{IMP}) and Verona integron-mediated MBL (bla_{VIM}) carbapanemase genes have also been reported in isolates from surveillance studies in the Southeast Asia region (Koh, 2008; Khosravi et al., 2010; Khuntayaporn et al., 2012; Khuntayaporn et al., 2013; Kim et al., 2013; Tang et al., 2014; Teo et al., 2015)

The successful global dissemination of multi-drug resistance (MDR) *Acinetobacter* spp is in association with the successful clonal lineage international clone I, II, III (Matsui et al., 2018) (previously named European clone, EU I, II, III or Global clone 1, 2) (Higgins et al., 2010). With respect to CRAB in SEA region, the dominant type of OXA-carbapenemase gene is mediated by bla_{OXA-23} belonging to global clone 2 (GC2), (Kim et al., 2013; Teo et al., 2015) although both GC1 and GC2 have been reported worldwide (Chaisathaphol and Chayakulkeeree, 2014). Apart from SEA region, Global clone 2 has been reported in Europe and now in South Korea, China and Australia (Kim et al., 2013). This shows the importance of clonal spread where *Acinetobacter baumannii* infections with acquired carbapenemase gene is a serious infection control problem (Higgins et al., 2010). Other OXA

carbapenemases reported in CRAB in this region includes the *bla*$_{OXA-40}$ and *bla*$_{OXA-58}$ (Koh, 2008; Kim et al., 2013; Tang et al., 2014; Teo et al., 2015). With respect to the MLST and plasmid acquired by GC2 in OXA-23 *A. baumannii* isolates in the SEA region, it is unknown.

With the global spread of CRAB, molecular tools such as PCRs targeting the genes encoding carbapenemases, clonal typing and other mobile genetic elements should be performed. For example, the use of multiplex PCR for detecting OXA carbapenemases (OXA-23-like, OXA-24-like, OXA-51-like, OXA-58-like) in *Acinetobacter* spp (Woodford et al., 2006).

Carbapenemase detection in carbapenem-resistant Gram-negative non-fermenters can be a problem in routine diagnostic microbiology laboratories due to limited diagnostic capacity, especially in developing countries (Zellweger et al., 2017). Although, other methods such as combined disk synergy test (Carbapenem Inactivation Method, CIM; ROSCO Kit) and biochemical/acidimetric tests (CarbaAcinetoNP, RAPIDEC® CARBA NP, Blue-Carba) have been developed and evaluated, their availability and costs will be the limiting factor for its use in routine diagnostic laboratories in developing countries (Osei Sekyere et al., 2015). In addition, most of these methods works best in Enterobacteriaceae and less likely with non-fermenters (Osei Sekyere et al., 2015).

Carbapenem inactivation method (CIM) is a method for detecting metallo-β-lactamase (MBL) specifically VIM carbapenemases and the OXA-type carbapenem-hydrolyzing class D β-lactamase, CHDL subgroups (OXA-23, OXA-40, OXA-58 and OXA-143) in *A. baumannii* (van der Zwaluw et al., 2015). CIM works by using 10µg MEM, meropenem (susceptibility-testing disk) immersed and incubated for 2 hours with the test bacterial isolate suspension and placed on Mueller-Hinton (MH) agar inoculated with the *E. coli* indicator control type strain (American Type Culture, Collection, ATCC 25922) as the background (van der Zwaluw et al., 2015). The only challenge with this method is detecting weak carbapenemase activities, especially with CHDL (van der Zwaluw et al., 2015).

CarbAcineto NP test is the updated version of the Carba NP (for enterobacteriaceae) used specifically for carbapenemase detection in *Acinetobacter* spp, that is based on the colorimetric changes and pH-based detection of hydrolysis of the β-lactam ring of imipenem, performed on Trypticase soy (TS) agar (Dortet et al., 2014a; Dortet et al., 2014b). The updated version uses NaCl solution replacing the lysis buffer and increasing the bacterial inoculum to a full calibrated loop in order to increase the enzyme quantity (Dortet et al., 2014b). The use of NaCl solution provides two advantages; it does not buffer enough to interfere with slight pH changes and efficient lysis of the bacteria due to its hyperosmotic properties (Dortet et al., 2014b). The presence MBL-producers will result in a color change of phenol red solution (red to yellow/orange) in less than

15 mins (Dortet et al., 2014b). However, the limitation of this test is the use of higher inoculum to produce a positive result.

RAPIDEC® CARBA NP test kit detects carbapenemase from bacterial colonies from a selective or non-selective agar plate (Garg et al., 2015). The time for detection is around 30 minutes to 2 hours with sensitivity and specificity of 97.8% (Garg et al., 2015). The test was performed using a loop full of bacterial colony picked up from overnight Muller-Hinton agar plates and mixed with API suspension medium (provided with the kit) and incubated at 37°C (Garg et al., 2015). Positive results showed a colour change from red to yellow/orange and negative results remain red (Garg et al., 2015).

In an evaluation study done by Poirel et al., they have documented RAPIDEC® CARBA NP with sensitivity and specificity of 96% when testing it against pre-characterized strains (Poirel and Nordmann, 2015). They have also reported false negative results with Group D carbapenemases; which is in accordance with the fact that Group D carbapenemases are known to lack significant carbapenemase activity (Poirel and Nordmann, 2015). Carbapenemase activity was detected in *A. baumannii* isolates with OXA-23, but no activity with carbapenem-resistant *A. baumannii* due to the overexpression of chromosomally-encoded oxacillinase gene, $bla_{\text{OXA-66}}$ (an OXA-51-like enzyme) (Poirel and Nordmann, 2015).

Blue-Carba test was a modification version of the Carba NP test, the aim being to contain costs and increase simplicity (Pires et al., 2013). Carbapenemase production detection with this method showed 100% sensitivity and specificity for all Gram-negative carbapenemase-producing bacteria (Pires et al., 2013). However, with OXA-48 producers higher inocula are required (Pires et al., 2013).

2.2 Objectives

- Molecular analysis (acquired resistance gene and other mobile genetic elements) of carbapenem-resistant *Acinetobacter baumannii* clinical isolates from Brunei Darussalam.

-To evaluate an acidimetric Blue-Carba method for the production of carbapenemases in carbapenem-resistant *Pseudomonas aeruginosa* clinical isolates from Brunei Darussalam.

2.3 Methods

2.3.1 Bacterial isolates collection

Carbapenem-resistant *Acinetobacter baumannii* (CRAB) (n=109) and *Pseudomonas aeruginosa* (CRPA) (n=22) clinical Isolates were collected from July 2014 – February 2015 and stored in Pro-Lab Diagnostics™ Microbank™ Bacterial and Fungal Preservation System, cryobeads (Fisher Scientific, Leicestershire, UK) at -70^{o}C in Microbiology Laboratory Services, Brunei Darussalam. Isolates were recovered as part of routine testing done in the Microbiology Laboratory Services, based on their carbapenem resistance profiles (resistance to either imipenem or meropenem or both).

2.3.2 Transportation of clinical isolates to the United Kingdom (UK)

Isolates from Pro-Lab Diagnostics™ Microbank™ Bacterial and Fungal Preservation System, cryobeads (Fisher Scientific, Leicestershire, UK) were seeded onto pre-poured Mueller Hinton II Agar (ISOLAB, Selangor, Malaysia) incubated at 37^{o}C for 24 hours and transported using Trans® Amies (MWE, Wiltshire, UK) to Queen Mary University of London, Blizard Institute, Centre for Immunobiology, UK with an estimated delivery of 3 days using FEDEX courier.

Isolates were inoculated back onto Mueller-Hinton 2 (MH2) agar (Sigma-Aldrich, Dorset, UK) in the UK for further testing.

2.3.3 Source of samples

Clinical samples were obtained mostly from the Intensive Care Unit (ICU) and medical wards in RIPAS hospital and also from other district hospitals in Brunei Darussalam. Source of specimens were from respiratory, pus and faecal samples unless otherwise stated in the methodology.

2.3.4 Media and broth preparation

Media used:

Mueller-Hinton 2 (MH2) agar (Sigma-Aldrich, Dorset, UK), Luria-Bertani (LB) agar (Sigma-Aldrich, Dorset, UK), MacConkey agar (Sigma-Aldrich, Dorset, UK).

Broth used:

Luria-Bertani (LB) broth (Sigma-Aldrich, Dorset, UK), Mueller-Hinton 2 (MH2) broth (Sigma-Aldrich, Dorset, UK), Trpyticase Soy Broth (TSB) (Oxoid, Basingstoke, UK)

All media and broth were weighed out following the manufacturer's instruction to a final volume of 500 ml in distilled water. Media and broth were then autoclaved at 15 lbs/sq inch at 121^{o}C for 15min.

2.3.5 Antibiotic Susceptibility Testing (AST) and Mininum Inhibitory Concentration (MIC)

AST and minimum inhibitory concentration (MIC) for both *A. baumannii* and *P. aeruginosa* clinical isolates were tested prior in Brunei using VITEK® 2XL machine, VITEK® 2 GN ID (Gram-Negative Identification) and AST card (GN-75) (bioMérieux, Marcy l'Etoile, France) according to CLSI guidelines (CLSI, 2014). AST results were interpreted as S, I or R (Sensitive, Intermediate or Resistance).

The antibiotic panel tested includes amikacin, ampicillin, aztreonam, cefepime, cefoxitin, ceftazidime, ceftriaxone, ciprofloxacin, colistin, gentamicin, imipenem, meropenem, netilmicin, piperacillin, piperacillin/tazobactam, ampicillin/sulbactam, co-trimoxazole, levofloxacin and ticarcillin/clavulanic acid.

2.3.6 Molecular Methodologies

2.3.6.1 DNA extraction

Analytical Grade Chelex® 100 (Bio-Rad, Hertfordshire, UK) were used for DNA extraction of isolates. Briefly, 5% of chelex were made by mixing 0.5 g of chelex powder with 10 ml of distilled water.

A 5 µl loop full obtained from a bacterial overnight culture on MH2 media was added into 100 µl of 5% Chelex solution in a 1.5 ml sterile Eppendorf tube. The mixtures were then heated in a heat block at 100°C for 15 mins. The mixtures were centrifuged using Heraeus Biofuge Pico centrifuge (DJB Labcare, Buckinghamshire,

UK) at 13000 rpm for 1 min. The DNA lysate (supernatant) was pipetted out and saved down in a new sterile Eppendorf tube and stored at 2-8°C for further testing.

2.3.6.2 DNA purification

Wizard® SV Genomic DNA Purification kit (Promega, Southampton, UK) was used for isolating genomic DNA from Gram-negative bacteria following manufacturers instruction.

1 ml of an overnight culture were pipetted into 1.5 ml sterile Eppendorf tubes. The tube was centrifuged using Heraeus Biofuge Pico centrifuge (DJB Labcare, Buckinghamshire, UK) at 13,000 rpm for 2 mins to pellet the cells. Supernatant was then removed. 600 µl of Nuclei Lysis Solution was added. The cells were then resuspended by gently pipetting. The mixture was then incubated at 80°C for 5 mins to lyse the cells and then cool down to room temperature. 3 µl of RNase solution was added to the cell lysate. Tubes were inverted 2–5 times to mix and then incubated at 37°C for 15–60 mins. The samples were cool down to room temperature.

200 µl of Protein Precipitation Solution was added to the RNase-treated cell lysate. The cell lysate was vortexed at high speed for 20 seconds. The mixture was incubated on ice for 5 mins and then centrifuged at 13,000 rpm for 3 mins. The supernatant containing the DNA was transferred to a clean 1.5 ml Eppendorf tube containing 600 µl of room temperature isopropanol.

The mixture was mixed gently by inversion until the thread-like strands of DNA form a visible mass and then centrifuged at 13,000 rpm for 2 minutes using Heraeus Biofuge Pico centrifuge (DJB Labcare, Buckinghamshire, UK). The supernatant was removed, and the tube drained on clean absorbent paper. 600 µl of 70% ethanol (at room temperature) was then added and gently inverted several times to wash the DNA pellet. The mixture was centrifuged at 13,000 rpm for 2 mins and ethanol carefully aspirated. The tube was then drained on clean absorbent paper and the pellet allowed to air-dry for 10–15 mins.

100 µl of DNA Rehydration Solution was added to the tube and DNA rehydrated by incubating at 65°C for 1 hour. The solution was periodically mixed by gently tapping the tube.

Alternatively, the DNA was rehydrated by incubating the solution overnight at room temperature or at 4°C. The DNA was then stored at 2–8°C for further testing.

2.3.7 PCR (Polymerase Chain Reaction)

2.3.7.1 Master Mix

ThermoPrime 2x ReddyMix PCR Master Mix, with 1.5 mM $MgCl_2$ (Thermofisher Scientific, Dartford, UK)

Final 1X reaction of this master mix contains 0.625 u ThermoPrime Taq DNA Polymerase, 75 mM Tris-HCl (pH 8.8 at 25°C), 20 mM $(NH4)_2SO4$, 1.5 mM $MgCl_2$, 0.01% (v/v) Tween® 20, 0.2 mM each of dATP, dCTP, dGTP and dTTP, precipitant and red dye for electrophoresis.

2.3.7.2 PCR preparation

ThermoPrime 2X ReddyMix PCR Master Mix, with 1.5 mM $MgCl_2$ (Thermofisher Scientific, Dartford, UK) were used unless otherwise stated in the methodology. For 1X concentration, 500 µl of the ThermoPrime 2X ReddyMix PCR Master Mix was added to 500 µl of nuclease free water to final volume of 1 ml.

25 µl of 1X ThermoPrime ReddyMiX PCR Master Mix were dispensed into 0.9 µl amplicon tubes. 1 µl of forward and reverse primers were added. 2 µl of DNA lysate (template) was then added to the mixture.

2.3.7.2.1 Gel electrophoresis

Bionic™ Buffer, 10X Concentrate

Bionic™ Buffer, 10X Concentrate (Sigma-Aldrich, Dorset, UK) was used for the electrophoresis buffer. Briefly, the 1X concentration was prepared by taking 100 ml of Bionic™ Buffer, 10X Concentrate diluted into 1 Litre of distilled water.

TBE buffer (Tris/Borate/EDTA), 10X Molecular Biology Grade

TBE buffer consists of a mixture containing Tris base, boric acid and EDTA. TBE buffer (Tris/Borate/EDTA), 10X Molecular Biology Grade (Promega, Southampton, UK) were used for gel preparation. Similarly, 1X concentration was made by diluting 100 ml of TBE buffer into 1 litre of distilled water.

2.3.7.2.1.1 Agarose gel preparation

1.5% agarose gel were prepared unless otherwise stated in the methodology. The gel was prepared 15-20 mins prior to the end of the PCR run. 1.5% of Ultrapure™ Agarose (Thermofisher Scientific, Dartford, UK) was added with 100 ml of 1X TBE buffer in a conical flask. The mixture was heated in a microwave and then cooled down. 5 µl of ethidium bromide solution (10 mg/ml), molecular grade (Promega, Southampton, UK) were added to the molten mixture. The molten mixture was then poured onto gel electrophoresis plastic cast together with a well comb and allowed to fully solidified.

2.3.7.2.1.2 Electrophoresis chamber

DNA ladders (100 bp or/and 1Kb bp) (NEW ENGLAND BioLabs, Hitchin, UK) were firstly loaded onto the gel's well immersed in 1X Bionic™ buffer in the chamber. DNA samples (controls and tests) were then loaded onto the remaining wells.

Positive and negative leads are connected to the chamber and a power supply. The voltage for running the gel electrophoresis in most of these studies was 120 V for approximately 45 mins.

2.3.7.2.1.3 Band visualization from gel electrophoresis

Bands were visualized using G:BOX (Syngene, Cambridge, UK) under UV transillumination and recorded.

2.3.8 DNA amplicon purification

The DNA amplicons of the positive bands were cleaned using MinElute® PCR purification Kit (Qiagen, Crawley, UK) following manufacturers instruction.

PCR sample was diluted 5-fold with Buffer PB. The mixture was then transferred to QIAquick spin column in a provided 2 ml collection tube. The tube was centrifuged for 1 min at 13,000 rpm using Heraeus Biofuge Pico centrifuge (DJB Labcare, Buckinghamshire, UK). The flow-through was discarded and placed back with the column. The DNA was then washed with 0.75 ml of Buffer PE and centrifuged for 1 min. The flow-through again discarded and placed into a 1.5 ml sterile Eppendorf tube. The DNA was eluted by adding 50 µl EB buffer (10 mM Tris-chloride, pH 8.5) or water, by centrifuging again for 1 min.

The concentration of the purified DNA (ng/µl) were measured using NanoDrop ND-1000 Spectrophotometer (Labtech International, Heathfield, UK). Following

the manufacturer's instruction, the DNA sample absorbance is measured at a wavelength of 260 nm. 2 sets of ratios are calculated. The first is 260/280, the ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA. A ratio of approximately 1.8 is generally accepted as pure DNA. Lower than 1.8, may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm.

The second is 260/230, the ratio absorbance at 260 nm and 230 nm, a secondary measure of nuclei acid purity. This ratio is often higher than the ratio of 260/280 and are commonly in the range of 1.8-2.2. Ratio lower than this value may indicate the presence of co-purified contaminants.

The final nuclei acid quantification is calculated using Beer-Lambert equation (modified to use an extinction coefficient with units ng-cm/ µl):

Beer-Lambert equation, c = (A * e)/b

c = the nucleic acid concentration in ng/microliter,

A = the absorbance in AU,

e = the wavelength-dependent extinction coefficient in **ng-cm/microliter**

b = the path length in cm.

2.3.9 Sanger sequencing

Purified DNA templates were sent for Sanger sequencing (SourceBioScience, Cambridge, UK). All sequencing reactions were performed on an ABI 3730Xl

analyzer (Applied Biosystems, Warrington, UK) with 50cm array using BigDye® Terminator v3.1 Cycle Sequencing Kit protocol (SourceBioScience, Cambridge, UK). Chromatograms were analysed and consensus sequence assembled using GeneTool Lite v1.0. Nucleotide and amino acid sequences were compared using blastn, blastp (NCBI, Rockville, Maryland) and ExPASy translate tool (Swiss Institute of Bioinformatics).

2.4 Molecular analysis of carbapenem-resistant *A. baumannii* clinical isolates

2.4.1 Molecular typing

2.4.1.1 RAPD typing and DiversiLab rep-PCR typing

RAPD (random amplification of polymorphic DNA) typing is a technique where one or more primers of variable length are arbitrarily selected and allowed to anneal to the DNA template at low stringency (Power, 1996). RAPD PCR was performed on 50 ng DNA template, as previously described (Renders et al., 1996). DNA extraction was performed as mentioned in section 2.3.6.1 and carried out in 25 µl volume. After PCR, amplicons are run on 2% agarose gel. Band profiles differing by one or two bands are considered not to be epidemiologically relevant genetic differences (Kersulyte et al., 1995).

Primers used are listed in Appendix A and PCR cycling conditions in Table 3.

For gel electrophoresis and band visualization see section 2.3.7.2

Based on preliminary results from RAPD typing, 4 CRAB clinical isolates (AB98, AB114, AB133 and AB143) were sent for Diversilab rep-PCR typing. DiversiLab rep-PCR typing (bioMérieux, Nürtingen, Germany) was done by our collaborators at the University of Cologne, Germany (Higgins et al., 2010). The results were analysed with the DiversiLab software using the modified Kullback–Leibler statistical method to determine distance matrices and the unweighted pair group method with arithmetic averages (UPGMA) to create dendrograms (Higgins et al., 2010). Isolates that clustered >95% were considered related (Saeed et al., 2006). The controls included representative isolates belonging to pan-European *A. baumannii* clonal complexes I, II and III (Higgins et al., 2010).

2.4.2 PCR for OXA carbapenemases, 16s rRNA methyltransferase and AmpC resistance genes

Multiplex PCR were used for the identification of OXA carbapenemases (OXA-23-like, OXA-24-like, OXA-51-like and OXA-58-like), 16s rRNA methyltransferase (*rmtA, rmtB, rmtC, rmtD, armA* and *npmA*), and single PCR were used to amplify AmpC as previously described (Corvec et al., 2003; Woodford et al., 2006; Fritsche et al., 2008).

DNA extraction was performed as mentioned in section 2.3.6.1 . 2 μl of the bacterial lysate was added to 25 μl of ThermoPrime 2x ReddyMix PCR Master Mix and 1 μl each of the forward and reverse primers in a 0.6 ml PCR tube.

For PCR preparation, gel electrophoresis and band visualization see section 2.3.7.2

DNA amplicon of single PCR producing positive band was cleaned (see section 2.3.8) and sent for Sanger sequencing (see section 2.3.9).

2.4.3 PCR for Integron, mapping of Class 1 integron and insertion element

Multiplex PCR was used for screening of integrons (int1, int2, and int3), PCR for mapping of class 1 integron in the 5' and 3' conserved segment and insertion element (IS*Aba1*) as previously described (Lévesque et al., 1995; Dillon et al., 2005; Ruiz et al., 2007).

DNA extraction was performed as mentioned in section 2.3.6.1 . 2 μl of the bacterial lysate was added to 25 μl of ThermoPrime 2x ReddyMix PCR Master Mix and 1 μl each of the forward and reverse primers in a 0.6 ml PCR tube.

For PCR preparation, gel electrophoresis and band visualization see section 2.3.7.2

DNA amplicon of single PCR producing positive bands was cleaned (see section 2.3.8) and sent for Sanger sequencing (see section 2.3.9).

PCR cycling conditions used in this study are listed in Table 4.

PCR reaction	PCR cycling conditions	References
RAPD typing	Initial denaturation 94^{o} C for 5min, following 4 cycles of 94^{o} C for 5 min, 40^{o} C for 5 min and 72^{o} C for 5 min; then 30 cycles of 94^{o} C for 1 min, 55^{o} C for 1 min and 72^{o} C for 2 min Final extension of 10 min at 72^{o} C.	(Kersulyte et al., 1995)
Multiplex OXA carbapenemases	Initial denaturation 94^{o} C for 5 min, following 30 cycles of 94^{o} C for 25 s, 52^{o} C for 40 s and 72^{o} C for 50 s. Final extension of 6 min at 72^{o} C.	(Woodford et al., 2006)
Multiplex 16s rRNA methyltransferase	Initial denaturation 96^{o} C for 1 min, following 25 cycles of 94°C for 15s, 58° C (45° C for those isolates found to be PCR negative at 58° C) for 30 s, 72° C for 60 s. Final extension of 5 min at 72°C.	(Fritsche et al., 2008)
AmpC PCR	Initial denaturation of 94°C for 90 s followed by 30 cycle of 30 s at 94° C, 30 s at 57°C and 1 min at	(Corvec et al., 2003)

	72°C. Final extension at 72°C for 10 min.	
Multiplex integron PCR	Initial denaturation 94^{o}C for 2 min, 35 cycle of 94^{o}C for 1 min, 59^{o}C for 1 min and 72^{o}C for 1 min. Final extension at 72^{o}C for 7 min.	(Dillon et al., 2005)
5' and 3' conserved segment (CS) PCR mapping of class 1 integron	Three-step profile: 35 cycles of 1 min at 94^{o} C, 1 min at 55^{o} C, and 5 min at 72^{o} C for a total of 35 cycles. 5 s added to the extension time at each cycle.	(Lévesque et al., 1995)
IS*Aba1* PCR	Initial denaturation of 94° C for 2 min, 30 cycles of 92^{o} C for 1 min, annealing 52^{o} C for 1 min and 72^{o} C for 1 min. Final extension of 72^{o}C for 10 min.	(Ruiz et al., 2007)

Table 4. PCR cycling conditions for each PCR reactions.

A. baumannii 12 (AB12) (EU clone 2/clinical isolate PFGE-UK defined UK lineage South East clone) (Betts et al., 2017), *A. baumannii* 14 (AB14) (EU clone 2/clinical isolate PFGE-UK defined UK lineage OXA-23 clone 1) (Coelho et al., 2006; Turton et al., 2009; Wareham et al., 2011), *A. baumannii* 16 (AB16) (EU clone 1/clinical isolate PFGE-UK defined UK lineage OXA-23 clone 2) (Coelho et al., 2006; Turton et al., 2009; Wareham et al., 2011) and type strain ATCC *A. baumannii* 19606 (AB19606) were used as control unless otherwise stated.

2.5 Blue-Carba test for carbapenemase detection in Carbapenem-resistant *P. aeruginosa* (CRPA) clinical isolates.

Blue-Carba test method was used for the detection of carbapenemase in CRPA clinical isolates as previously described (Pires et al., 2013). Blue-Carba test was performed by direct inoculation of bacterial colonies growing on Mueller-Hinton agar plates and by direct inoculation of bacterial colonies into Mueller-Hinton (MH2) broth.

The bacterial isolates were then added to the test and control solution.

2.5.1 Test solution

Test solution is a mixture comprising of 10 ml of 0.04% bromothymol blue sodium salt (Thermo Fisher Scientific, Lanchashire, UK), 0.06 g of Imipenem powder (Sigma-Aldrich, Dorset, UK), 10 µl of Zinc sulfate, $ZnSO_4$ solution (Sigma-Aldrich, Dorset, UK) and 2 drops of 0.5 M Hydrochloric acid, HCL (Sigma-Aldrich, Dorset, UK) adjusted to a final pH 7.0.

2.5.2 Control solution

Negative control solution is a mixture comprising of 10 ml of 0.04% bromothymol blue sodium salt and drops of 0.5 M HCL adjusted to a final pH 7.0.

2.5.3 Blue-Carba set up for direction inoculation from MH plates

96 well sterile microtiter plates were used for the test and control solution.

100 µl of test and control solution was added to the wells. A 5 µl loop of bacteria were then added to the wells. Plates were incubated at 37 °C on shaking incubator for a total of 2 hours.

2.5.4 Blue-Carba setup for direct inoculation of bacterial colonies into Mueller-Hinton (MH2) broth

MH2 broth was prepared as mentioned in section 2.3.4

Briefly, 3 ml of MH2 broth was pipetted out into a sterile 25 ml universal tube. A 5 µl loop full of isolates were inoculated into the broth. The universal tubes were then incubated at 37° C for 24 hours on innova 2100 platform shaker (New Brunswick Scientific; Eppendorf, Stevenage UK).

750 µl of the bacterial inoculum from the turbid MH2 broth was pipetted out into 1.5 ml sterile Eppendorf tube. The tubes were centrifuged at 13000 rpm for 1 min using Heraeus Biofuge Pico centrifuge (DJB Labcare, Buckinghamshire, UK).

100 µl of supernatant were dispensed into test and control solution.

Two positive controls *Klebsiella pneumoniae* [KP19 (2015)] (NDM-1-like) and *P. aeruginosa* [PA25] (imipenem and meropenem resistance) and American Type Culture Collection (ATCC) *P. aeruginosa* (PA27853) as negative control were used in this study.

2.5.5 Interpretation of Blue-Carba Assays

Positive results

Test group yellow and control remains blue or green or test group green and control remains blue (Figure 9)

Negative results

No colour change (remains blue) in the test and control group (Figure 9).

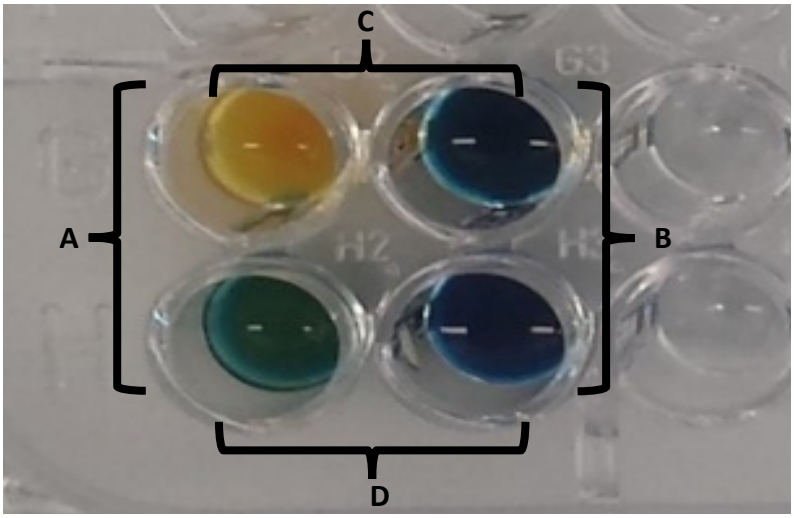


Figure 9. Blue-Carba assay result interpretations. A- Positive results, B- Negative results, C- Test group and D- Control group

2.6 Results

2.6.1 Carbapenem-resistant *Acinetobacter baumannii* (CRAB)

2.6.1.1 Antibiotic Susceptibility Testing (AST)

One hundred and nine carbapenem-resistant *A. baumannii* (CRAB) clinical isolates were tested using VITEK 2XL (bioMérieux, Marcy l'Etoile, France).

Co-resistance were observed with penicillin (ampicillin, piperacillin) cephalosporins (ceftazidime, cefoxitin, cefepime, ceftriaxone), aminoglycosides (gentamicin, netilmicin), fluoroquinolones (ciprofloxacin, levofloxacin), piperacillin/tazobactam, ticarcillin/clavulanic acid, ampicillin/sulbactam, co-trimoxazole and aztreonam. Colistin remained susceptible (Figure 10).

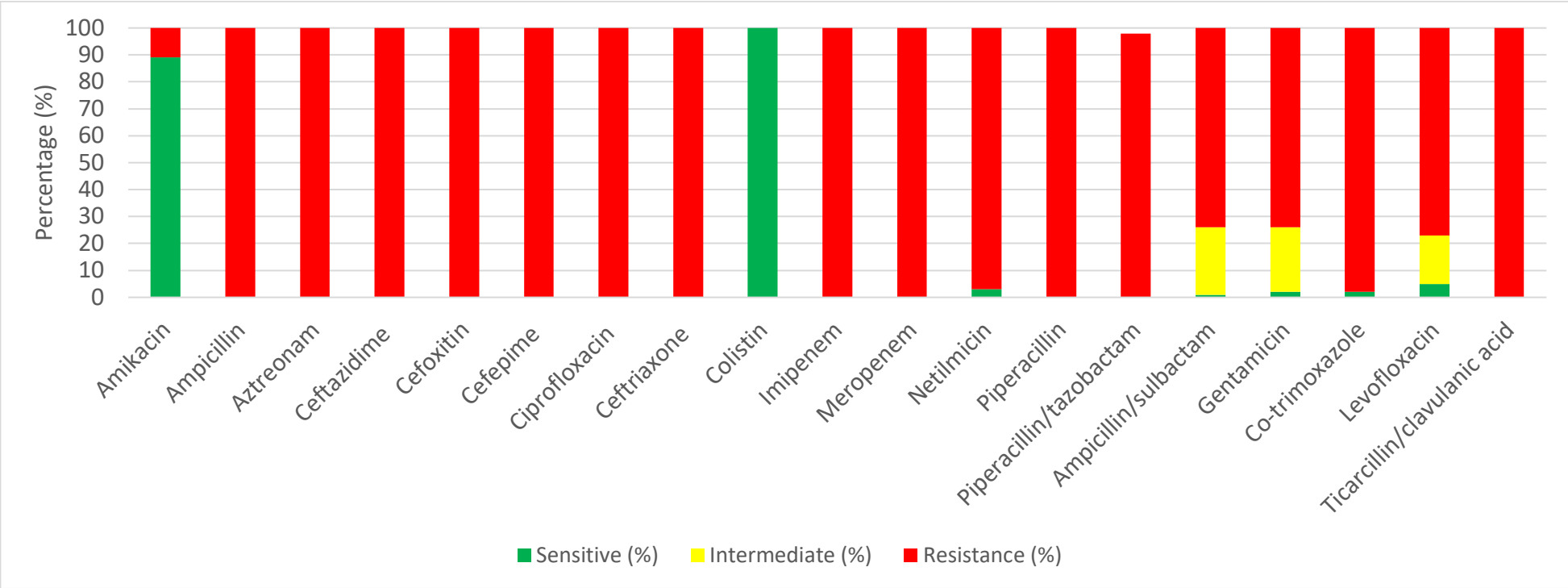


Figure 10. Co-resistance in carbapenem-resistant *A. baumannii* clinical isolates.

2.6.1.2 Molecular typing

2.6.1.2.1 RAPD PCR

RAPD analysis showed clonal diversity belonging to either EU clone 2/OXA-23 clone 1 and EU clone 1/OXA-23 clone 2 (Figure 11) (Table 5). Four isolates (AB98, AB114, AB133, AB143) were chosen as a representative for all isolates and sent for further confirmation using DiversiLab rep-typing on the basis of indistinguishable profiles or those with different RAPD profiles.

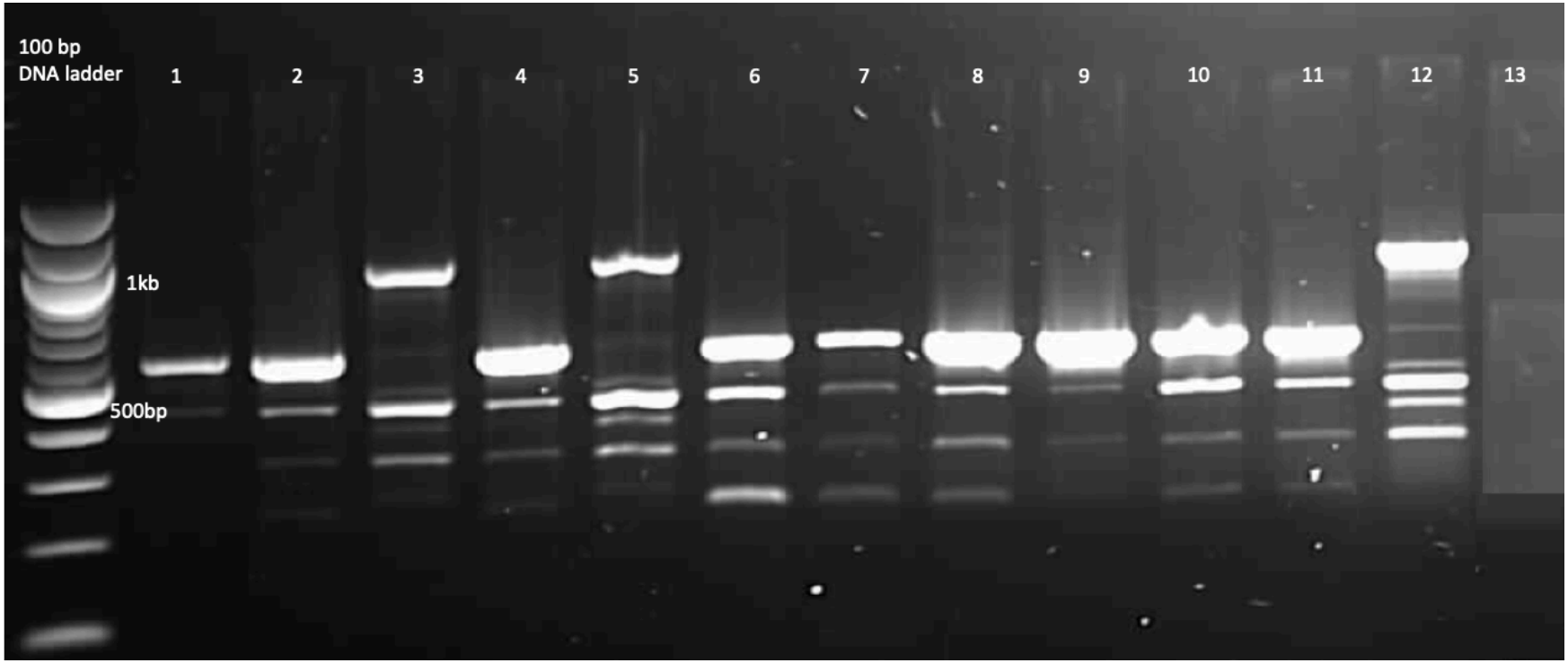


Figure 11. RAPD analysis of carbapenem-resistant *A. baumannii* clinical isolates from Brunei against reference strains EU clones. Lane 1- AB 12 (EU clone 2/ South East clone), Lane 2- AB 14 (Eu clone 2/OXA-23 clone 1), Lane 3- AB 16 (EU clone 1/OXA-23 clone 2), Lane 4- AB143, Lane 5- AB 114, Lane 6- AB186, Lane 7- AB117, Lane 8- AB210, Lane 9- AB133, Lane 10- AB103, Lane 11- AB116, Lane 12- AB98, Lane13- Negative control

Isolates	Characteristics	Comments
AB12	Reference strain	EU clone 2/ South East clone
AB14	Reference strain	Eu clone 2/OXA-23 clone 1
AB16	Reference strain	EU clone 1/OXA-23 clone 2
AB143	Test isolate	Eu clone 2/OXA-23 clone 1
AB114	Test isolate	EU clone 1/OXA-23 clone 2
AB186	Test isolate	Eu clone 2/OXA-23 clone 1
AB117	Test isolate	Eu clone 2/OXA-23 clone 1
AB210	Test isolate	Eu clone 2/OXA-23 clone 1
AB133	Test isolate	EU clone 2/ South East clone
AB103	Test isolate	Eu clone 2/OXA-23 clone 1
AB116	Test isolate	Eu clone 2/OXA-23 clone 1
AB98	Test isolate	EU clone 1/OXA-23 clone 2

Table 5. RAPD profiles comparison of carbapenem-resistant *A. baumannii* clinical isolates from Brunei against reference strains EU clones.

2.6.1.2.2 DiversiLab rep-PCR typing

Four of the CRAB clinical isolates (AB98, AB114, AB133 and AB143) DiversiLab analyses are shown in Figure 12 and Figure 13. Susceptibility testing of these isolates is shown in Table 6. All isolates had similar AST profiles, except for netilmicin and gentamicin.

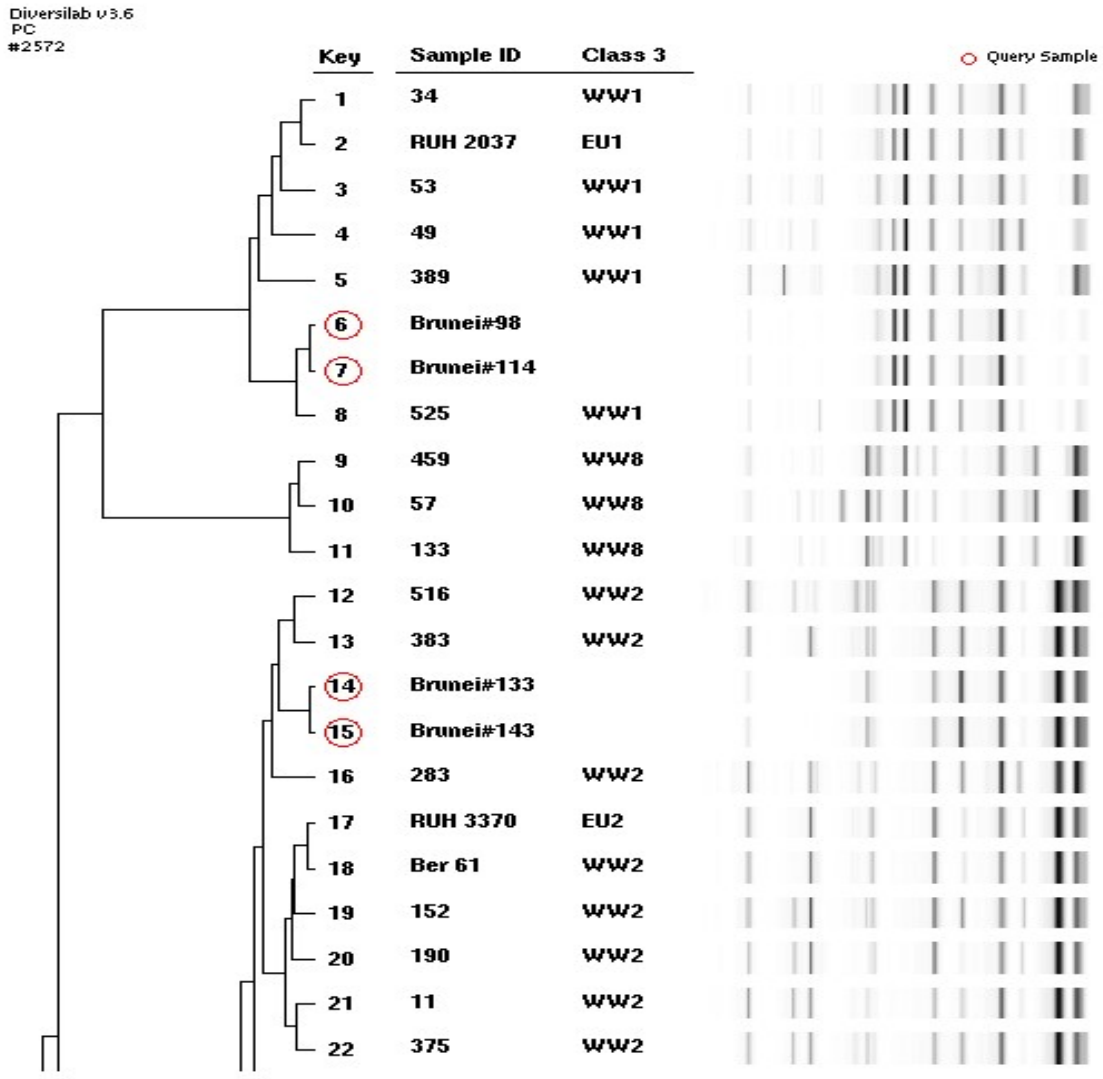


Figure 12. DiversiLab rep-PCR analysis. Dendogram of four CRAB isolates (labelled Brunei#98 (AB98), Brunei#114 (AB114), Brunei#133 (AB133), Brunei#143 (AB143))

and generated image of rep-PCR banding patterns showing representative of Worldwide clones (WW) and European clone (EU). WW- an old nomenclature used in this DiversiLab system program, however now renamed as IC, International Clone.

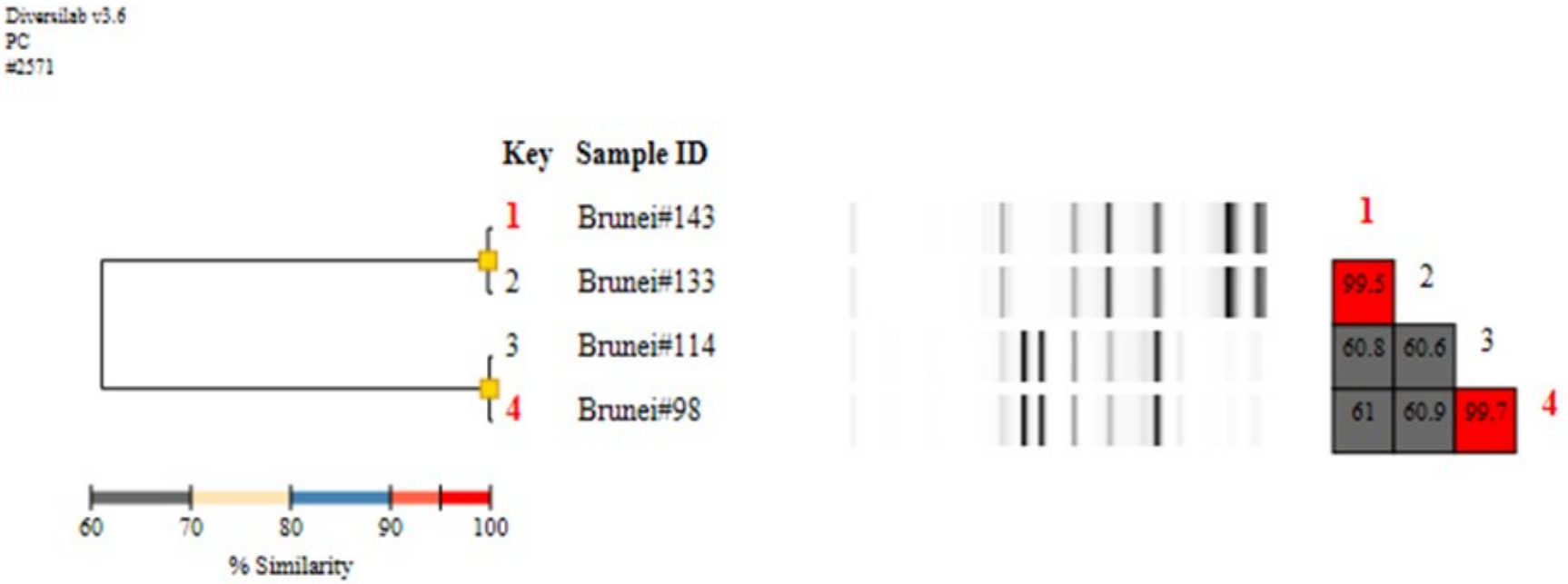


Figure 13. Dendogram showing the % similarity of the four Brunei CRAB isolates. Based on this, isolates 143 (AB143) and 133 (AB133) are identical, showing 99.5% similarity. Isolate 114 (AB114) is identical to isolate 98 (AB98) showing 99.7% similarity. And between the two clones, there is ~60% similarity, hence unrelated.

Isolates	Source of specimen	AK	CAZ	FEP	CIP	CRO	COL	IMI	MEM	NET	TZP	CN
AB98	Tracheal aspirate	≤ 16	≥ 32	≥ 32	≥ 4	≥ 64	≤ 2	≥ 8	≥ 8	≤ 8	≥ 128/4	≥ 16
AB114	Sputtum	≤ 16	≥ 32	≥ 32	≥ 4	≥ 64	≤ 2	≥ 8	≥ 8	≤ 8	≥ 128/4	≤ 4
AB133	Endotracheal aspirate	≤ 16	≥ 32	≥ 32	≥ 4	≥ 64	≤ 2	≥ 8	≥ 8	≥ 32	≥ 128/4	8
AB143	Tracheal aspirate	≤ 16	≥ 32	≥ 32	≥ 4	≥ 64	≤ 2	≥ 8	≥ 8	≥ 32	≥ 128/4	≥ 16

Table 6. Characteristics of carbapenem-resistant *A. baumannii* isolates sent for DiversiLab rep-typing. Susceptibility testing results are based on MIC breakpoints according to CLSI guidelines. AK- amikacin, CAZ- ceftazidime, FEP- cefepime, CIP- ciprofloxacin, CRO- ceftriaxone, COL- colistin, IMI- imipenem, MEM- meropenem, NET- netilmicin, TZP- piperacillin/tazobactam and CN- gentamicin. ≥ - resistance, ≤ - sensitive, no symbol - intermediate

2.6.1.2.3 OXA β-lactamase and mobile genetic elements (MGE)

All CRAB clinical isolates carried *bla*$_{OXA-51}$-like and *bla*$_{OXA-23}$-like genes (Figure 14), *armA*, *ampC* were positive for Class 1 integron and insertion sequence (IS*Aba1*). Mapping of the integron gene cassette, although not characterized showed two (AB92, AB101) belonged to integron family In634 according to the integron database (http://integrall.bio.ua.pt/). In634 comprises of *aacA4 (*aminoglycoside 6'-N-acetyltransferase)*, catB8* (chloramphenicol acetyltransferase)*, aadA1a* (aminoglycoside 3'-adenyltransferase)*, dfrA1b* (dihydrofolate reductase type I)*, aacA3* (aminoglycoside 3 -N-acetyltransferase) and *blaP2* (carbenicillin-hydrolysing penicillinase) (Ito and Hirano, 1997) which was first reported in *Salmonella enterica* subsp. *enterica* serovar Typhi (Salmonella typhi) GenBank accession number AY123251 (Pai et al., 2003). Susceptibility profiles, resistance gene determinant and genetic environment of AB92 and AB101 are shown in Table 7.

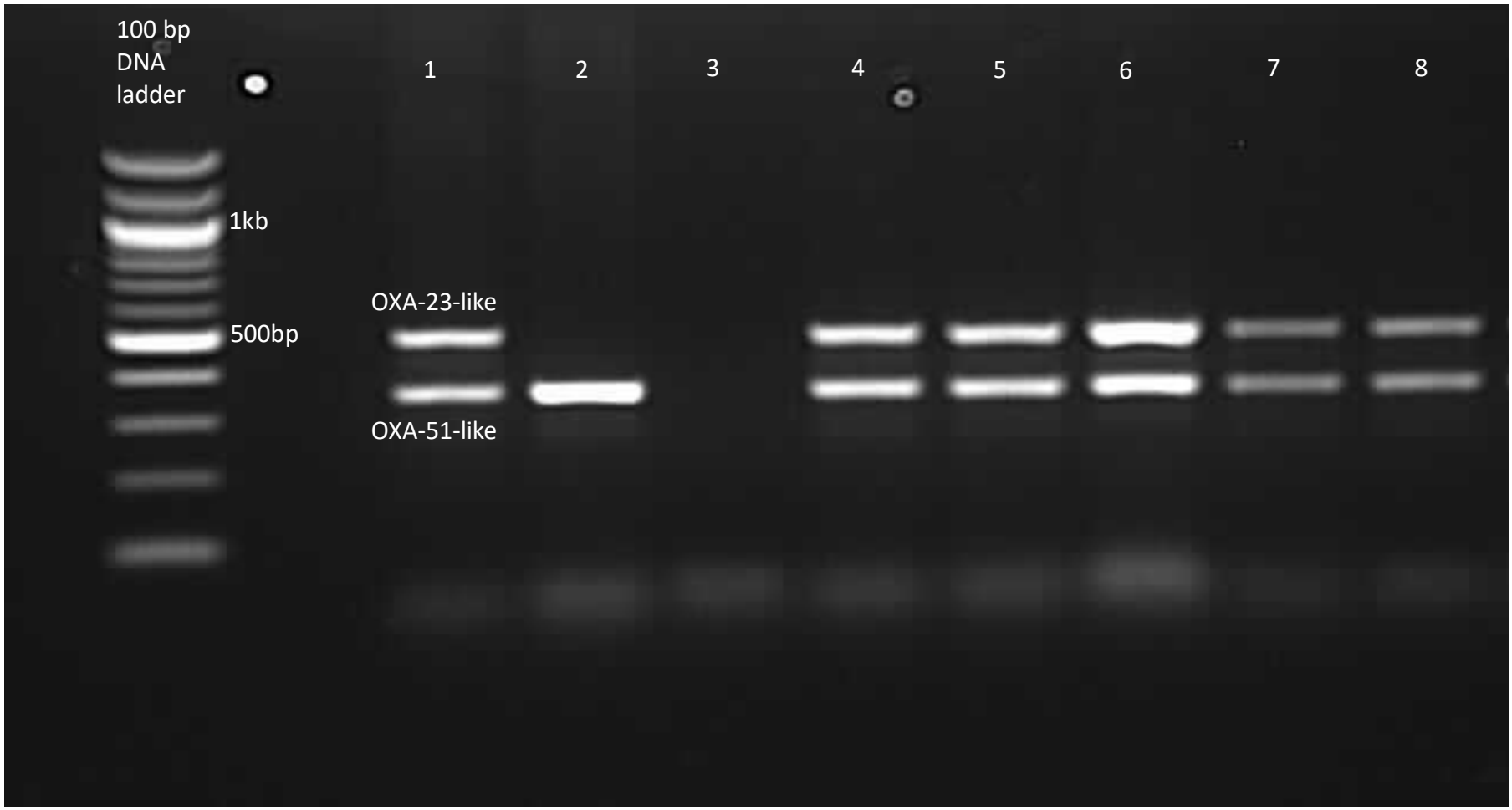

100 bp
DNA
ladder
1
2
3
4
5
6
7
8
1kb
500bp
OXA-23-like
OXA-51-like

Figure 14. Multiplex PCR of OXA beta-lactamases in CRAB clinical isolates. Lane 1- AB14 (positive control for both OXA-23-like and OXA-51-like), Lane 2- AB19606 (positive control for OXA-51-like), Lane 3- water (negative control), Lane 4- AB84, Lane 5- AB98, Lane 6- AB114, Lane 7- AB133, Lane 8- AB143. Lane 4-7 – all were positive for OXA-23-like (501 bp) and OXA-51-like (353 bp).

Isolate	Source of specimen	AK	CAZ	FEP	CIP	CRO	COL	IMI	MEM	NET	SAM	TZP	CN	Resistance determinant	Mobile genetic element
AB92	Endotracheal aspirate	≤ 16	≥ 32	≥ 32	≥ 4	≥ 64	≤ 2	≥ 8	≥ 8	≤ 8	≥ 32/16	≥ 128/4	≥ 16	*bla*$_{\text{OXA-23-like}}$, *bla*$_{\text{OXA-51-like}}$, *armA*, *ampC*	IS*Aba1*, In634
AB101	Endotracheal aspirate	≤ 16	≥ 32	≥ 32	≥ 4	≥ 64	≤ 2	≥ 8	≥ 8	≤ 8	≥ 32/16	≤ 16/4	≤ 4	*bla*$_{\text{OXA-23-like}}$, *bla*$_{\text{OXA-51-like}}$, *armA*, *ampC*	IS*Aba1*, In634

Table 7. Susceptibility testing results (MICs), resistance determinant and genetic environment of CRAB clinical isolates. AK- amikacin, CAZ- ceftazidime, FEP- cefepime, CIP- ciprofloxacin, CRO- ceftriaxone, COL- colistin, IMI- imipenem, MEM- meropenem, NET- netilmicin, SAM- ampicillin/sulbactam, TZP- piperacillin/tazobactam and CN- gentamicin.

2.6.2 Carbapenem-resistant *Pseudomonas aeruginosa* (CRPA)

2.6.2.1 Antibiotic susceptibility testing (AST)

Twenty-two carbapenem resistance *P. aeruginosa* (CRPA) clinical isolates were tested using VITEK 2XL (bioMérieux, Marcy l'Etoile, France).

Co-resistance were observed with ampicillin and co-trimoxazole. Amikacin, ciprofloxacin, netilmicin, levofloxacin and colistin were susceptible (Figure 15).

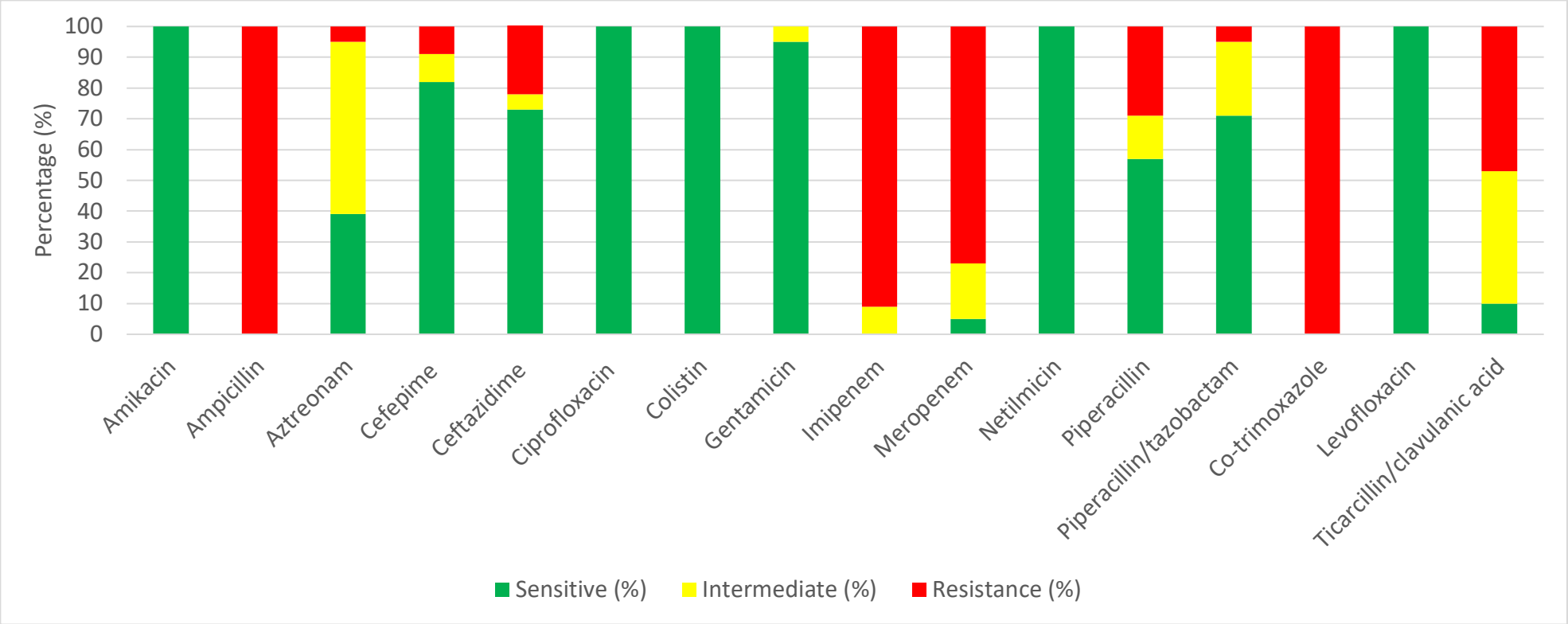


Figure 15. Co-resistance in carbapenem-resistant *P. aeruginosa* (CRPA) clinical isolates.

2.6.2.2 Carbapenemase production

Twenty-two of the carbapenem-resistant *P. aeruginosa* clinical isolates were negative for carbapenemase production using direct bacterial colonies and MH2 broth with the Blue-Carba method.

2.6 Summary

Carbapenem-resistant *A. baumannii* clinical isolates

- 109 CRAB clinical isolates were collected from endotracheal aspirate, tracheal aspirate, ETT-tip, sputum and pus.
- AST and MICs were performed on these isolates with VITEK® 2XL system, prior in Brunei's Microbiology Laboratory.
- All *A. baumannii* clinical isolates were resistance to carbapenems (imipenem, meropenem), penicillin (ampicillin, piperacillin) cephalosporins (ceftazidime, cefoxitin, cefepime, ceftriaxone), aminoglycosides (gentamicin, netilmicin), fluoroquinolones (ciprofloxacin, levofloxacin), piperacillin/tazobactam, ticarcillin/clavulanic acid, ampicillin/sulbactam, co-trimoxazole and aztreonam. Colistin remained susceptible.
- Molecular typing (RAPD and DiversiLab rep-typing) showed isolates belonged to either International clone 1 or 2.
- All CRAB clinical isolates carried OXA-23-like and the intrinsic OXA-51-like carbapenemase genes.
- Other resistance genes include *armA* 16S rRNA methylase determinant, *ampC,* IS*Aba1* and a class 1 integron.
- MDRAB is prevalent in Brunei and similar to epidemic strains (acquiring OXA-23-like carbapenemases) reported globally.

Carbapenem-resistant *P. aeruginosa* clinical isolates

- 22 *P. aeruginosa* clinical isolates collected from endotracheal aspirate, tracheal aspirate and sputum were resistance to carbapenems (imipenem and meropenem) ampicillin and co-trimoxazole. Amikacin, ciprofloxacin, netilmicin, levofloxacin and colistin remained susceptible.
- All CRPA clinical isolates recovered in Brunei were tested for carbapenemase production.
- Blue-Carba test was negative for carbapenemase production. This could be due to the absence of acquired carbapenemase genes and the presence of other resistance mechanisms such as porin loss mediating the carbapenem resistance.

Chapter 3

Carbapenem-Resistant Enterobacteriaceae (CRE) in Brunei Darussalam

3.1 Introduction

In Chapter 2, phenotypic and genotypic methods were performed as an introductory diagnostic tool on carbapenem-resistant Gram-negative Non-fermenters (*P. aeruginosa* and *A. baumannii*), an important WHO critical pathogen. In this chapter, using tools that have been performed previously and additional new diagnostic tools, carbapenem-resistant Enterobacteriaceae (CRE) (another important WHO critical pathogen) in particular *Klebsiella pneumoniae* causing an outbreak in Brunei's Hospital will be investigated.

The prevalence of MDR *Klebsiella pneumoniae* is a global public health threat with high mortality and morbidity, especially those harbouring Extended Spectrum βeta-lactamases (ESBLs) and more importantly carbapenemases (CRKP) (Tumbarello et al., 2006). These pathogens can be hospital-associated or community-acquired causing urinary tract infection (UTI), pneumonia, intra-abdominal infection and bloodstream infections (Tumbarello et al., 2006).

Carbapenems, drugs of last resort used to treat multi-drug resistant Gram-negative bacteria have become ineffective due to the emergence of CRE (Iovleva and Doi, 2017). More worryingly are those CRE acquiring plasmid-encoded carbapenemases of class A (KPC, IMI, GES), B (IMP, VIM, NDM) and D (OXA-48-like producers) (Iovleva and Doi, 2017). In Southeast Asia, Class D carbapenemases ($bla_{\text{OXA-48-like}}$ gene and its variants) are now the commonest carbapenemase reported in Enterobacteriaceae (Evans and Amyes, 2014). The OXA-48 enzyme

was first identified in 2001 in *K. pneumoniae* isolates from Turkey, since then many variants have been described, this includes OXA- 162 in Turkey, OXA-163 in Argentina, OXA-181 in India, OXA-204 in Tunisia and OXA-232 from France and in Singapore (Castanheira et al., 2011; Nordmann et al., 2011a; Dimou et al., 2012; Poirel et al., 2012; Potron et al., 2013; Teo et al., 2013).

More importantly, CRE infections are extremely difficult to control in hospitals and the community, hindered by limited therapeutic options, spread between bacterial species and dissemination of resistance through successful epidemic clones. Therefore, rapid and effective diagnostics are required in tackling the problem of CRE.

Novel assays using both genotypic and phenotypic approaches have been developed which includes genetic detection of resistance gene profiles (PCR, LAMP, microarrays, genome sequencing) (Osei Sekyere et al., 2015), selective culture media (chromogenic/supplemented) (Hornsey et al., 2013b), combination disc testing (van Dijk et al., 2014) and direct or indirect detection of carbapenem hydrolyzing enzymes (MALDI-TOF, acidometric Carba NP and Blue-Carba) (Pires et al., 2013; Dortet et al., 2015; Mirande et al., 2015). However, each of these methods requires variable levels of technical skill, investment into equipment and quality assurance optimization. Strains producing OXA-48-like carbapenem hydrolyzing class D enzymes (CHDL) have proven particularly difficult to detect in clinical laboratories, due in part to relatively low MICs, conflicting interpretive

rules associated with automated systems (Woodford et al., 2010) and a lack of suitable inhibitor compounds to use in confirmatory tests.

Recent development of OXA-48-like enzyme detection (OXA-48 *K*-SeT) using an antibody-mediated approach (Ote et al., 2015) was designed to detect current CHDL OXA-48-like variants (OXA-48, 181, 204, 232 and 244) (Potron et al., 2013). The principle of the test was immunological capture of two epitopes specific to the OXA-48 enzyme using colloidal gold nanoparticles bound to a nitrocellulose membrane within a lateral flow device (Ote et al., 2015).

3.2 Objectives

- To investigate the mechanism of resistance amongst carbapenem-resistant *K. pneumoniae* clinical isolates from Brunei Darussalam.

-To characterize CRKP by multiple phenotypic (RAPID CARB Blue kit, Micronaut-S Beta Lactamase VII, OXA-48 K-SeT) and molecular methods. Evaluation of the OXA-48 K-SeT for the detection of OXA-48 carbapenemase family in CRKP clinical isolates (Brunei) and CRE islolates (UK collection).

3.3 Methods

3.3.1 Bacterial collection and transportation

Carbapenem-resistant *K. pneumoniae* [KP41 (2015), KP42 (2015), KP43 (2015), KP44 (2015), KP45 (2015)] were recovered from 5 patients admitted over a 3-month period (May – July 2015) to general and intensive care units (ICU) at the main (RIPAS) and Tutong district hospital (PMMPMHAB) in Brunei. Isolates were recovered from blood culture (n = 1), endotracheal samples (n = 1), pus (n = 2) and sputum samples (n = 1). These isolates were found carbapenem-resistant in Brunei and stored as part of Microbiology routine laboratory for CRE collection.

For wide evaluation of the OXA-48 K-SeT assay, 82 additional enterobacterial isolates were collected from The Royal London Hospital, UK. 78 clinical isolates resistant to one or more carbapenems were collected (*K. pneumoniae*, n= 60; *Escherichia coli*, n= 11; *Enterobacter cloacae*, n= 6; *Enterobacter aerogenes,* n=1) and 4 susceptible type strains as control for each species (*K. pneumoniae* NCTC 9633, *E. coli* NCTC 12241, *E. cloacae* 13380, and *E. aerogenes* NCTC 9375).

Further details on the collection and transportation, see Chapter 2 section 2.3.1 and 2.3.2

3.3.2 AST and MIC Determination

AST and MIC of CRKP clinical isolates were tested prior in Brunei as mentioned in Chapter 2, section 2.3.5

Disc diffusion testing, MIC using Micronaut-S Beta Lactamase VII (BioConnections, Knypersley, UK) (cefotaxime, ceftazidime, cefepime and meropenem) and Etest strip (colistin, polymyxin B and ceftazidime/avibactam) were performed again in the UK.

AST was performed using Kirby-Bauer disc diffusion method. Antibiotic discs were sourced from Oxoid, Basingstoke, United Kingdom. The MICs were determined by Etest strips (BioMérieux, Basingtoke, UK and Launch Diagnostic, Kent, UK), broth microtiter dilution (BMD) and agar dilution on Mueller-Hinton 2 (MH2) agar.

AST and MICs follow guidelines from European Committee on Antimicrobial Susceptibility Testing (EUCAST) (EUCAST, 2015) and/or Clinical & Laboratory Standards Institute (CLSI) (CLSI, 2015).

The discs used on the CRKP clinical isolates includes colistin (CT, 25 μg), cefoxitin (FOX, 30 μg), minocycline (MH, 30 μg), imipenem (IPM, 10 μg), azithromycin (AZM, 15 μg), doxycycline (DO, 30 μg), spectinomycin (SH, 100 μg), apramycin (APR, 15 μg) and sulfonamide (S, 10 μg).

Antibiotic discs were sourced from Oxoid, Basingstoke, United Kingdom. Etest strips were sourced either from BioMérieux, Basingtoke, UK or Launch Diagnostic, Kent, UK.

3.3.3 Micronaut-S Beta Lactamase VII testing on CRKP clinical isolates

Micronaut-S Beta Lactamase VII (BioConnections, Knypersley, UK) (Figure 16) was used for the phenotypic identification of ESBL, AmpC, and carbapenemases using a range of antibiotics with and without inhibitors as well determination of MICs according to CLSI guidelines.

Bacterial inoculum was prepared according to the manufacturer's instruction. Briefly, 0.5 MacFarland standard was made up in 5 ml of 0.85% NaCl (Oxoid, Basingstoke, UK). 50 µl of the aliquot was then transferred into 11 ml MH 2 broth provided and vortexed. 100 µl of the suspension was dispensed into the wells. The plate was then sealed with an unperforated plate sealer and incubated at 37^{o} C for 18-24 h. The plate was read visually by looking at the turbidity of the wells. Phenotypic confirmatory test for ESBL, AmpC and carbapenemases follows the calculation and algorithm according to the manufacturer's protocol.

	1	2	3	4	5	6	7	8	9	10	11	12
A	CEP 128	CMC 32/4	CAZ 128	CZC 32/4	CZB 32	CTX 128	C/C 32/4	CTB 32	MER 128	MEE 32	MEB 32	
B	CEP 64	CMC 16/4	CAZ 64	CZC 16/4	CZB 16	CTX 64	C/C 16/4	CTB 16	MER 64	MEE 16	MEB 16	
C	CEP 32	CMC 8/4	CAZ 32	CZC 8/4	CZB 8	CTX 32	C/C 8/4	CTB 8	MER 32	MEE 8	MEB 8	
D	CEP 16	CMC 4/4	CAZ 16	CZC 4/4	CZB 4	CTX 16	C/C 4/4	CTB 4	MER 16	MEE 4	MEB 4	
E	CEP 8	CMC 2/4	CAZ 8	CZC 2/4	CZB 2	CTX 8	C/C 2/4	CTB 2	MER 8	MEE 2	MEB 2	
F	CEP 4	CMC 1/4	CAZ 4	CZC 1/4	CZB 1	CTX 4	C/C 1/4	CTB 1	MER 4	MEE 1	MEB 1	GC/B
G	CEP 2	CMC 0.5/4	CAZ 2	CZC 0.5/4	CZB 0.5	CTX 2	C/C 0.5/4	CTB 0.5	MER 2	MEE 0.5	MEB 0.5	GC/E
H	CEP 1	CMC 0.25/4	CAZ 1	CZC 0.25/4	CZB 0.25	CTX 1	C/C 0.25/4	CTB 0.25	MER 1	MEE 0.25	MEB 0.25	GC

Figure 16. Micronaut-S Beta Lactamase VII plate layout adapted from manufacturer's protocol. [CEP (Cefepime), CMC (Cefepime/ Clavulanic acid), CAZ (Ceftazidime), CZC (Ceftazidime/ Clavulanic acid), CZB (Ceftazidime/ 3-APB), CTX (Cefotaxime), C/C (Cefotaxime/ Clavulanic acid), CTB (Cefotaxime/ 3-APB, *3*-aminophenylboronic acid), MER (Meropenem), MEE (Meropenem/ EDTA, Ethylenediaminetetraacetic acid), MEB (Meropenem/ 3-APB, *3*-aminophenylboronic acid), GC (Growth Control), GC/B (Growth Control/ 3-APB, *3*-aminophenylboronic acid), GC/E (Growth Control/ EDTA, Ethylenediaminetetraacetic acid)]. Black shaded area contains no antibiotic or growth control and not used in this experiment.

3.3.4 Detection of carbapenemases using Rapid CARB Blue kit in CRKP clinical isolates

Rapid CARB Blue kit (ROSCO, Tasstruo, Denmark) is a commercial version of Blue-Carba test. This method is based on the identification of the hydrolysis of the beta-lactam ring of a carbapenem in the presence of an indicator and does not require lysis buffer.

Rapid CARB Blue kit was performed according to the manufacturer's instruction. Briefly, 10 µl loop of bacterial isolate was added to 200 µl 0.9% NaCl solution adjusted to pH 8.5 (8.3 - 8.7) using 0.01 N NaOH in a sterile tube. The mixture was vortexed for 1 min and maintained at room temperature for 30 min. 1 Imipenem (2) + Bromothymol Blue tablets were added to the mixture. The mixture was again vortexed for 1-2 seconds to disintegrate the tablet. The tube was then incubated at 37^{o} C for 15 min, 30 min or 1 h, respectively.

The control was performed in the same process using CARB Negative Control Blue Diatab.

Interpretation of results:

Positive for carbapenemase

- Test suspension color change from blue to yellow.
- Test suspension green to yellow and negative control is blue, or
- Test suspension yellow and negative control is green

Negative for carbapenemase

-No colour change in test suspension and negative control, remains blue for both.

Uninterpretable

If the negative control shows light-yellow color, no matter the result of Imipenem + Bromothymol Blue, result reported as uninterpretable.

3.3.5 OXA-48-like detection in CRKP clinical isolates from Brunei using lateral flow assay, CORIS OXA-48 K-SeT

Rapid testing using CORIS OXA-48 K-SeT (CORIS BioConcept, Gembloux, Belgium) was used to confirm the presence of OXA-48-like variant in CRKP Brunei clinical isolates according to the manufacturer's protocol. Single colony (using 5 μl loop) of the CRKP clinical isolates grown overnight on MH2 media was re-suspended in 10 drops of LY-A buffer (TRIS-HCl, NaN_3, pH7.5) in tubes provided. Three drops of the homogenized solution were then applied to the sample well on the cassette. Tests were read by eye within 15 minutes.

3.3.6 Evaluation of OXA-48 K-SeT for the detection of carbapenemase OXA-48-like variants using clinical isolates from The Royal London Hospital

Eighty-two enterobacterial isolates were used in the evaluation. Seventy-eight were clinical isolates (*Klebsiella pneumoniae* n=60, *Escherichia coli* n=11, *Enterobacter cloacae* n=6, *E. aerogenes* n=1) with resistance to one or more carbapenems (ertapenem, imipenem, meropenem) along with a susceptible type strain as a representative control for each bacterial species (*K. pneumoniae* NCTC

9633, *E. coli* NCTC 12241, *E. cloacae* 13380, *E. aerogenes* NCTC 9375). Resistance or reduced susceptibility to carbapenems in the clinical isolates was identified by disc diffusion and/or Etest (bioMerieux, Macy L'Etoile, France) and confirmed by broth microtitre dilution (MIC of ertapenem ≥1 µg/ml) according to Clinical Laboratory Standards Institute (CLSI) methodology.

The lower limit of detection (CFU/ml) of the OXA-48 K-SeT device was determined using *K. pneumoniae* NCTC 9633 and KP41 (bla_{OXA-48}) grown in trypticase soy broth, TSB (Oxoid Basingtoke, UK). Cells from 1 ml of overnight cultures were harvested by centrifugation. The supernatant was removed, and the pelleted cells were re-suspended in phosphate buffered saline (PBS). Serial dilutions (10^{-1} - 10^{-9}) were made by dispensing 100 µl into 900 µl of PBS in a 1.5 ml sterile Eppendorf tubes. The dilutions were then plated onto MH agar with viable counts (CFU/ml) recorded following 18 h incubation.

OXA-48-like detection using CORIS OXA-48 K-SET (CORIS BioConcept, Gembloux, Belgium) was performed according to the manufacturer's protocol as mentioned in section 3.3.6

3.3.6.1 Evaluation of OXA-48 K-SeT in blood culture simulation

Similar clinical isolates from The Royal London Hospital were used for blood culture simulation. Heparinised horse blood (Oxoid, Basingstoke, United Kingdom) was pre-warmed to 37° C prior to use. 10ml of heparinised horse blood was supplemented into Aerobic FA Plus 30 ml blood culture bottle. 0.1 ml of test organism (10^2 CFU/ml) was inoculated aseptically into the same blood culture bottle. Blood culture bottle was then incubated at 37°C for 18 h. 0.1 ml aliquot of blood was withdrawn from the overnight blood culture bottle into sterile 1.5 ml Eppendorf tube without further dilution. The tube was centrifuged at 12000 RPM for 3 minutes. The supernatant was discarded. Ten drops of LY-A buffer were added to the pellet. Three drops of the mixture were dispensed onto OXA-48 K-set cassettes.

Adequate growth of each isolate under simulated blood culture conditions was confirmed by subculture on MH II agar.

3.3.7 Molecular analysis of CRKP clinical isolates

3.3.7.1 Clonal typing of CRKP clinical isolates

RAPD PCR (see Chapter 2, section 2.4.1.1) using ERIC2 primer was performed on all 5 CRKP clinical isolates from Brunei.

3.3.7.2 Multilocus sequence typing (MLST) of CRKP clinical isolates

Multilocus sequence typing (MLST) using Pasteur scheme of seven conserved *K. pneumoniae* housekeeping genes (*gapA, infB, mdh, pgi, phoE, rpoB and tonB*) was performed as previously described (Diancourt et al., 2005) (Brisse et al., 2009).

DNA extraction was performed as mentioned in section 2.3.6.1 . 2 μl of the bacterial lysate was added to 25 μl of ThermoPrime 2x ReddyMix PCR Master Mix and 1 μl each of the forward and reverse primers in a 0.6 ml PCR tube.

Primers for MLST are found in Appendix A.

PCR cycling conditions:

Initial denaturation at 94^{o} C for 2 min, followed by 35 cycles of 94^{o} C for 30 s, 50^{o} C for 1 min and 72^{o} C for 30 s. Final extension of 72^{o} C for 5 min.

For gel electrophoresis and band visualization see section 2.3.7.2

DNA amplicons producing positive bands were cleaned (see section 2.3.8) and sent for Sanger sequencing (see section 2.3.9).

3.3.7.3 PCR for virulence and capsular types

PCR was used for the detection of *K. pneumoniae* virulence (*kfu, fimH, uge, wabG*, *ureA*) determinants and the K1 hypermucoviscous (cps) phenotype as previously described. (Compain et al., 2014) Primers used are found in Appendix A.

DNA extraction was performed as mentioned in section 2.3.6.1 . 2 μl of the bacterial lysate was added to 25 μl of ThermoPrime 2x ReddyMix PCR Master Mix and 1 μl each of the forward and reverse primers in a 0.6 ml PCR tube.

PCR conditions:

Initial denaturation at 95° C for 15 min, followed by 30 cycles at 94° C for 30 s, 60° C for 90 s, and 72° C for 60 s, and a final extension at 72° C for 10 min.

For gel electrophoresis and band visualization see section 2.3.7.2

DNA amplicons producing positive bands were cleaned (see section 2.3.8) and sent for Sanger sequencing (see section 2.3.9).

3.3.7.4 Molecular characterisation of resistance determinants in CRKP clinical isolates

Characterisation of CRKP clinical isolates was initially performed using a commercial ARM-D™ for β-Lactamase ID kit and further confirmation using

multiplex PCR for β-lactamase detection and other resistant gene determinants that have been previously described.

3.3.7.4.1 ARM-D™ for β-Lactamase ID

The ARM-D™ for β-Lactamase ID kit (Alpha laboratories, Hampshire, UK) is a commercial kit providing two control reactions that detect genes encoding members of the β-lactamase families: IMP-1, NDM, OXA-48, CTX-M-14, CTX-M-15, CMY-2, DHA, VIM, and KPC. And an endogenous internal control is also included to reduce false negatives; it targets a conserved region common in Gram-negative bacteria. The kit also consists of single tube containing all primers in a 10X Primer Mix and a custom DNA polymerase supermix (ARM-D 2X Supermix). The ARM-D 2X Supermix includes DNA polymerase, PCR reaction buffer, dNTPs, and agarose gel loading dye.

PCR was carried out as per the manufacturer's instructions for the ARM-D for β-Lactamase ID kit. DNA extraction was performed as mentioned in section 2.3.6.1 . 1 μl of template DNA or control is added to the ARM-D 2X Supermix.

PCR cycling conditions:

Hot start of 98° C for 30 seconds, followed by 25 cycles of 98° C for 5 seconds, 60° C for 10 seconds, and 72° C for 20 seconds, and a final extension of 72° C for 30 seconds.

PCR amplicons were stained with ethidium bromide and resolved on a 2.5% agarose gel.

For gel electrophoresis and band visualization see section 2.3.7.2

3.3.7.4.2 Multiplex PCR for detecting resistance determinants

Mulitplex PCR were performed for the detection of beta-lactamase genes (bla1-6) (Dallenne et al., 2010), BIC/NDM beta-lactamase genes (Poirel et al., 2011b), quinolone resistance gene (Cattoir et al., 2007) and aminoglycoside modifying enzyme (Noppe-Leclercq et al., 1999) as previously described.

DNA extraction was performed as mentioned in section 2.3.6.1 . 2 μl of the bacterial lysate was added to 25 μl of ThermoPrime 2x ReddyMix PCR Master Mix and 1 μl each of the forward and reverse primers in a 0.6 ml PCR tube.

PCR set up mentioned in Chapter 2, section 2.3.7. Primers used are found in Appendix A.

PCR cycling conditions:

Aminoglycoside multiplex PCR

Initial denaturation for 30 s at 94° C, followed by 45 cycles of an annealing step for 40 s (at 58 °C for triplex assay 1, 49 °C for triplex assay 2, and 55 °C for duplex assay 3) and an extension step for 90 s at 72 °C.

Beta-lactamases (bla1-6)

Initial denaturation at 94° C for 10 min, followed by 30 cycles of 94° C for 40 s, 60° C for 40 s and 72° C for 1 min. Final extension at 72° C for 7 min. For the carbapenemase gene multiplex PCR assays, the annealing temperature was optimal at 55° C for amplification of bla_{VIM}, bla_{IMP} and bla_{KPC} genes, and optimal at 57° C for amplification of bla_{GES} and bla_{OXA-48} genes.

Carbapenemase (BIC/NDM)

Initial denaturation 10 min at 94° C, followed by 36 cycles of amplification consisting of 30 s at 94° C, 40 s at 52° C, and 50 s at 72° C. Final extension of 5 min at 72° C.

Quinolone multiplex PCR

Initial denaturation of 10 min at 95° C, followed by 35 cycles of amplification consisting of 1 min at 95° C, 1 min at 54° C and 1 min at 72° C. Final extension of 10 min at 72° C.

For gel electrophoresis and band visualization see section 2.3.7.2

3.3.7.4.3 Cloning of β-lactamase

Full coding sequences of genes identified in KP41 (2015) isolates were cloned using Zero Blunt™ TOPO™ PCR Cloning Kit, with pCR™-Blunt II-TOPO™ Vector (Figure

17), One Shot™ TOP10 Chemically Competent *E. coli*, and PureLink™ Quick Plasmid Miniprep Kit (Thermo Fisher Scientific, Lancashire, UK) according to the manufacturer's protocol.

TOPO® cloning reaction was set up using the following reagents in order; fresh PCR product (0.5-4 µl), salt solution (1 µl), water (add to a volume of 5 µl) and pCR™II-Blunt-TOPO® (1 µl) to a final volume of 6 µl in a 0.9 ml microcentrifuge.

The mixture was incubated at room temperature for 5 mins (22^{o} C- 23^{o} C). The reaction mixture was then placed on ice.

Transformation steps were then performed using Transform One Shot® competent cells. 2 µL of the TOPO® Cloning reaction was added into a vial of One Shot® chemically competent *E. coli* and was mixed gently in a sterile 1.5 ml Eppendorf tube. The tube was then incubated on ice for 5–30 minutes. The One Shot® chemically competent *E. coli* cell was heat-shocked for 30 seconds at 42°C without shaking. The tubes were immediately transferred to ice. 250 µL of room temperature S.O.C. medium was added and incubated horizontally on an innova 2100 platform shaker (New Brunswick Scientific; Eppendorf, Stevenage UK) (200 rpm) at 37°C for 1 hour.

10–50 µL were seeded and spread on a prewarmed selective plate (LB plates containing 50 mg/L kanamycin) and incubated overnight at 37°C. Transformant colonies grown on the plate were selected for analysis.

Transformants were analysed by PCR using the M13 Forward (−20) and M13 Reverse primers. Briefly, 48 µL of PCR SuperMix High Fidelity (22 U/ml DNA polymerase mixture in 66 mM Tris-SO4 (pH 9.1 at 25° C), 19.8 mM (NH4)2SO4, 2.2 mM MgSO4, 220 µM dGTP, 220 µM dATP, 220 µM dTTP, 220 µM dCTP and stabilizers) were added with 1 µL each of the M13 forward and M13 reverse PCR primer into a 0.9 ml microcentrifuge tube. Individual colonies were then resuspended in the mixture.

PCR cycling conditions were as follows:

10 minutes at 94°C to lyse the cells and inactivate nucleases. Amplification for 20−30 cycles and final extension, incubate at 72°C for 10 minutes.

Since the PCR SuperMix High Fidelity is colourless, 5 µl of Gel Loading Dye, Purple 6X (NEW ENGLAND BioLabs, Hitchin, UK) were added.

For gel electrophoresis and band visualization see section 2.3.7.2

Positive gel band was cleaned (as mentioned in Chapter 2, section 2.3.8) and sent for Sanger sequencing (as mentioned in Chapter 2, section 2.3.9)

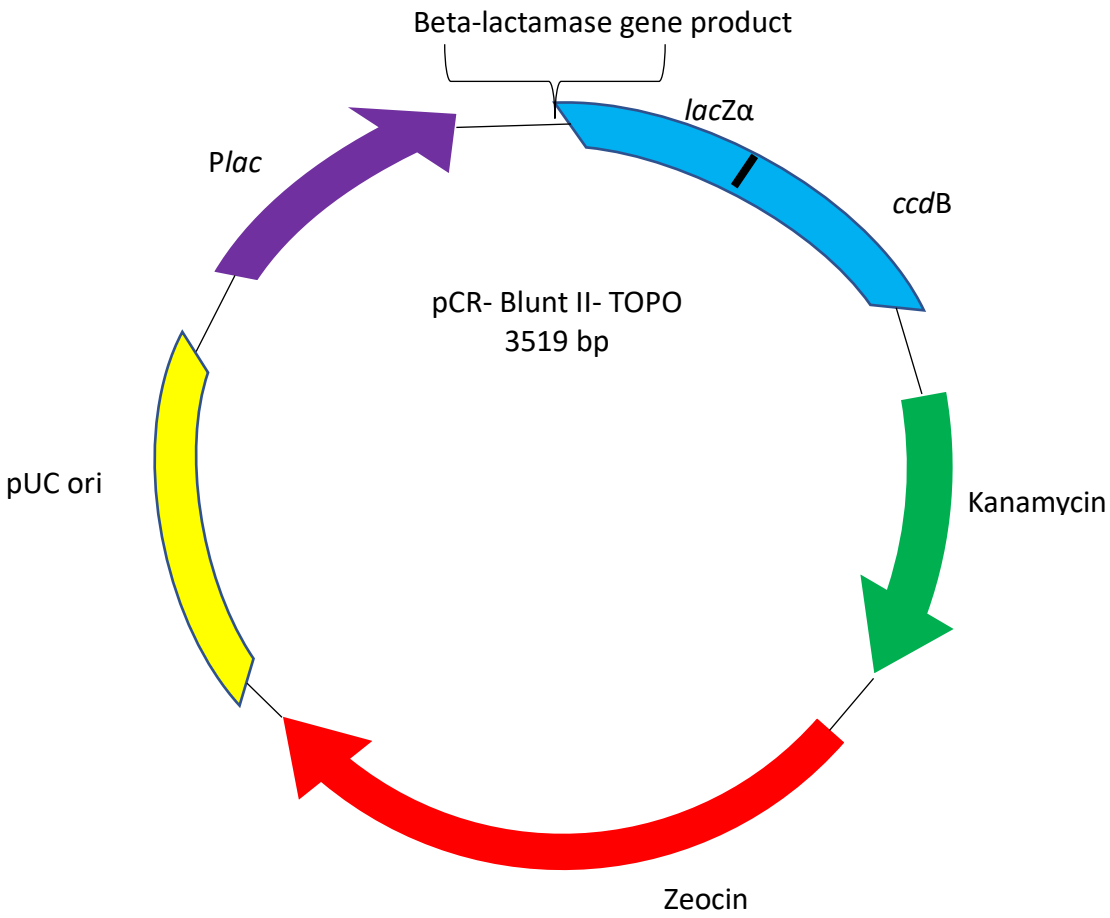

Beta-lactamase gene product
lacZα
ccdB
Kanamycin
Zeocin
pUC ori
Plac
pCR- Blunt II- TOPO
3519 bp

Figure 17. Schematic diagram of a vector constructed for the expression of beta-lactamase gene. pCR-Blunt II- TOPO vector encodes the pUC origin of replication, *lac*Zα, *ccd*B lethal gene, kanamycin resistance and zeocin resistance gene. The beta-lactamase gene insert is expressed under the lac promoter (P*lac*).

3.3.7.5 Plasmid analysis

3.3.7.5.1 PCR-based replicon typing

Plasmid replicon typing on CRKP clinical isolates was performed using a commercial PCR-based replicon typing kit (DIATHEVA, Cartoceto, Italy) with additional single PCRs for IncX4 and ColE-like plasmid replicons (Potron et al., 2011; Johnson et al., 2012; Potron et al., 2013). Briefly, 24 μl of the amplification mixes was aliquoted into each of the PCR vials. 1 μl DNA samples to be tested were then added into the corresponding PCR vial containing amplification mixes and vortexed. PBRT positive controls were thawed and vortexed for 30- 40 seconds. 1μl of each positive control was added into the corresponding PCR vial containing amplification mix and vortexed briefly.

PCR-based replicon typing kit PCR conditions:

Initial denaturation at 95° C for 10 min, followed by 30 cycles at 95° C for 60 s, 60° C for 30 s, and 72° C for 60 s, and a final extension at 72° C for 5 min.

5μl of DNA loading buffer was added directly to amplified samples and ethidium bromide on a 2.5% agarose gel was prepared.

ColE-type PCR conditions:

Initial denaturation at 95° C for 10 min, followed by 30 cycles at 95° C for 60 s, 60° C for 30 s, and 72° C for 60 s, and a final extension at 72° C for 5 min.

IncX4 replicon PCR conditions

initial denaturing step at 95° C for 5 min, followed by 25 cycles of 95° C for 1 min, 52° C for 30 sec, and 72° C for 1 min, and a final extension of 72° C for 5 min.

The amplified products were electrophoresed in 1% agarose gels, stained with ethidium bromide.

For gel electrophoresis and band visualization see section 2.3.7.2

3.3.7.5.2 Plasmid sizing

Plasmid location of each β-lactamase gene and sizing of KP41 (2015) was performed by pulsed-field gel electrophoresis (PFGE) of S1-digestion DNA and Southern hybridisation with specific intragenic DNA probes as previously described (Barton et al., 1995) done by our collaborators in Lieden University, Netherlands.

3.3.7.5.3 Sequencing of the entire *bla*$_{OXA-232}$-encoding plasmid (pKP41-OXA-232)

The sequence of the entire *bla*$_{OXA-232}$-encoding plasmid (pKP41-OXA-232) was determined using a primer walking approach (Figure 18). Inverse PCR, using long-range Taq polymerase (Invitrogen™ Platinum™ SuperFi™ DNA Polymerase; Thermo Fisher Scientific, Lanchashire, UK), in combination with the primers OXA232invL and OXA-232invR was used to amplify the entire sequence. Additional

primers were used to sequence the extremities flanking *bla*$_{OXA-232}$. All the primer sequences used are found in Appendix A.

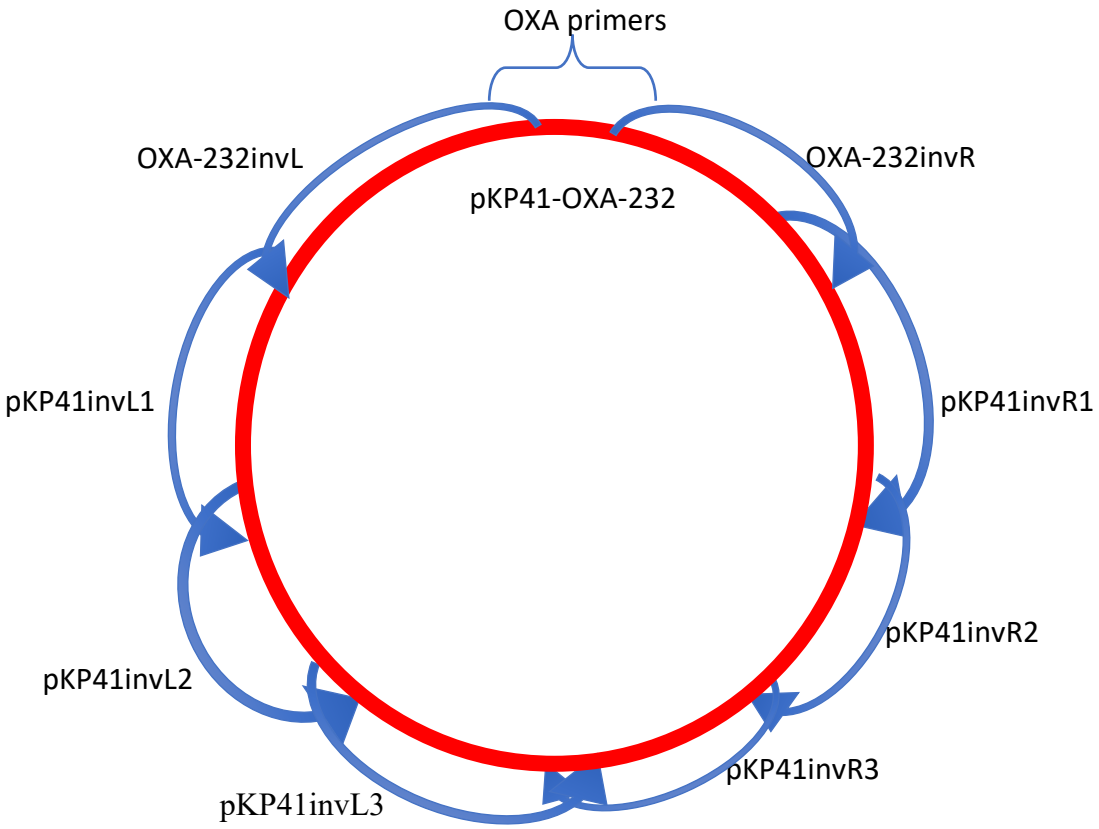


Figure 18. Inverse PCR primer walking approach for pKP41-OXA-232. Amplification starts from OXA-232 coding sequence and the amplification moves from the right and left to cover the whole plasmid. R- right side, L- Left side.

PCR conditions of long-range Taq polymerase:

Initial denaturation of 98° C for 30 s followed by 35 cycles of 10 s at 98° C, 10 s at 51.2° C and 3 m at 72° C. Final extension at 72° C for 5 min.

PCR conditions for extremities flanking *bla*$_{OXA-232}$:

Initial denaturation of 94° C for 2 min followed by 35 cycles of 10 s at 94° C, 1 min at 55° C and 1 min at 72° C. Final extension at 72° C for 7 min.

3.3.8 Bacterial conjugation

Transfer of plasmid-mediated resistance genes by conjugation was investigated using rifampicin-resistant CSH26 and sodium azide-resistant J53 strains of *Escherichia coli* as recipients. Conjugation experiments were set up by mixing 0.5 mL of exponential-phase cultures in 4 mL of Luria–Bertani (LB) (Sigma-Aldrich, Dorset, UK) broth and then incubating overnight at 37^{o} C without shaking. Transconjugants were selected by growth on LB agar supplemented with rifampicin (128 mg/L) and either ampicillin (32 mg/L), cefotaxime (16 mg/L) or ertapenem (1 mg/L) for conjugation to E. coli CSH26 or with sodium azide (100 mg/L) and cefotaxime (0.0625–2 mg/L) using E. coli J53.

3.3.9 Whole Genome Sequencing (WGS)

3.3.9.1 Sample preparation

A single colony of the strain from overnight culture plate was mixed in 100 µl sterile saline. The mixture is inoculated onto MH 2 agar plate and incubated at 37 ^{o}C for 24 h. 1/3 plate lawn of the strain were mixed into barcoded bead tube supplied by MicrobesNG (Birmingham) using 5 µl loop. The bead tubes were mixed by inverting 10 times. The tubes were sent at room temperature back to MicrobesNG (Brimingham).

3.3.9.2 Packaging and transportation of samples

Samples are packaged and transported according to Category B infectious substance assigned to UN3373 (http://www.who.int/csr/resources/publications/biosafety/WHO_CDS_EPR_2007_2cc.pdf)(WHO, 2007).

3.3.9.3 Sequencing of isolates

The genomic DNA from KP41 (2015) was subjected to whole-genome sequencing using 2 x 250-bp paired-end reads on an Illumina MiSeq platform (Illumina, Inc., San Diego, CA) This generated a total of 338,242 reads with an average length of 521 bp (Figure 22). Trimmomatic algorithm (version 0.36) (Bolger et al., 2014) were performed to trim the generated reads and their quality assessed by in-house scripts combined with SAMtools (version 1.3.1) (Li et al., 2009), BedTools (version 2.25.0) (Quinlan and Hall, 2010), and BWA-mem (version 2) (Li and Durbin, 2009) algorithms. SPAdes algorithm (version 3.7.1) (Bankevich et al., 2012) were performed to subsequently assemble *de novo* high-quality filtered reads into 98 scaffolds, with a minimum length of 211 bp and an N_{50} of 316,659 bp. Additionally, the sequence coverage of the *de novo* assemblies was approximately 190 reads per assembled base.

3.3.9.4 *In silico* of resistance determinants of WGS

Contigs and the draft genome assembly of KP41 (2015) isolate were anlaysed *in silico* using online tools (http://www.genomicepidemiology.org/) available at the Centre for Genomic Epidemiology (Lygby, Denmark). Genes encoding genotypic and phenotypic resistance (ResFinder 3.1), chromosomal (MLST 2.0) and plasmidic (PlasmidFinder 2.0) epidemiological markers were compared to reference sequences in Pubmed (NCBI). Sequences with < 100 % nucleotide identity were analysed by blastx (NCBI) to identify potential new alleles or non-synonymous mutations.

3.3.9.5 Capsule and lipopolysaccharide serotype prediction (Kaptive Web)

A user-friendly program Kaptive web (http://kaptive.holtlab.net/) was used to predict the capsule and lipopolysaccharide serotype in *Klebsiella* genomes. WGS data of KP41 (2015) were uploaded into this program in a single compressed data directory and analyzed against the *Klebsiella* primary K locus and the *Klebsiella* O locus databases.

3.4 Results

3.4.1 Phenotypic analysis

3.4.1.1 AST, MIC and carbapenemase detection

Susceptibility testing identified resistance to azithromycin, cephalosporins, carbapenems, fosfomycin, minocycline and nitrofurantoin in all isolates. Isolates remained susceptible (Etest, bioMérieux, Marcy l'Etoile, France) to colistin (COL <1 mg/L), polymyxin B (POL <1 mg/L) and the combination of ceftazidime (CAZ) and avibactam (AVI) (1 mg/L) (Table 6). The MICs of cefotaxime (CTX >128mg/L), cefepime (FEP >128mg/L), ceftazidime (CAZ >128mg/L) and meropenem (MEM >32mg/L), determined using a Micronaut-S Beta Lactamase VII panel (Merlin Diagnostika, Bornheim-Hersel, Germany) confirmed resistance to all β-lactams (Table 8). In addition, phenotypic identification with this test showed AmpC and ESBL positive.

Antibiotic	Zone of inhibition (mm)	Antibiotic profile (S, I, R or Unknown)	MIC (mg/L)	Antibiotic zone diameter and MIC breakpoint guidelines
Apramycin (15 μg)	16	Unknown	-	-
Azithromycin (15 μg)	6	R	-	-
Colistin (25 μg)	13	Unknown	-	-
*Colistin	-	S	< 1	(EUCAST, 2015)
#Cefepime	-	R	> 128	(EUCAST, 2015)
#Cefotaxime	-	R	> 128	(EUCAST, 2015)
Cefoxitin (30 μg)	6	R	-	(EUCAST, 2015)
#Ceftazidime	-	R	> 128	(EUCAST, 2015)
*Ceftazidime/avibactam	-	S	1	(EUCAST, 2017)
Doxycycline (30 μg)	11	I	-	(CLSI, 2015)
Fosfomycin (200 μg)	18	R	-	(EUCAST, 2015)
Imipenem (10 μg)	10	R	-	(EUCAST, 2015)
#Meropenem	-	R	> 32	(EUCAST, 2015)
Minocycline (30 μg)	10	R	-	(CLSI, 2015)

Nitrofurantoin (200 μg)	6	R	-	(EUCAST, 2015)
*Polymyxin B	-	S	< 1	-
Spectinomycin (100 μg)	20	Unknown	-	-
Sulfonamide (10 μg)	16	Unknown	-	-

Table 8. AST and MICs for CRKP clinical isolate, KP41 (2015). This result is identical to other CRKP clinical isolates tested [KP42 (2015), KP43 (2015), KP44 (2015), KP45 (2015)]. S- sensitive, I- intermediate, R- resistance, Unknown – no available breakpoints. *- MICs tested with Etest strips, #- MICs tested with Micronaut-S Beta Lactamase VII.

All isolates were positive for carbapenemase production using RAPID CARB blue kit. (Figure 19)

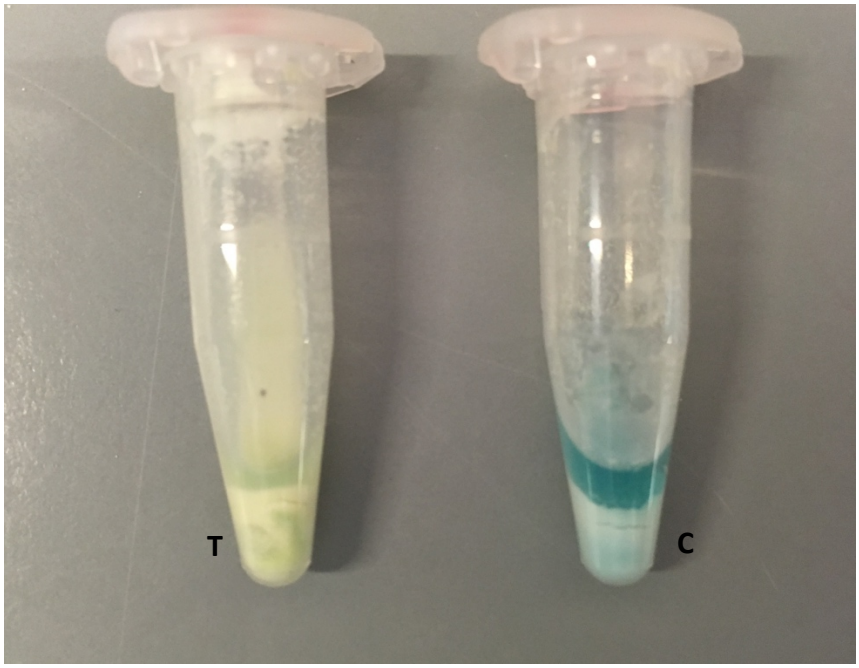


Figure 19. Rapid CARB Blue kit test against CRKP clinical isolates. Positive colour change from blue to yellow was observed. T- test, C- control

3.4.1.2 OXA-48 K-SeT lateral flow in CRKP clinical isolates, OXA-48 K-SeT evaluation and blood culture simulation

All CRKP clinical isolates were positive for OXA-48-like on the lateral flow assay (identified by PCR as OXA-232) (Figure 20).

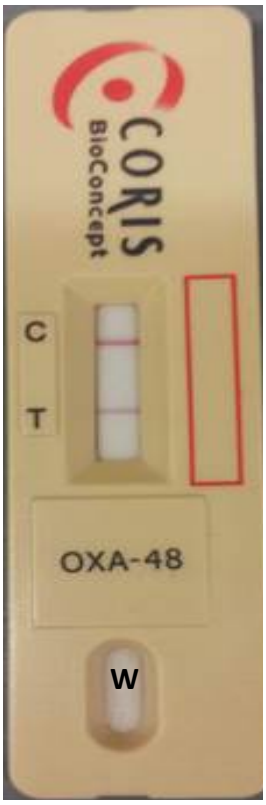


Figure 20. Detection of OXA-48-producing CRKP clinical isolates using OXA-48 K-SeT cassettes. C- control and T – test organism. 2 x 10^6 CFU/ml of CRKP clinical isolates were inoculated (3 drops) into the well (W) and read after 5 mins.

3.4.1.2.1 Evaluation of OXA-48 K-SeT assay

Eighty-two additional enterobacterial isolates were tested from The Royal London Hospital, UK. Molecular analysis of these isolates confirmed that 53 produced an OXA-48-like carbapenemase including 2 *K. pneumoniae* with the OXA-181 and

OXA-232 variant (Table 7). Twenty-five isolates were carbapenem resistant due to the production of either KPC, VIM or NDM (n=13) β-lactamases or had hyper ampC and/or permeability lesions. The OXA-48 K-SeT assay gave a positive result for all 53 OXA-48 producing strains and was negative with all of the other isolates tested, including 5 isolates positive for *bla*$_{OXA-1}$. Correlation of viable colony counts with a positive result for OXA-48 using the NCTC 9633 and KP41 strains assessed the lower limit of detection of the assay as 2.41 x 10^6 CFU/ml. In this evaluation, the sensitivity, and specificity for the detection of OXA-48 using the OXA-48 K-SeT was therefore 100% (Table 9). Positive and negative results could be differentiated within 10 minutes. Results were identical whether the cassettes were inoculated with lysates prepared from MH II plates or BacT/ALERT blood culture bottles.

OXA-48 *K*-SeT	Carbapenem-Resistant Enterobacteriaceae (78)		
	OXA-48-like carbapenemase	**Other carbapenemase**	**No carbapenemase***
Positive (53)			
K. pneumoniae	46; OXA-48 (44), OXA-181 (1), OXA-232 (1)	0	0
E. coli	5	0	0
E. cloacae	2	0	0
Negative (25)			
K. pneumoniae	0	8 KPC (2), NDM (3), VIM (2), KPC+NDM (1)	6
E. coli	0	2 KPC (1), NDM (1)	4
E. cloacae	0	3 NDM (2), VIM (1)	1
E. aerogenes	0	0	1

Table 9. Evaluation for the detection of OXA-48 production in CRE using OXA-48 K-SeT cassette.

3.4.2 Genotypic analysis

3.4.2.1 Clonal typing and virulence determinants

Molecular typing using RAPD PCR revealed the isolates were clonally related (Figure 21). And MLST identified CRKP clinical isolates belonged to ST 231 (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html) (Table 10).

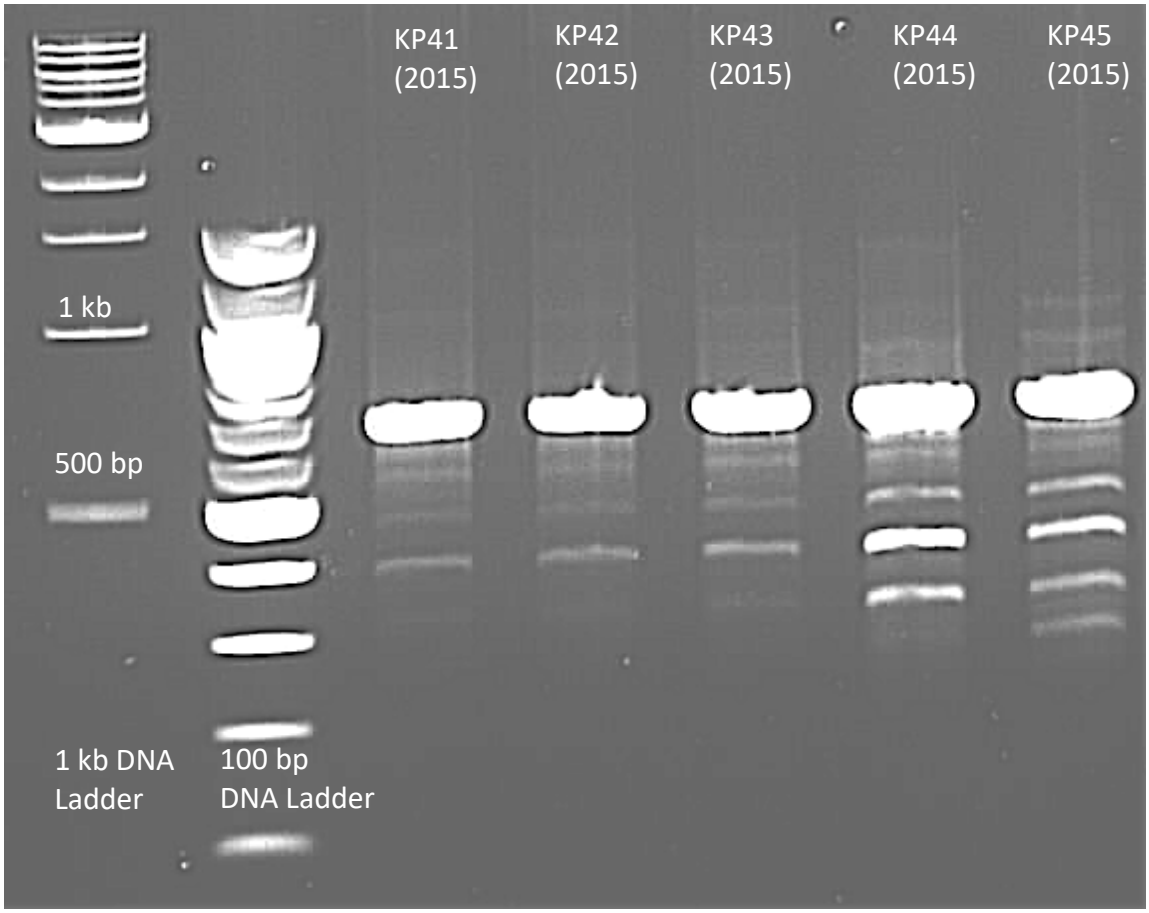


Figure 21. RAPD typing of CRKP clinical isolates. All CRKP isolates showed indistinguishable profiles (clonally related).

Isolates	Housekeeping gene	PCR	Allele
KP41-KP45 (2015)	*gapA*	+	2
	infB	+	6
	pgi	+	3
	rpoB	+	1
	phoE	+	26
	mdh	+	1
	tonB	+	77

Table 10. MLST Pasteur scheme allele results from PCR of seven housekeeping genes (*gap A*, *infB*, *pgi*, *rpoB*, *phoE*, *mdh*, *tonB*). + genes were present. All isolates belonged to sequence type, ST 231.

Genes associated with virulence showed iron uptake (*kfu*), fimbrial mediated adhesion (*fimH*), lipopolysaccharide biosynthesis (*uge* and *wabG*) and urease production (*ureA*). And negative for K1 hypermucoviscous (*cps*) phenotype associated with heightened virulence (Compain et al., 2014).

3.4.2.2 Detection of resistance gene determinants

ARM-D™ for β-Lactamase ID kit revealed all isolates were positive for $bla_{\text{OXA-48–like}}$. Further PCR confirmed all isolates were positive for bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-M}}$, $bla_{\text{OXA-48–like}}$ and *qnrS* alleles. No other carbapenemase genes were detected. BLASTN analysis of the assembled sequence showed 100% nucleotide identity with the $bla_{\text{OXA-232}}$ sequence (accession number JX423831.1) that encodes a carbapenemase hydrolyzing class D enzyme (CHDL) (Potron et al., 2013). The class A β-lactamases in each isolate were similarly identified as $bla_{\text{TEM-1b}}$, $bla_{\text{SHV-11}}$ and

bla$_{CTX-M-15}$.

3.4.2.3 Plasmid typing, sizing and sequencing

PCR-based replicon typing documented the presence of A/C, FIA, FIIK and ColE-like replicons. The plasmidic location of each β-lactamase gene by pulsed field gel electrophoresis (PFGE) of S1-digestion DNA and Southern hybridization with specific intragenic DNA probes showed *bla*$_{OXA-232}$ gene was carried on a small ColE-like plasmid of ~6Kb. The *bla*$_{CTX-M-15}$ gene was localized to a ~161.7Kb IncF plasmid containing fused FIA-FIIk replicons and the *bla*$_{TEM-1b}$ to a non-typeable plasmid of ~8.4Kb.

The sequence of the entire *bla*$_{OXA-232}$-encoding plasmid carried several mobilization genes (*mob*A-D), a replication gene (*rep*A), truncated part of genes encoding a putative LysR-type transcriptional regulator (Δ*lysR*), an erythromycin esterase (Δ*ereA*), and part of an Δ*ISEcp*1 upstream of the *bla*OXA-232 gene (Figure 22).

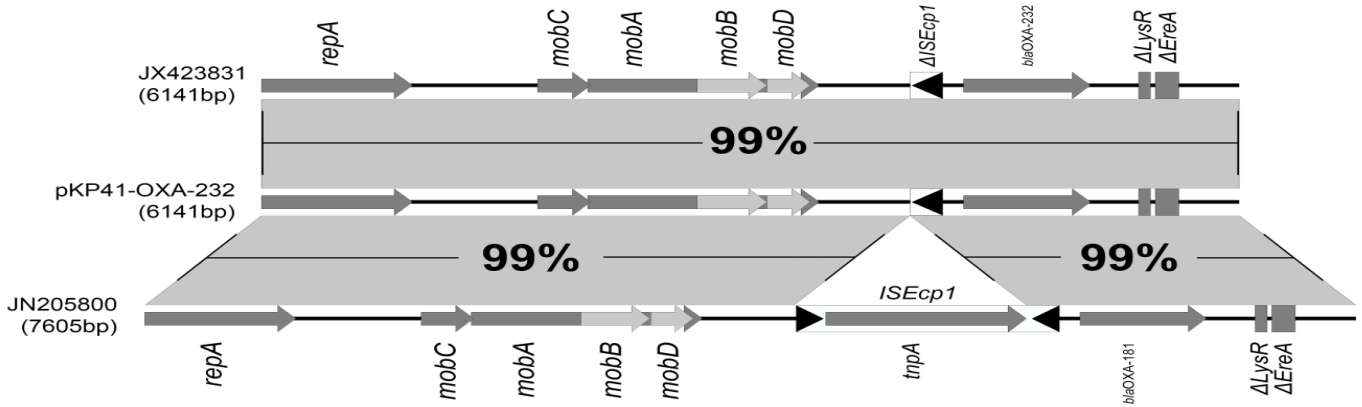


Figure 22. Genetic structure of pKP41-OXA-232 in comparison with other ColE-like plasmids encoding *bla*$_{\text{OXA-232}}$ (GenBank accession no. JX423831) and *bla*$_{\text{OXA-181}}$ (GenBank accession no. JN205800). The entire *bla*$_{\text{OXA-232}}$-encoding plasmid with several mobilization genes (*mob*A-D), a replication gene (*rep*A), truncated part of genes encoding a putative LysR-type transcriptional regulator (Δ*lysR*), an erythromycin esterase (Δ*ereA*), and part of an Δ*ISEcp*1 upstream of the *bla*OXA-232 gene. The reading frames are shown as grey shaded arrows, with the arrowhead indicating the direction of transcription. The truncated reading frames are shown as grey shaded rectangles. The mobile genetic elements are shown as white shaded rectangles flanked by two (complete) or one (truncated) black triangles. The areas shaded in grey indicate nucleotide identity in plasmid sequences, with the percentage indicated.

3.4.2.4 Bacterial conjugation

Transfer of plasmid-mediated resistance genes by conjugation was investigated using rifampicin (RIF) resistant CSH26 and sodium azide (AZ) resistant J53 strains of *E. coli* as recipients. Neither, the $bla_{OXA-232}$ or $bla_{CTX-M15}$ containing plasmids could be mobilized to either *E. coli* strain under the antibiotic selection methods used.

3.4.2.5 WGS analysis

The draft genome sequence of KP41 (2015) revealed a genome size of 5,692,661 bp, with an average G+C content of 56.94%. Provisional annotation using the *ab initio* gene finder algorithm Prokka (version 1.11) (Seemann, 2014) revealed a total of 5,570 coding sequences (CDSs), including at least 82 tRNAs, 23 rRNAs (8 complete and 15 partial), and 19 ncRNAs from the complete genome.

Comparative analysis of the WGS data *in-silico* and the phenotypic susceptibility data was used to define and characterise the entire resistome of KP41. Multiple genes encoding resistance to aminoglycosides (*aph(3')-Ia, aac(6')-Ib3, rmtf*), β-lactams ($bla_{OXA-232}$, bla_{TEM-1B}, $bla_{CTX-M-15}$, bla_{SHV-12}, bla_{LEN-12}), fluoroquinolones (*qnrS1*, *oqxA*, *oqxB*, *aac(6')-Ib-cr*), fosfomycin (*fosA*), macrolide (*erm(42)*), phenicols (*catA1, floR*), rifamipicin (*ARR-2*) and sulphonamides (*sul2*) were identified using ResFinder and correlated entirely with the susceptibility profile of KP41 determined *in-vitro*. PlasmidFinder confirmed the presence of the A/C_2, $ColK(P_3)$, FIB, and $FII_{(K)}$ replicon types, as we have shown earlier based on PCR-

based replicon typing (Diatheva, Fano, Italy), while PHAST (Zhou et al., 2011) revealed the presence of four intact, one incomplete, and two putative prophages. *K. pneumoniae* KP41 (2015) contains genes for iron acquisition, such as *kfuA* and *kfuC* and several virulence genes including *fyuA*, *irp1*, *irp2 ybtA*, *ybtE*, *ybtP*, *ybtQ*, *ybtS*, *ybtT*, *ybtU* and *ybtX* (yersiniabactins), as well as *mrkD* and *mrkH* (type 3 fimbriae formation). *In silico* analysis of OXA-232 CRKP also revealed the absence of rspj mutation (He et al., 2018) causing tigecycline resistance and *aac(6')-Ian* amikacin resistance gene (Troyano-Hernaez et al., 2018).

Additionally, from Kaptive Web K locus result, the assembly of KP41(2015) isolate matches with KL51 (K locus 51) and the O locus typing identified as O1v2. In terms of the diversity in both K and O locus in our KP41 (2015) compared to the core chromosomal phylogeny of the 309 global genomes targeting the globally distributed MDR clones showed our isolate belonged to ST 231 clone.

3.5 Summary

- Five CRKP clinical isolates were recovered from Brunei Hospital. Isolates were recovered from blood culture, endotracheal samples, pus and sputum samples.
- All isolates were resistant to, meropenem (MIC > 32 mg/L), cephalosporins, fosfomycin, minocycline, nitrofurantoin but remained susceptible to colistin (MIC < 1 mg/L), polymyxin B (MIC < 1 mg/L) and ceftazidime/avibactam (MIC = 1 mg/L).
- Rapid CARB Blue kit identified carbapenemase producers in all CRKP isolates.
- OXA-48 K-SeT lateral flow assay performed in all CRKP clinical isolates were positive for $bla_{\text{oxa-48}}$ alleles
- Further evaluation of the OXA-48 K-SeT lateral flow assay with 82 enterobacterial isolates showed sensitivity and specificity of detecting OXA-48 producing isolates to be 100% (95% CI, 91.9-100% and 84.2-100%, respectively). With the blood simulation, results were identical whether the cassettes were inoculated from lysates prepared on MH II plates or BacT/Alert blood culture bottles.
- Molecular typing showed all CRKP isolates were assigned to sequence type (ST) 231. ST231 is also an important clone in *K. pneumoniae* isolates acquiring important ß-lactamases including OXA-232 with diverse plasmid backbones. Other important ST include ST14 acquiring OXA-232 and NDM-1 enzymes.

- Virulence factor genes; *Kfu* (iron uptake), *fimH* (fimbrial-mediated adhesion), *uge* and *wabG* (lipopolysaccharide biosynthesis) and *ureA* (urease production) were found in all isolates except for *cps* (K1 hypermucoviscous) phenotype associated with heightened virulence.
- Molecular detection of acquired genes encoding Class A, B, C and D beta-lactamases were performed by a commercial ARM-D™ for β-Lactamase ID kit and multiplex PCR.
- All isolates were carbapenem-resistant harbouring OXA-232 carbapenemases and carried class A beta-lactamases $bla_{\text{TEM-1b}}$, $bla_{\text{SHV-11}}$ and $bla_{\text{CTX-M-15}}$.
- Plasmid replicon typing showed all strains contained the plasmids A/C, FIA, FIIK and ColE-like replicons.
- The $bla_{\text{OXA-232}}$ was carried on small ColE-like plasmid of ca. 6141 kb. While $bla_{\text{CTX-M-15}}$ ESBL gene was localised to an IncF plasmid containing fused FIA-FIIk replicons, and the $bla_{\text{TEM-1b}}$ to a non-typeable plasmid.
- Conjugation showed neither $bla_{\text{OXA-232}}$- nor $bla_{\text{CTX-M-15}}$ - containing plasmids could be mobilised to either of the *E. coli* recipient strains.
- WGS analysis of KP41 (2015) further identified genes encoding resistance to aminoglycosides (*aph(3')-Ia, aac(6')-Ib3, rmtf*), β-lactams ($bla_{\text{OXA-232}}$, $bla_{\text{TEM-1B}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{SHV-12}}$, $bla_{\text{LEN-12}}$), fluoroquinolones (*qnrS1*, *oqxA*, *oqxB*, *aac(6')-Ib-cr*), fosfomycin (*fosA*), macrolide (*erm(42)*), phenicols (*catA1, floR*), rifamipicin (*ARR-2*) and sulphonamides (*sul2*) using ResFinder that correlates to susceptibility testing *in-vitro*.

Chapter 4

Emergence of plasmid-mediated polymyxin resistance (PMPR), *mcr* genes in Brunei Darussalam

Part 1: Development and evaluation of novel CHROMagar™ COL-*APSE* media

Part 2: Surveillance of PMPR from poultry farms and human clinical faecal samples in Brunei Darussalam

Part 3: A novel PMPR determinant recovered from poultry farm in Brunei Darussalam

4.1 Introduction

In Chapter 3, CRE (carbapenem-resistant OXA-232 producing *Klebsiella pneumoniae*) in Brunei Darussalam which poses a global health threat was characterized. In this chapter, another important global public health issue arises with the emergence of plasmid-mediated colistin resistance *mcr-1* gene reported in China in 2015 (Liu et al., 2016). Since it was first reported, plasmid-mediated colistin resistance (*mcr* genes) have been reported globally and has been identified in animals, humans and the environment (Zhi et al., 2016).

Lack of new antibiotics to treat highly resistant Gram-negative clinical infections has resulted in the repurposing of an old drug such as colistin (Falagas and Kasiakou, 2005). However, the downside to colistin is the neurotoxicity and nephrotoxicity effects (Falagas and Kasiakou, 2005).

Our understanding with colistin resistance was initially limited to a chromosomal-mediated mechanism involving in the activation of the two-component systems (TCSs) PhoP/PhoQ and PmrA/PmrB by specific mutations or environmental stimuli leading to an overexpression of LPS-modifying genes as mentioned in Chapter 1, section 1.6.2.1.1. However, with plasmid-mediated colistin resistance, the *mcr-1* gene encodes phosphoethanolamine transferase (pEtN) enzyme through the addition of pEtN to the lipid A component of the lipopolysaccharides (LPS) conferring resistance to colistin (Liu et al., 2016). (Figure 23)

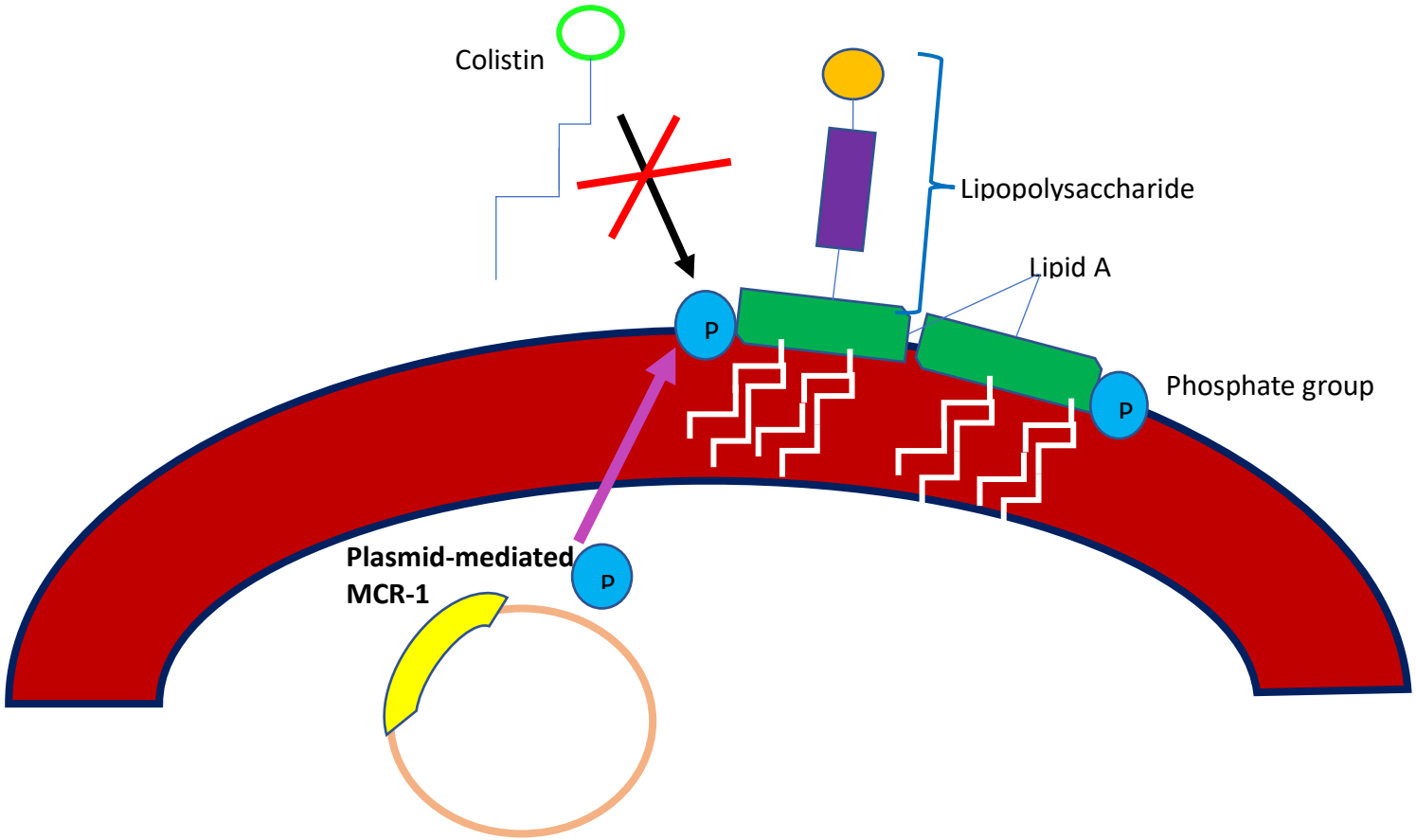


Figure 23. Schematic diagram of plasmid-mediated colistin resistance mechanism. The *mcr-1* gene encodes phosphoethanolamine transferase (pEtN) adding pEtN to Lipid A component of the Lipopolysaccharides (LPS), which in turn cause the negatively charge phosphate group (P) of the Lipid A subunits to become positively charge not allowing the cationic colistin to alter the LPS, leading to resistance.

In 2012, WHO has reclassified colistin as critically important for human medicine (WHO, 2011). Unfortunately, the use of colistin is not only in clinical settings but also in veterinary medicine for prophylactic and treatment purposes (Rhouma et al., 2016). In addition, colistin is used as in-feeds antibiotic growth promoters and has been going on for decades, especially in swine and veal calves (Catry et al., 2015; Kempf et al., 2016; Rhouma et al., 2016). This was further shown, in a retrospective study done by Jianzhong Shen and colleagues, (Shen et al., 2016) where MCR-1 was present in *Escherichia coli* from food-producing animals as far back as the mid-1980s, suggestive of the use of colistin in animal feeds.

In 2006 the European Union (EU) banned antibiotics for growth promotion while the United States (US) implemented legislation based on a voluntary cessation of antibiotic use for growth-promotion as well as re-labelling of antibiotics (Founou et al., 2016). However, in China colistin is widely used in agriculture and the largest user worldwide, with an astounding 11,942 tonnes per year by the end of 2015 (Liu et al., 2016). With increasing production of animal husbandry, by 2021 the amount of colistin use will continue to increase to 16,000 tonnes per year, a 4.75% average annual increase use in this country (Liu et al., 2016). Colistin use was eventually put to a halt in 2016, where an announcement was made by the formal Ministry of Agriculture in China to ban colistin as growth promoter (feed additive) in animals, a major big step for the country (Walsh and Wu, 2016). Resistance to colistin in humans was believed to be linked to colistin ingestion; however, there is evidence of independent emergence of colistin-resistant bacteria in humans without colistin usage, that likely suggests such bacteria may pre-exist in the

human gut and be selected upon colistin therapy (Rolain and Olaitan, 2016; Olaitan et al., 2016b).

As of 2018, multiple *mcr* gene variants (*mcr-1- 8)* have been reported (Partridge et al., 2018; Wang et al., 2018b). Mostly, the reported variants are from human and veterinary specimens in different bacterial species (*E. coli, K. pneumoniae, Salmonella sp, Moraxella sp, Aeromonas sp, R. ornithinolytica*) (Schwarz and Johnson, 2016) (Wang et al., 2019) and on different plasmid types such as IncX4, IncI2, IncFIB and IncHI2 (Zurfluh et al., 2016; Wang et al., 2018a). Moreover, *mcr-1* plasmids have been reported in a variety of different MLST clades and only some of which fall into the B2 group of human pathogens (He et al., 2017; Wang et al., 2017). Most worryingly, *mcr-1* is now starting to emerge in ST131, a virulent strain associated with a variety of clinical infections (Ortiz de la Tabla et al., 2017; Wang et al., 2017). Now, a new highly virulent *mcr-1*-mediated colistin resistance *Escherichia coli* lineage, sequence type 95 (ST95), associated with meningitis and severe avian infection has emerged. In contrast to ST131, ST95 is a global pandemic clone of ExPEC (Extra-intestinal pathogenic *Escherichia coli*), and this lineage is the low frequency of MDR among clinical isolates (Forde et al., 2018).

With the Increasing number of *mcr* cases, the need to implement screening strategies such as the use of selective media or rapid testing kit is crucial, an important step in AMR surveillance. Hence, it necessary to screen for *mcr* genes in animals, humans and environment to understand its dissemination throughout

the world. Now with the evolving CRE and the emergence of plasmid-mediated colistin resistance, the greatest concern is the development of pan-drug resistance leaving no treatment options. Although there has been unprecedented research in new drugs and using an unorthodox drug combination for combatting plasmid-mediated colistin resistance Gram-negative infections, it is still far from reality.

Therefore, prevention is key and tackling AMR through One Health approach would hopefully allow AMR containment and stop their dissemination. The One Health approach involves a network of animal health, human health and environment working together to produce policies, legislation and research to achieve better public health outcome following existing guidelines from the WHO, the Food and Agriculture Organization of the United Nations (FAO) and World Organization for Animal Health (OIE) (WHO, 2017b). With prompt recognition of MCR-1 producers in the animal, human and environment sectors, hopefully, it will prevent future epidemic spread.

4.2 Objectives

Part 1:

- To develop and evaluate our CHROMagar™ COL-*APSE* media for the detection of PMPR and compare it with existing SuperPolymyxin medium.

Part 2:

- To determine the prevalence by performing antibiotic resistance surveillance for colistin resistance *E. coli mcr* genes from chicken farms and human clinical faecal samples in Brunei Darussalam.

Part 3:

- Molecular analysis of novel *mcr-1* variants found during surveillance.

Part 1: Development and evaluation of novel CHROMagar™ COL-*APSE* media

4.3 Methods

4.3.1 CHROMagar™ COL-*APSE* media preparation

CHROMagar™ COL-*APSE* media is a selective media for the isolation and differentiation of colistin-resistant Gram-negative pathogens which is important in antimicrobial resistance surveillance.

CHROMagar™ COL- *APSE* plates were prepared in-house using dehydrated CHROMagar™ base media (CHROMagar™, Paris, France) supplemented with the CHROMagar™ COL- *APSE* supplement (X192) and growth supplement S1. The X192 contains antimicrobials (colistin sulfate, 4 mg/L and linezolid, 1 mg/L) at concentrations designed to enhance the growth of polymyxin resistant (PR) Gram-negative species and suppress the growth of Gram-positive bacteria, while avoiding significant synergy between the active compound (Table 11).

Compound	Amount/L[#]
CHROMagar™ base media	42.5 g
Distilled water	1000 ml
CHROMagar™ COL-*APSE* supplement (X192)	2 ml
CHROMagar™ growth supplement S1	4 ml

Table 11. CHROMagar™ COL-*APSE* preparation. [#]For 50 x 20 ml plates.

The medium was not autoclaved but instead sterilized by boiling at 100 °C for 10 mins to preserve the chromogenic compounds included in the mixture, prior to the addition of all of the supplements.

4.3.2 SuperPolymyxin™ media preparation

SuperPolymyxin™ is the first selective culture medium for the screening of polymyxin-resistant Gram-negative bacteria, which was developed by Nordmann and colleagues (Nordmann et al., 2016b).

SuperPolymyxin plates were prepared according to the protocol described by Nordmann and colleagues (Nordmann et al., 2016b). Briefly, 15 g of EMB agar powder manufactured by Sigma-Aldrich (Dorset, UK) were mixed in 400 ml distilled water at a final concentration 3.75% (w/v) and autoclaved at 15 lbs/sq inch at 121^{o}C for 15min. After autoclaving, the EMB medium was cooled down for 1 h at 56^{o}C. Colistin (3.5 µg/ml), daptomycin (10 µg/ml) and amphotericin B (5 µg/ml) were then added to the medium. The poured plates were then stored in a cold room protect from direct light exposure at 4^{o}C for 1 week.

All of the additional antibiotics used were sourced from Sigma-Aldrich (Dorset, UK) or Cambridge Bioscience (Cambridge, UK). Preparation of antibiotic stock solution preparation see section 4.3.3 below.

4.3.3 Antibiotic stock solution preparation

Antibiotic stock solution was prepared using the formula previously described: (Andrews, 2001)

1000/P x V x C = W

P = potency given by the manufacturer (μg/mg)

V = volume required (mL),

C = final concentration of solution (multiples of 1000) (mg/L)

W = weight of antibiotic in mg to be dissolved in volume V (mL)

Preparation of further stock solutions, from the initial 10,000 mg/L solution, hence the following was prepared:

1 mL of 10,000 mg/L solution + 9 mL diluent* = 1000 mg/L

100 μl of 10,000 mg/L solution + 9.9 mL diluent* = 100 mg/L

*diluent used in this study are all distilled water

For colistin and polymyxin B, both are prepared in 25 ml sterile glass bottle instead of universal tubes to avoid its binding to polystyrene.

4.3.4 Evaluation of CHROMagar™ COL-*APSE* and SuperPolymyxin media

Sixty-one MCR-1 veterinary clinical Isolates provided by Denmark and France, 12 type strains (8 ATCC and 4 NCTC) and 11 human clinical isolates (6 from Clinical Microbiology Laboratory, the Royal London Hospital and 5 from Microbiology Laboratory Services, RIPAS Hospital, Brunei).

4.3.5 Determination of Lower Limit of Detection (LLD)

The lowest limit of detection was assessed using serial dilutions (10^{-1} – 10^{-9}) of an overnight culture grown at 37 °C for 24 h in 3 ml of Luria-Bertani (LB) broth. Ten-fold serial dilutions (100 µl in 900 µl) were made in phosphate-buffered saline (PBS) and 20 µl of each dilution plated onto unsupplemented MH 2 (control) and CHROMagar COL-*APSE* plates using the Miles and Misra procedure.

Colony counts obtained on CHROMagar COL-*APSE* were subtracted from the number recovered on MH 2 agar to quantify the total number of COL resistant organisms (CFU) within the total population plated, required for viable growth on each selective media.

4.3.6 Performance testing using bacterial organisms in mixed culture

Two pools containing mixtures of COL resistant (R) and COL susceptible (S) organisms were used to assess the performance of the media with complex polymicrobial samples.

Pool 1 - *A. baumannii* AB205 (COL resistant), *E. coli* E7 (COL resistant), *E. cloacae* NCTC 10005 (COL resistant), *E. faecalis* ATCC 2912 (COL resistant), *K. pneumoniae* KP19 (COL resistant), *M. morgannii* MM2 (COL resistant), *P. aeruginosa* ATCC 27853 (COL heteroresistant), *S. marcescens* NCTC 10211 (COL resistant), *P. mirabilis* NCTC 13376 (COL resistant).

Pool 2 - *A. baumannii* ATCC 19606 (COL susceptible), *E. coli* ATCC 25922 (COL susceptible), *E. faecalis* ATCC 2912 (COL resistant), *P. aeruginosa* ATCC 27853 (COL heteroresistant), *K. pneumoniae* ATCC 9633 (COL susceptible)

Internal and external quality control of the stability of the media was assessed using *P. mirabilis* NCTC 13376*, E. coli* ATCC 25922*, K. pneumoniae* ATCC 9633 and *E. faecalis* ATCC 2912. Media for use with pools containing *P. mirabilis* NCTC 13376 was supplemented with PNPG.

4.3.7 Agar dilution method for determining colistin and polymyxin B MICs using multi-point inoculator

Isolates included in the CHROMagar™ COL-*APSE* study underwent agar dilution method for colistin and polymyxin B MICs**.** The multi-point inoculator delivers approximately 1µL spot of inoculum onto the agar surface. The final inoculum on the agar will approximately be 10^4 CFU per spot (Figure 24).

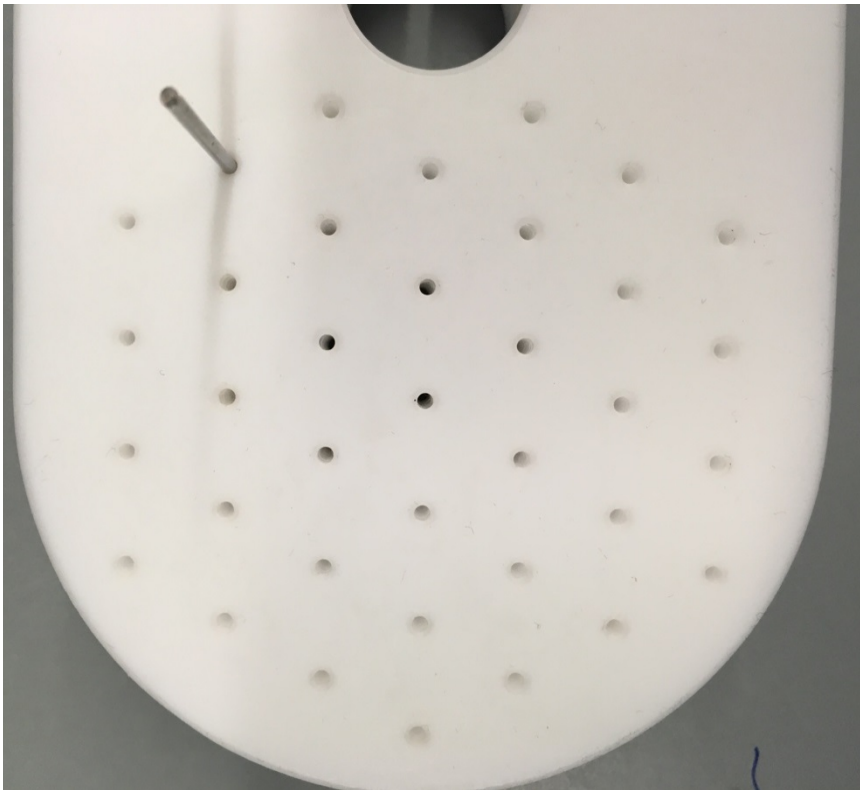


Figure 24. Multi-point inoculator. Individual inoculating pins (delivering approximately 1 µl spot of inoculum onto agar) are arranged into the existing holes.

4.3.7.1 Inoculation preparation

2-3 colonies were selected from MH 2 agar plates grown overnight. These colonies were then inoculated into 3ml of pre-poured 0.9% sterile saline (Oxoid, Basingstoke, UK) and mixed until 0.5 McFarland standard was reached. The 0.5 McFarland suspension is diluted 10-fold by transferring 100 µl to 900 µl saline in Eppendorf tubes. The tubes were mixed twice by inversion. 400 µl were then transferred into the multi-point inoculator wells (Figure 25).

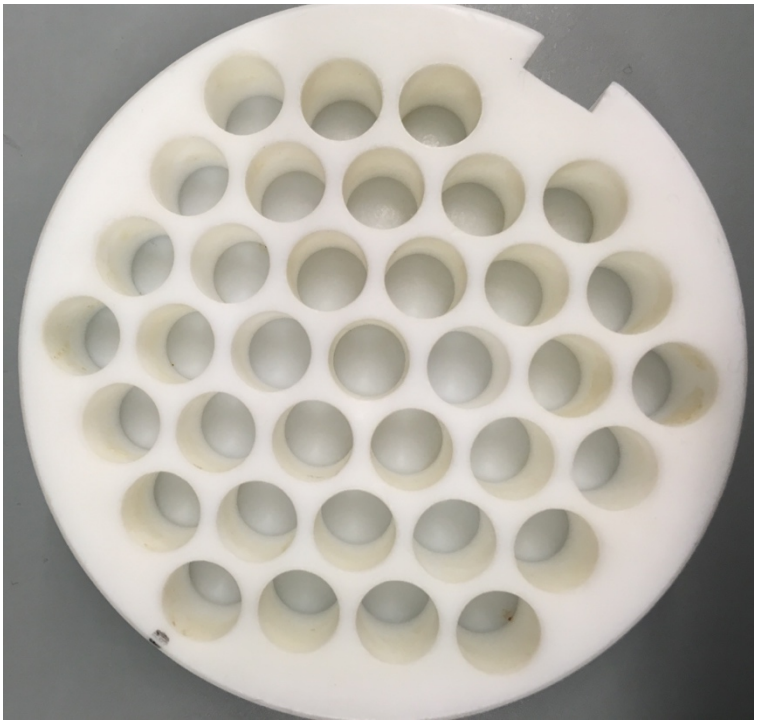


Figure 25. 36-well multi-point inoculator. 400 µl of bacterial inoculum was dispensed individually into these wells.

Antibiotic plates were then inoculated starting with the lowest concentration. The multi-point inoculators are then autoclaved after each use.

As a purity control, the bacterial suspension was seeded onto a media and incubated at 37^{o} C for 24 h.

4.3.7.2 Preparation of MH2 agar containing antibiotics

MH 2 agar plates were prepared as mentioned in Chapter 2, section 2.3.4

Colistin and Polymyxin B stock solutions were prepared as mentioned in section 4.3.3

Colistin and Polymyxin B were added onto MH 2 agar separately from the lowest to the highest concentrations (0.15 – 32 mg/L) according to CLSI guidelines for agar dilution method.

The purity of each inoculum suspension was first checked. If the suspension is not pure, results were not reported. The MIC of each isolate is read as the lowest concentration without visible growth.

4.4 Results

4.4.1 CHROMagar COL-*APSE* media versus SuperPolymyxin agar

The *in vitro* activity (MIC, mg/L) of both COL and POL B when determined by agar dilution was comparable (+/- 1 dilution) for all Enterobacteriaceae isolates tested (Table 12). However, the MICs of COL and POL B differed by at least three dilutions for *S. maltophilia* NCTC 10258 and *A. baumannii* AB205. The *mcr-1* gene was present in 61 of the *E. coli*, 58 (95 %) of which were also phenotypically resistant to COL (MIC > 2 mg/L). Three isolates containing *mcr-1* had colistin MICs below the pharmacodynamic breakpoint when the MIC was determined by agar dilution. Three COL-resistant *A. baumannii* and two *K. pneumoniae* (MIC 8 - >256 mg/L) clinical isolates were also identified and included in the evaluation of both media. The mechanism of resistance in these three strains is yet to be determined, despite sequencing for mutations in *mgrB*, pmrA/B and the phoP/Q regulatory genes known previously to be associated with mutational polymyxin resistance. (Jayol et al., 2015) When susceptibility to COL was determined by BMD, the MIC of COL was comparable to that determined by agar dilution (+/- 1 dilution vs all strains).

Isolates	MIC (mg/L)/ Agar dilution		Polymyxin Resistance Mechanism	Lowest Limit of Detection (C.F.U)	
	Colistin	Polymxyin B		CHROMAgar COL-*APSE*	SuperPolymyxin
Intrinsic Resistance to Polymyxins					
P. mirabilis NCTC 13376	>256	>256	Intrinsic	10^1	10^1
S. marcescens NCTC 10211	>256	>256	Intrinsic	10^1	10^1
M. morgannii MM2	>256	>256	Intrinsic	10^1	10^1
E. faecalis ATCC 2912	>256	256	Intrinsic	$>10^9$	$>10^9$
E. gallinarum ATCC 49573	>256	>256	Intrinsic	$>10^8$	$>10^8$
C. albicans ATCC 10231	256	256	Intrinsic	$>10^7$	$>10^7$
E. cloacae NCTC 10005	128	256	Intrinsic	10^2	10^1
S. maltophilia NCTC 10258	8	64	Intrinsic	10^{1*}	$10^{2‡}$
Susceptible to Polymyxins					

P. aeruginosa ATCC 27853	2	2	NA	10^{2*}	10^{5}
Salmonella Group D (non-Typhi) Sal3	2	2	N/A	10^{4*}	10^{6}
E. coli E17	1	2	MCR-1	10^{7}	10^{7}
Salmonella enterica Subspecies *diarizonae* Sal1	1	2	N/A	10^{5*}	10^{6}
A. baumannii ATCC 19606	1	2	NA	10^{6}	10^{6}
E. coli E44	1	1	MCR-1	$>10^{9}$	$>10^{9}$
Salmonella enterica Subspecies *diarizonae* Sal4	1	1	N/A	10^{5*}	10^{6}
K. pneumoniae KP32	0.5	1	NA	10^{6}	10^{6}
Salmonella enterica Subspecies *diarizonae* Sal2	0.5	1	N/A	10^{5*}	10^{6}
Salmonella Group D (non-Typhi) Sal5	0.5	1	N/A	10^{3*}	10^{5}
E. coli ATCC 25922	0.5	0.5	NA	10^{6}	10^{6}

E. coli 40875 (calf)	0.5	0.5	MCR-1	10^6	10^6
K. pneumoniae ATCC 9633	0.5	0.5	NA	$>10^{9*}$	10^5
Acquired Resistance to Polymxins					
A. baumannii AB219	>256	>256	Unknown	10^1	10^1
A. baumannii AB205	>256	32	Unknown	10^1	10^1
A. baumannii AB287	8	4	Unknown	10^{1*}	10^1
K. pneumoniae KP6	128	256	Unknown	10^1	10^1
K. pneumoniae KP19	64	64	Unknown	10^1	10^1
#*E. coli* 35095 (calf)	>256	>256	MCR-1	10^1	10^1
#*E. coli* 35175 (calf)	64	32	MCR-1	10^1	10^1
#*E. coli* 29881 (calf), 33907 (calf), 34936 (calf), 35593 (calf)	32	32	MCR-1	10^1	10^1
^*E. coli* E10	32	16	MCR-1	10^{1*}	10^4

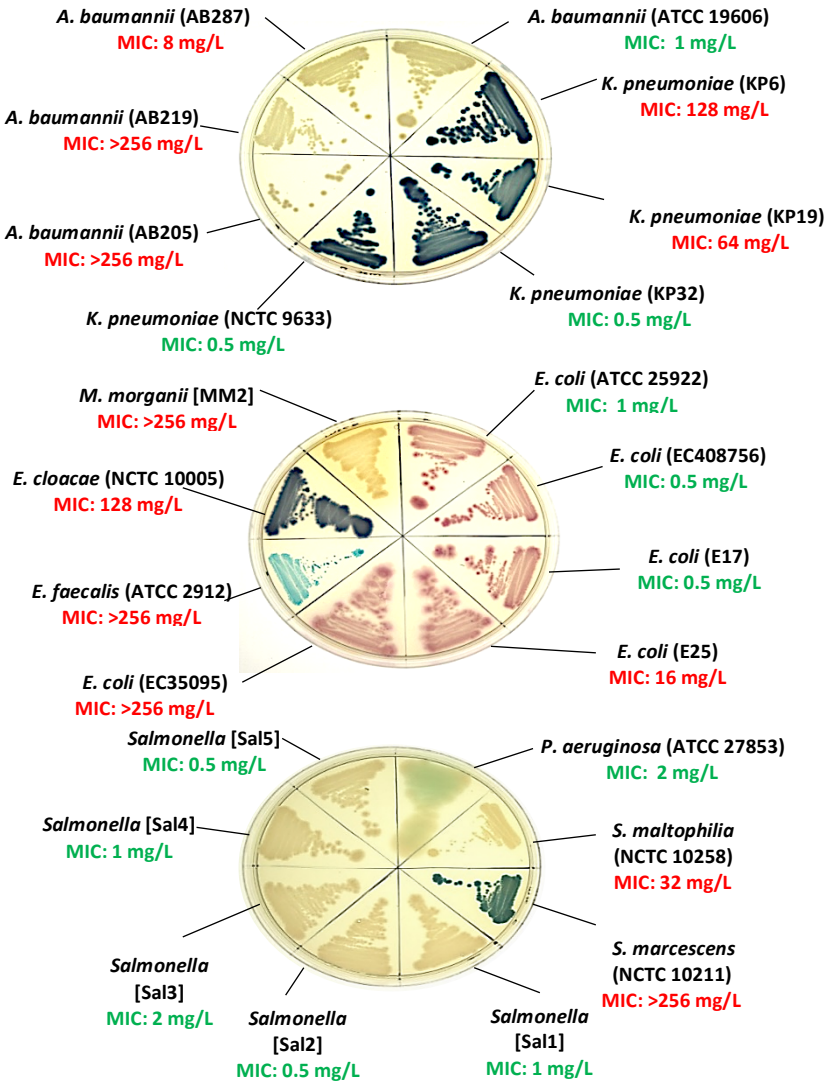
^*E. coli* E9, E33, E3, E1, E35, E29, E32, E13, E24, E20, E25	32	16	MCR-1	10^1	10^1
^*E. coli* E27	16	32	MCR-1	10^1	10^1
^*E. coli* E8, E11, E39, E40, E34, E2, E37, E41, E36, E31 and #*E. coli* 412049521	16	16	MCR-1	10^1	10^1
^*E. coli* E30	16	16	MCR-1	10^{1*}	10^3
^*E. coli* E14, E12, E22, E19, E7, E47, E16, E18, E43, E46, E28, E26,14042624, 412016126 and #*E. coli* 27852 (calf), 37914 (calf), 41323 (chicken), 41339 (chicken), 41848 (calf)	16	16	MCR-1	10^1	10^1
^*E. coli* E49	16	16	MCR-1	10^{1*}	10^3
^*E. coli* E5	8	16	MCR-1	10^{1*}	10^3

#*E. coli* 35062 (calf)	16	8	MCR-1	10^{1*}	10^{3}
#*E. coli* 412044854 and 413040864	16	8	MCR-1	10^{1}	10^{1}
#*E. coli* 34692 (calf)	16	8	MCR-1	10^{1*}	10^{2}
^*E. coli* E45	8	16	MCR-1	10^{1}	10^{1}
#*E. coli* 32218 (calf)	8	8	MCR-1	10^{1}	10^{1}

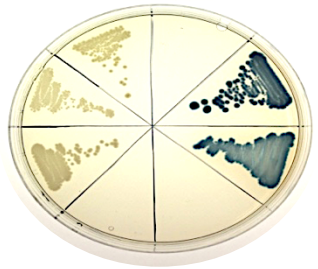
Table 12. Lowest limit of detection (C.F.U) comparison between CHROMagar COL-*APSE* and SuperPolymyxin media. ^isolates were supplied by a group from Denmark and #isolates were supplied by a group from France. *CHROMagar COL-*APSE* sensitivity enhanced and ‡SuperPolymyxin sensitivity enhanced

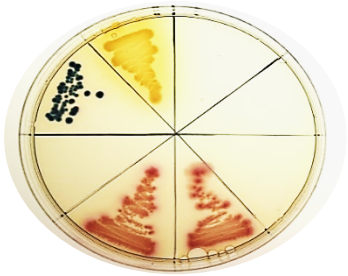
Growth on CHROMagar COL-*APSE* on plates inoculated with 10^3 CFU was observed for all COL R Gram-negative isolates. All of the COL-susceptible Enterobacteriaceae, COL-resistant Gram-positive (*Enterococci* spp) and fungal (*Candida* spp) isolates failed to grow at these inocula (Figure 26). The growth of the *P. aeruginosa* ATCC 27853 type strain (COL MIC 2 mg/L), however, was supported on CHROMagar COL-*APSE* at 10^3 CFU. Although this isolate is deemed susceptible to COL according to current CLSI breakpoints and is recommended as a control strain for antimicrobial susceptibility testing, it should be noted that it has frequently been shown to exhibit heteroresistance to COL *in vitro* when population analysis profiling (PAP) is used as the gold standard. (Bergen et al., 2011) The -70° C stock of ATCC 27853 held in our laboratory and used in this study consistently demonstrates heteroresistance to COL when assessed using the PAP method.

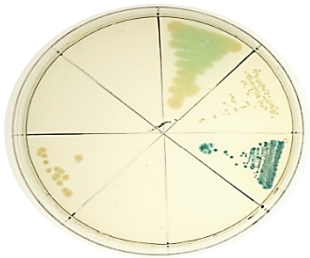
CHROMagar Unsupplemented



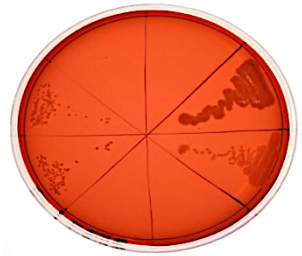
CHROMagar COL-*APSE*







SuperPolymyxin Agar



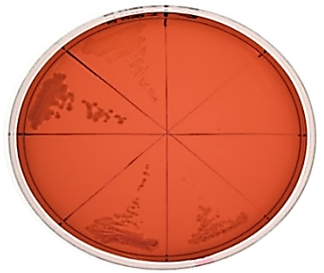
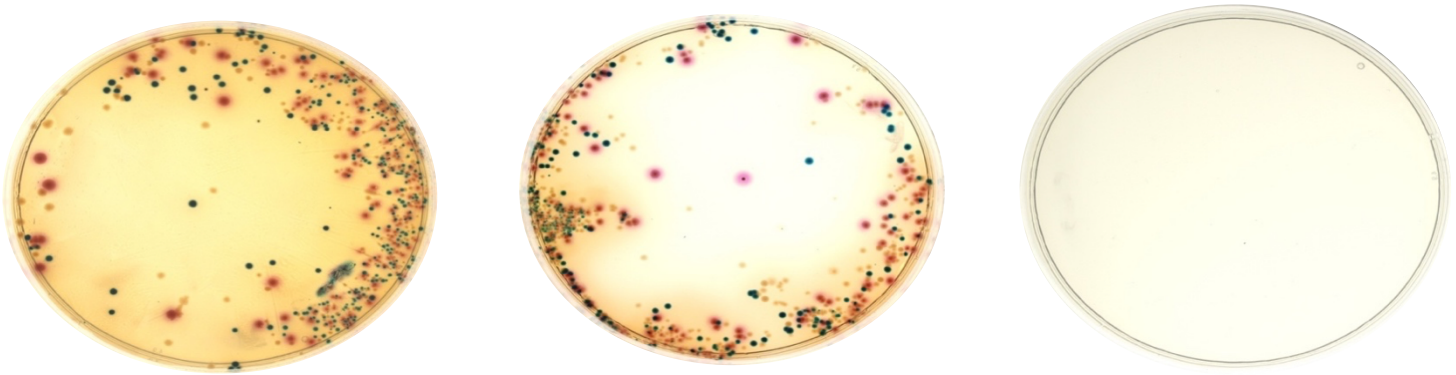


Figure 26. Growth of isolates on CHROMagar Unsupplemented, CHROMagar COL-*APSE* and SuperPolymyxin agar. Growth of isolates observed on CHROMagar COL-*APSE* were AB287, AB219, AB205, KP6, KP19, MM2 (intrinsic resistance to colistin), *E. cloacae* NCTC 10005 (intrinsic resistance to colistin), EC35095, E25, *P. aeruginosa* ATCC 25922 (heteroresistance), *S. maltophilia* NCTC 10258 (intrinsic resistance to colistin), *S. marcescens* NCTC 10211 (intrinsic resistance to colistin) and SAL3. With SuperPolymxin, growth of AB219, AB205, KP6, KP19, MM2 (intrinsic resistance to colistin), *E. cloacae* NCTC 10005 (intrinsic resistance to colistin), EC35095, E25 and *S. marcescens* NCTC 10211 (intrinsic resistance to colistin) were observed.

The growth on SuperPolymyxin media with an inoculum of 10^3 CFU was similar but visibly weaker for each of the COL-resistant non-fermenters. The growth of one strain of COL-resistant *A. baumannii* (MIC 8 mg/L) and *S. maltophilia* (MIC 32 mg/L) was not supported at all (Figure 26). It is possible that the antimicrobial synergy is known to exist between COL and daptomycin (Phee et al., 2013) against *A. baumannii* could account for the poor growth or inhibition of these species that we observed on SuperPolymyxin agar. In contrast to growth on SuperPolymyxin, the chromogenic properties of the CHROMagar COL-*APSE* media enabled the clear differentiation of COL-resistant Enterobacteriaceae from non-fermenters. Phenotypic identification as either dark pink to reddish (*E. coli*), metallic blue (*Klebsiella*, *Enterobacter* and *Serratia* spp) or colourless natural pigmentation (*Morganella*) was also possible, while on SuperPolymyxin only COL-resistant *E. coli* could be identified by their metallic green appearance. In the limit of detection studies, COL-resistant strains could be recovered on both media at 10^1 CFU, while the growth of strains deemed susceptible to COL was only possible when using an inoculum of $>10^4$ (Table 10).

The stability of the media confirmed that there was no reduction in performance (10^3 CFU/ml) for up to 4 weeks. Swarming of *P. mirabilis* NCTC 13376 was observed when it was used to inoculate both CHROMagar COL-*APSE* and SuperPolymyxin plates. The addition of 50 mg/L p-nitro-phenyl glycerol (PNPG) to the CHROMagar COL-*APSE* plates reduced this significantly (Figure 27).



A B C

Figure 27. Growth of simulated bacterial pools inoculated using L-spreader method. A) CHROMagar COL-*APSE* without PNPG containing A. *baumannii* AB205 (COL R), *E. coli* E7 (COL R). *E. cloacae* NCTC 10005 (COL R), *E. faecalis* ATCC 2912 (COL R), *K. pneumoniae* KP19 (COL R), *M. morgannii* MM2 (COL R), *P. aeruginosa* ATCC 27853 (COL heteroresistant), *S. marcescens* NCTC 10211 (COL R) and *P. mirabilis* NCTC 13376 (COL R) B) CHROMagar COL-APSE + 50 mg/L PNPG containing *A. baumannii* AB205 (COL R), *E. coli* E7 (COL R). *E. cloacae* NCTC 10005 (COL R), *E. faecalis* ATCC 2912 (COL R), *K. pneumoniae* KP19 (COL R), *M. morgannii* MM2 (COL R), *P. aeruginosa* ATCC 27853 (COL heteroresistant), *S. marcescens* NCTC 10211 (COL R) and *P. mirabilis* NCTC 13376 (COL R) C) CHROMagar COL-APSE + 50 mg/L PNPG simulated pool containing *A. baumannii*

ATCC 19606 (COL susceptible), *E. coli* ATCC 25922 (COL susceptible), *E. faecalis* ATCC 2912 (COL resistant), *P. aeruginosa* ATCC 27853 (COL heteroresistant), *K. pneumoniae* ATCC 9633 (COL susceptible)

Part 2. Prevalence and surveillance of PMPR from poultry farms and clinical human faecal samples in Brunei Darussalam

4.5 Methods

4.5.1 Chicken cloacal samples

Three hundred cloacal swabs obtained from three different farms (A, B and C) from different geographic location were selected in this study in Brunei in July 2016. Consent was given by these farms for sample collection. The selected farms are the major poultry producers and food distributor for supermarkets in Brunei. Three hundred cloacal swabs from healthy chickens were collected before undergoing slaughter using a transport swab (Transwab® Amies, Corsham, UK).

4.5.2 Human faecal samples

Two hundred and twenty-four human faecal samples were collected during a period in July 2017 and January 2018 as part of Brunei's Microbiology Laboratory routine testing. Faecal samples obtained were from different hospitals and clinics around Brunei. Similarly, faecal samples were collected using a transport swab (Transwab® Amies, Corsham, UK).

Swabs were then transported to the UK (mentioned in Chapter 2, section 2.3.2).

4.5.3 Sample processing and bacterial identification

On arrival, chicken cloacal swabs were inoculated directly onto CHROMagar™ COL-*APSE* media. However, with the human faecal samples, it was first enriched with colistin 50 µg disc in 10 ml of TSB broth (Oxoid, Basingtoke, UK) (TSB) at a final concentration of 5 mg/L in 25 ml sterile universal tube. The enriched human faecal samples were incubated at 37^{o} C for 24 h without shaking. Turbid universal tubes were then seeded onto CHROMagar COL-*APSE* media.

CHROMagar™ COL-*APSE* media from both chicken and humans were incubated at 37^{o} C for 24 h. Red colonies (colonial morphology) isolated from the media were identified as *E. coli* using MALDI-Tof MS (Bruker, Coventry, UK) according to the manufacturer's instructions. Briefly, individual colonies were lightly smeared on to the target plate and overlaid with matrix (α-cyano-4-hydroxycinnamic acid in 50 % acetonitrile/2.5 % trifluoroacetic acid). Once dried, the plates are loaded into the mass spectrometer, where each sample is vaporised (desorption) by the laser and ionised. The mass analyser detects these ions, creating a spectral image unique to bacterial and fungal genera and most species (Clark et al., 2013).

The Isolates were saved at -70˚C in Pro-Lab Diagnostics™ Microbank™ Bacterial and Fungal Preservation System, cryobeads (Fisher Scientific, Leicestershire, UK) until further testing.

4.5.4 Isolates used in prevalence studies

One hundred and seventy-four positive colistin-resistant *E. coli* isolates recovered from CHROMagar COL-*APSE* media from chicken cloacal swabs and 106 from human faecal swabs were chosen for the period prevalence studies.

4.5.5 Isolates used in surveillance analysis

RAPD PCR with ERIC2 primers (as mentioned in Chapter 2, Section 2.4.1.1) were performed on 60 *E. coli mcr-1-like* positive isolates from chickens and 102 from humans. Fifty-two distinguishable profiles of colistin-resistant *E. coli* isolates were then selected for surveillance from both chicken (n= 26) and human samples (n= 26) (Figure 29 and Figure 31).

4.5.6 Susceptibility testing and MICs

Susceptibility to β-lactams; ampicillin (2 μg), augmentin (30 μg), aztreonam (30 μg), cefpirome (30 μg), cefotaxime (30 μg), ceftriaxone (30 μg), cefoxitin (10 / 30 μg), cefpodoxime (10 μg), cephalothin (30 μg), cefepime (30 μg), cefuroxime (30 μg), ceftazidime / avibactam (50 μg), ceftolozane / tazobactam (30 / 10 μg), piperacillin / tazobactam (75 / 10 μg), ertapenem (10 μg), imipenem (10 μg), meropenem (10 μg); amikacin (30 μg), gentamicin (10 μg), kanamycin (30 μg), neomycin (30 μg), netilmicin (30 μg), tobramycin (10 μg), spectinomycin (100 μg), colistin (25 μg), chloramphenicol (30 μg), tetracycline (10 μg), tetracycline (30 μg), levofloxacin (5 μg), minocycline (30 μg), norfloxacin (10 μg), ciprofloxacin (1 / 5 μg), moxifloxacin (5 μg), trimethroprim (5 μg), tigecycline (5 μg), doxycycline (30

μg) and streptomycin (10 μg) was assessed in disc diffusion assays on Mueller-Hinton II (MH II) agar (Oxoid, Basingstoke, UK). The activities of apramycin (15 μg), florfenicol (30 μg), pefloxacin (5 μg), enrofloxacin (5 μg) and ceftiofur (30μg), antimicrobials often used in veterinary practice, were also investigated.

Minimum Inhibitory Concentrations (MICs) of colistin were determined by agar dilution (as mentioned in section 4.3.7) and/or the E-test method (bioMérieux, Marcy l'Etoile, France). Clinical Laboratory Standards Institute (CLSI), CLSI VET08 and European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints were used to define resistance where available (EUCAST, 2018; CLSI, 2018; CLSIVET08, 2018)

4.5.7 PCR amplification of *mcr* genes

DreamTaq Green PCR Master Mix (2X) (Thermofisher Scientific, Dartford, UK) was used for the PCR reaction. Master mix contains DreamTaq DNA polymerase, 2X DreamTaq Green buffer, dATP, dCTP, dGTP and dTTP, 0.4 mM each, and 4 mM $MgCl_2$. And it also contains a density reagent and two dyes for monitoring electrophoresis progress: the blue dye migrates with 3- 5 kb DNA fragments in a 1 % agarose gel and the yellow dye migrates faster than 10 bp DNA fragments in 1 % agarose gel with absorption peaks at 424 nm and 615 nm, respectively.

PCR amplification was performed using primers targeting *mcr* genes (1-2) as described previously (Cavaco et al., 2016).

DNA extraction was performed as mentioned in section 2.3.6.1 . 2 μl of the bacterial lysate was added to 25 μl of DreamTaq Green PCR Master Mix (2X) and 2 μl of primers (multiplex *mcr-1* and *mcr-2*) in a 0.6 ml PCR tube.

For gel electrophoresis and band visualization see section 2.3.7.2

See Appendix A for list of primers used.

4.5.7.1 Cycling condition

PCR cycling conditions used were as follows:

Initial denaturation 15 min at 94^{o} C, followed by 25 cycles of 30 s at 94^{o} C, 90s at 58^{o} C and 60s at 72^{o} C. Final extension of 72^{o} C at 10 min.

4.5.8 WGS analysis

Contigs and draft genome assembly of all 52 colistin-resistant *E. coli* isolates were determined *in silico* using online tools from Centre for Genomic Epidemiology (CGE) as mentioned in Chapter 3, section 3.3.9.4 with additional tools SerotypeFinder 2.0, VirulenceFinder 2.0 and pMLST 2.0.

With regards to MLST, those isolates not assigned a sequence type (ST) from the CGE database are then directed to the EnteroBase database for ST assignment (available at http://enterobase.warwick.ac.uk/species/index/ecoli) (Zhou et al., 2018).

4.5.9 Phylotyping

Phylotypes A, B1, B2 or D of 46 *E. coli mcr-1-like* isolates from chicken samples were determined by triplex PCR phylogroup targeting *chuA*, *yjaA* and TspE4.C2 (putative lipase esterase) genes (Clermont et al., 2000). Phylotyping are performed to identify the virulent extra-intestinal *E. coli* strains belonging to mainly group B2 and, to a lesser extent, to group D (Clermont et al., 2000). While the commensals belonged to either group A or B1 (Clermont et al., 2000).

DNA extraction was performed as mentioned in section 2.3.6.1 . 2 μl of the bacterial lysate was added to 25 μl of DreamTaq Green PCR Master Mix (2X) and 2 μl of primers in a 0.6 ml PCR tube.

For gel electrophoresis and band visualization see section 2.3.7.2

Primers are listed in Appendix A.

Phylotypes of 52 colistin-resistant *E. coli* isolates for surveillance analysis were performed by *in silico* ClermonTyping (Beghain et al., 2018). The ClermonTyping tool allows a given strain sequence to be assigned to *E. albertii*, *E. fergusonii*, *Escherichia* clades I–V, *E. coli sensu stricto* as well as to the seven main *E. coli* phylogroups (A, B1, B2, C, D, E and F) (Beghain et al., 2018). The phylogroup E is formerly a small set of unassigned strains of which O157:H7 is the best-known

member and is now well recognised (Tenaillon et al., 2010). While phylogroup F consists of strains that form a sister group to phylogroup B2 (Jaureguy et al., 2008; Clermont et al., 2011). Phylogroup C has been proposed for strains that is closely related to, but distinct from, phylogroup B1 (Moissenet et al., 2010; Clermont et al., 2011).

Contigs and the draft genome assembly of all isolates were analysed using this online tool available at **http://clermontyping.iame-research.center/**

4.5.10 Serotyping

Serotyping of O antigens was performed on 46 colistin-resistant *E. coli* isolates from chicken samples using antisera pools obtained from Statens Serum Institut (SSI Diagnostica, Denmark), specific for human enterotoxigenic *E. coli* (ETEC) (O11, O20, O21, O27, O32, O48, O78, O80) and invasive O serotypes common to poultry, pigs and sheep (O10, O18ab, O35, O83, O87, O95, O116, O120). Antisera (80 µl) was added to equal volumes of boiled bacterial culture in microtitre plates and incubated at 50-52 °C for 18-24hr. A positive reaction for any serotype was recorded as a precipitate covering the entire bottom of each well. Negative O serotypes were reported when the bacterial suspension produced only small white spots confined to the center of wells.

Serotypes of 52 colistin-resistant *E. coli* isolates for surveillance analysis were determined *in silico* using online tools from Centre for Genomic Epidemiology (CGE) as mentioned in section 4.5.8

4.6 Results

4.6.1 Prevalence *mcr* genes

4.6.1.1 In chicken cloacal samples

Three hundred cloacal swabs collected from three different chicken farms showed 58% (174/300) recovery of colistin-resistant *E. coli* on CHROMagar COL-*APSE* media. The *mcr-1* gene was detected in every strain by PCR. Co-resistance was highest with aminoglycosides (apramycin, 100% and kanamycin, 72%) quinolones (pefloxacin, 92%), phenicols (chloramphenicol, 98% and florfenicol, 100%), β-lactams (amoxicillin/clavulanic, 86% and cefpodoxime, 52%) antibiotics (Figure 28). No reference (EUCAST/CLSI) zone diameter breakpoints are available for apramycin or florfenicol. Isolates with no zone of inhibition are considered resistant.

RAPD analysis revealed considerable diversity (Figure 29). There were 6 different profiles identified out of 60 colistin-resistance *E. coli* isolates tested. However, only 12 colistin-resistance *E. coli* isolates was shown in Figure 29.

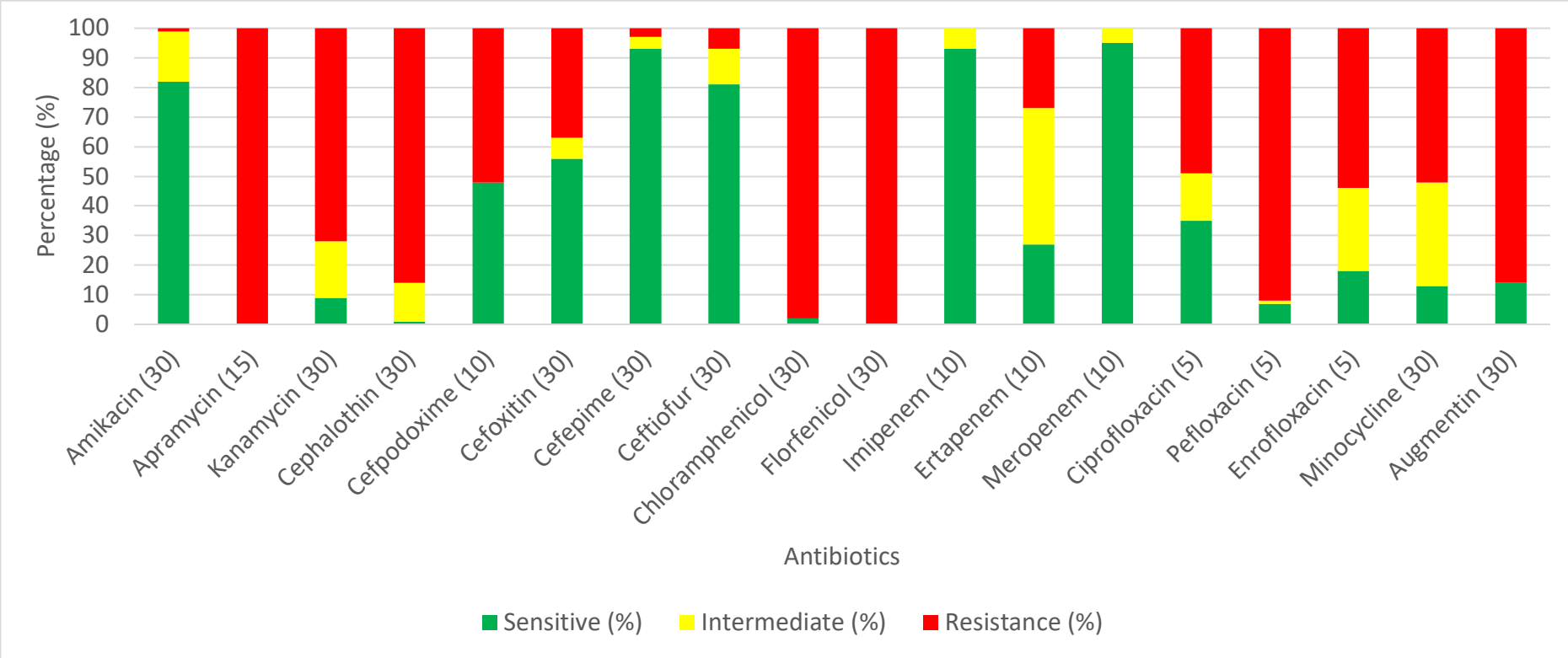


Figure 28. Percentage of co-resistance in *E. coli mcr-1-like* isolates in chickens.

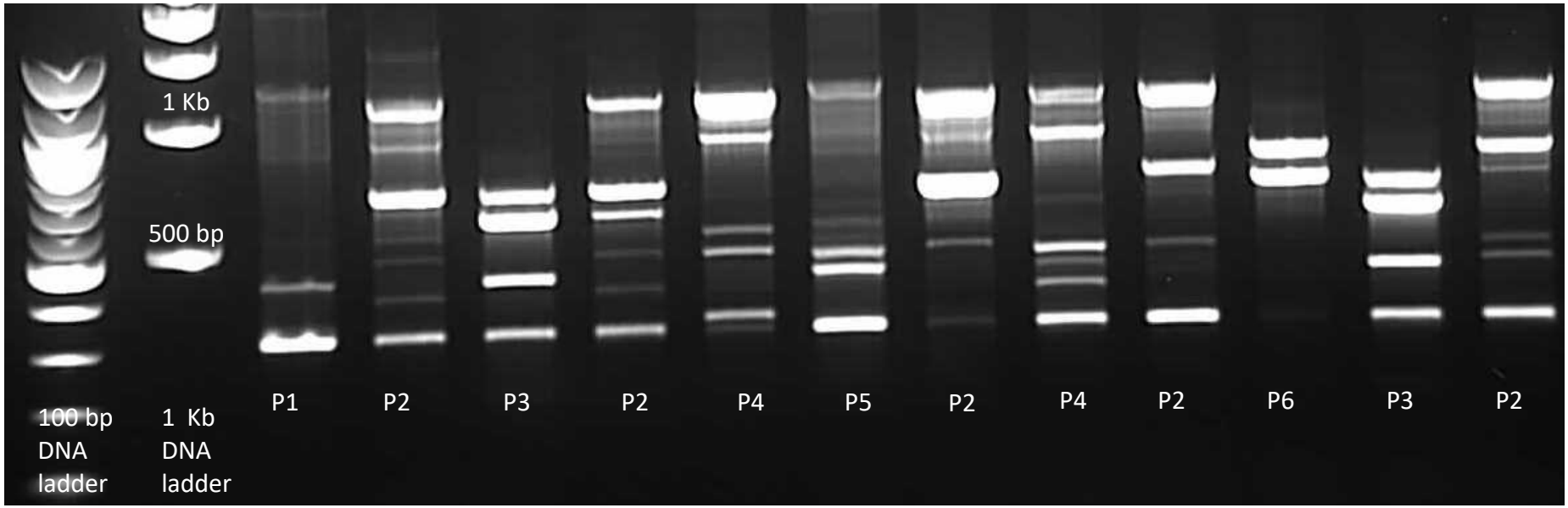


Figure 29. Representative RAPD profiles of colistin-resistant *E. coli* (12/60) isolated from chickens. Considerable diversity is seen with different RAPD profiles (P).

4.6.1.2 In humans faecal samples

41.7 % (106/254) of the faecal samples grew colistin-resistant *E. coli on* CHROMagar COL-*APSE media*. And all of the 106 colistin-resistant *E. coli* isolates were positive for *mcr-1* gene by PCR. The MICs for Polymyxins were in the range of 3-32 mg/L.

Co-resistance was highest with aminoglycoside (apramycin, 100%), quinolones (pefloxacin, 83%), phenicols (chloramphenicol, 83% and florfenicol, 100%), β-lactams (amoxicillin/clavulanic, 84%) antibiotics (Figure 30).

RAPD analysis (Figure 31) revealed considerable diversity, with 26 distinguishable profiles.

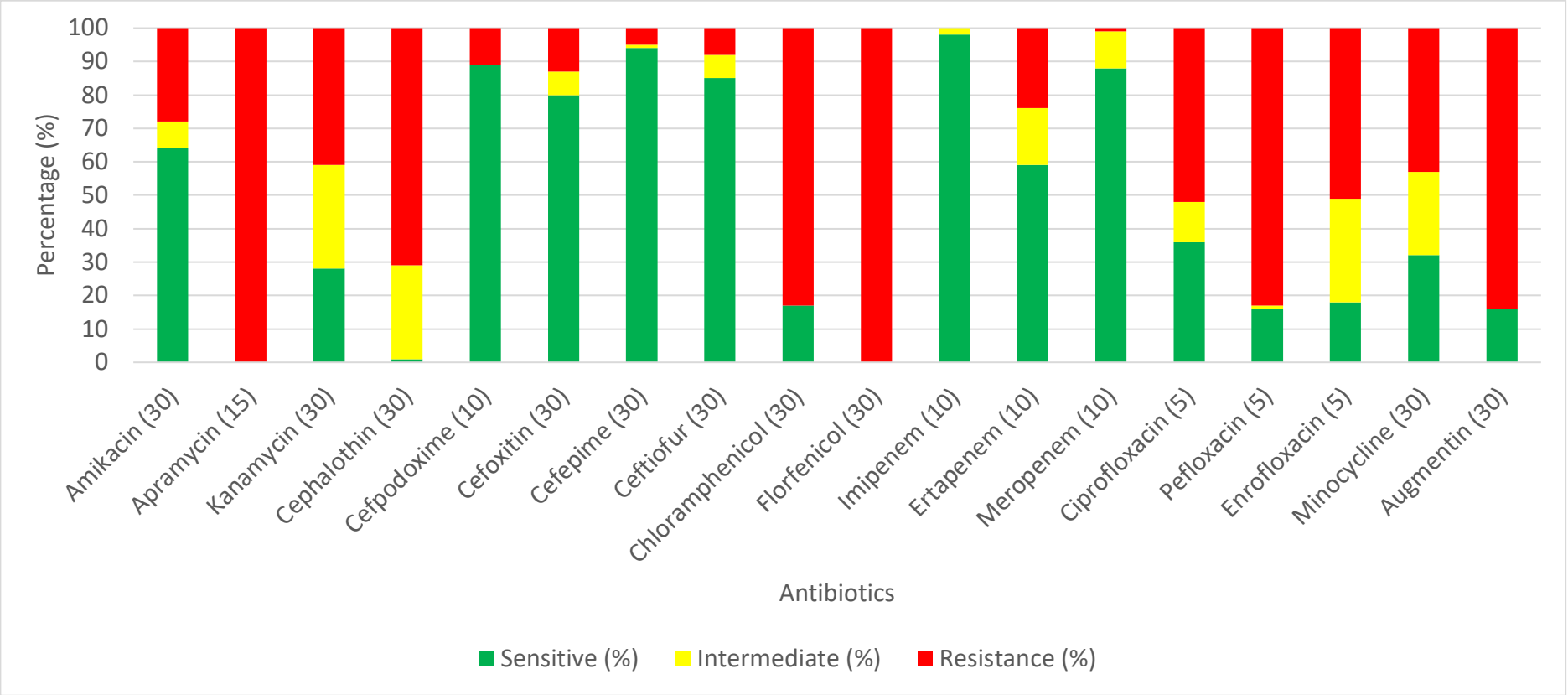


Figure 30. Percentage of co-resistance in *E. coli mcr-1-like* isolates in humans.

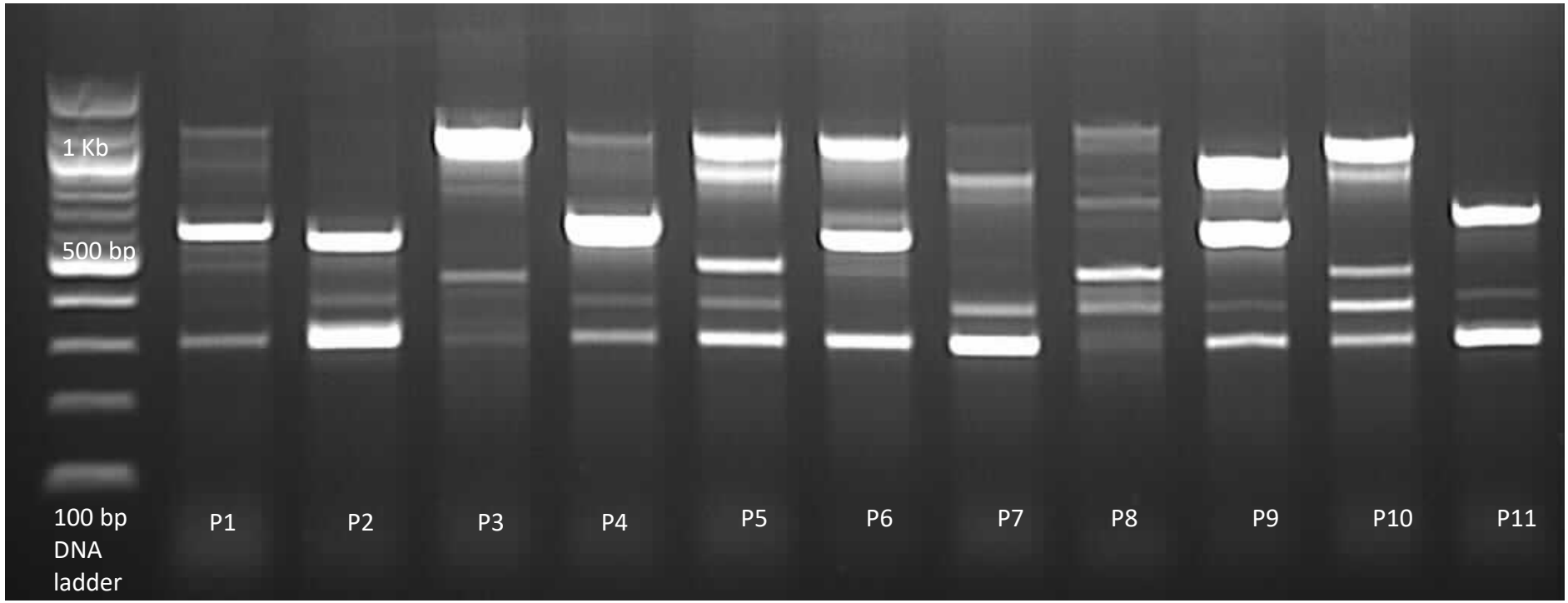


Figure 31. Representative RAPD profiles of colistin-resistant *E. coli* (11/102) isolated from humans. Considerable diversity is seen with different profiles (P).

4.6.2 Surveillance of colistin-resistant *E. coli* isolates

Contigs of 52 colistin-resistant *E. coli* isolates were anlaysed *in silico* using online tools (http://www.genomicepidemiology.org/). The characteristics of each colistin-resistant *E. coli* isolates from chickens and humans are shown in Table 13. Susceptibility testing results for all isolates are found in Appendix B. There were common *E. coli* ST clones observed between chicken farms and human faecal samples (Table 15) and variation in the number of virulence factors observed in all isolates (Table 13). There was no carriage of carbapenemase genes in all of the isolates. However, ESBL genes (CTX-M-3 and CTX-M-65) were identified in 2 of the isolates.

There were differences in the sequence type, ST using alleles to type each of the plasmids by pMLST 2.0 except for B1 and B42 shown in Table 14. Most of the colistin-resistant *E. coli* isolated from chickens and humans are non-typable. There were missing alleles with respect to the plasmid scheme (IncI1, InHI1 and IncN), indicative of novel or undescribed plasmids. Hence, there is no statistical association between the plasmid types and the *mcr-1* carriage.

The most prevalent phylotype performed by PCR in colistin-resistant *E. coli* isolated from chickens were group D (50% n=23/46), followed by B1 (26%, n=12/46), A (15%, n= 7/46) and B2 (9% n=4/46). While results from *in silico* ClermonTyping showed the most prevalent phylotype in chickens were phylogroup B1 (35%, n=9/26), followed by D (23%, n=6/26), A (19%, n=5/26), E (8%, n=2/26), F (8%, n=2/26), B2 (3.5%, n=1/26) and unknown/novel (3.5%,

n=1/26). In humans, the most prevalent phylotype was phylogroup A (38%, n=10/26), D (31%, n=8/26), B1 (15%, n=4/26), unknown/novel (7%, n=2/26), B2 (4.5%, n=1/26) and F (4.5%, n=1/26). However, there are phylogroup B2 (extra-intestinal infection) observed in the chickens using the triplex PCR phylogroup assignment (n=4). While by ClermonTyping method; chickens (n=1) and humans (n=1) (Table 15). Interestingly, discrepancies were reported in 50% (10/20) of colistin-resistant *E. coli* isolates tested with triplex PCR phylotype and *in silico* Clermontyping (Table 16).

Serotypes associated with animal (avian, porcine, ovine) infections were found in 42 % (19/45), but no avian (APEC) or human enterotoxigenic (ETEC) serogroups were found.

Lab ID	Species	Colistin MIC (mg/L)	*mcr* genes	ST	Resistance determinant	Virulence factor genes	Serotype	Incompatibility group
Farm A								
A8	*E. coli*	4	*mcr-1.1*	ST115	*strA, strB, aadA1, aph(3')-Ia, bla*$_{CMY-2}$*, bla*$_{TEM-1B}$*, qnrS1, fosA4, cmlA1, floR, sul3, tet(A), dfrA1*	*iss, celb, astA, iroN, iha, lpfA, gadA, eilA, invasin*	O21:H15	IncFI, ColpVC, Col8282, IncHI1A, IncFIA(HI1)
*A12	*E. coli*	>16	*mcr-1.1*	ST2705	*aadA1, aph(3')-1a, bla*$_{TEM-1B}$*, qnrS1, floR, sul3*	*gadB*	:H10	p0111, IncI2, IncX1
*A13	*E. coli*	2	*mcr-1.1*	ST2473	*aadA2, aadA1, aph(3')-Ia, bla*$_{TEM-1B}$*, qnrS1, fosA4, cmlA1, floR, sul3, tet(A), tet(M), dfrA15*	*iss, lpfA, gad*	O168:H28	IncFIA(HI1), IncHI1A, IncHI1B(R27)
*A25	*E. coli*	2	*mcr-1.1*	ST2705	*bla*$_{TEM-1B}$*, qnrS1, floR, sul3,*	*gad*	:H10	p0111, IncX1, IncI2
A28	*E. coli*	>32	*mcr-1.1*	ST226	*aph(6)-Id, aadA2, bla*$_{TEM-1B}$*, fosA4,cmlA1, floR, sul2, sul3, tet(A), dfrA5*	*gad, ireA*	O18:H10	IncI1

*A31	*E. coli*	2	*mcr-1.1*	ST8262	*aph(6)-Id, fosA4, sul2, sul3*	*air, eilA*	O166:H23	IncFIB(pLF82), IncHIA, IncFIA(HI1)
A33	*E. coli*	1	*mcr-1.1*	ST2732	*aph(4)-Ia, strA, aph(3')-Ia, aadA1, strB, bla*$_{\text{TEM-1B}}$*, bla*$_{\text{CTX-M-3}}$*, oqxA, oqxB, fosA4, floR, catA2, catA1, cmlA1, dfrA12, dfrA14, mph(A)*	*astA, cma, eilA, gad, iha, lpfA, iatB, mchF*	O33:H28	IncHI2, IncFII
A41	*E. coli*	2	*mcr-1.1*	ST117	*aph(3')-Ia, aadA1, aadA2, bla*$_{\text{TEM-1B}}$*, qnrB4, fosA4, floR, cmlA1, sul1, sul3, tet(A), dfrA5, dfrA12, dfrA17, mph(A)*	*iss, iroN, cma, astA, pic, eilA, gad, iha, lpfA, gadB, ireA, invasin*	O8:H19	col(BS512), IncHI1B(R27), IncI1, IncHI1A, IncFIA(HI1)
A53	*E. coli*	2	*mcr-1.1*	ST2223	*aph(4)-Ia, aadA2, strA, strB, bla*$_{\text{TEM-1B}}$*, qnrS1, qnrB4, fosA4, floR, cmlA1, catA2, cat, sul1, sul2, sul3, tet(A), dfrA12*	*iroN, astA, gad, cma*	O75:H42	IncI2, IncN, IncX4, IncX1, p0111, IncI, IncHI2A, IncHI2, IncFII(pSE11), InFII, IncFIB(AP00191

								8), IncB/O/K/Z, ColpVC, Col156
Farm B								
*B1	*E. coli*	4	*mcr-1.1*	ST711	*aph(4)-Ia, aac(3)-Iva, strA, bla*$_{DHA-1}$*, qnrS1, qnrB4, fosA4, florR, cmlA1, sul1, sul3, tet(A), tet(M), dfrA14, mph(A)*	*iroN, lpfA, cma*	O8:H10	IncI1, IncX4, IncHI1B(R27), IncN, IncHI1A, IncFII, IncFIA(HI1)
*B5	*E. coli*	2	*mcr-1.1*	ST117	*aph(4)-Ia, aph(3')-Ia, aadA2, bla*$_{TEM-1B}$*, fosA4, cmlA1, floR, sul2, sul3, tet(A),*	*cma, iss*	O71:H52	IncN, IncI1
B7	*E. coli*	2	*mcr-1.1*	ST1011	*aph(4)-Ia, bla*$_{TEM-176}$*, fosA3, florR, cmlA1, catA1, sul1, sul3, dfrA12, mph(A)*	*iss, iatB*	:H25	IncN, IncHI2, IncI2
*B11	*E. coli*	4	*mcr-1.1*	ST4684	*aph(4)-Ia, bla*$_{TEM-1B}$*, qnrS1, fosA4, floR, sul2, sul3,*	*lpfA*	O9:H7	IncHI1B(R27), IncHI1A, IncX4, IncFIA(HI1)
*B12	*E. coli*	2	*mcr-1.1*	ST1011	*aph(4)-Ia, aadA1, bla*$_{TEM-1B}$*, fosA3, catA1, cmlA1, sul3,*	*iss*	:H25	IncN, IncI2, IncHI2

					sul1, dfrA12 , mph(A)			
*B23	*E. coli*	2	*mcr-1.1*	ST117	*aph(4)-Ia, bla*$_{TEM-1B}$*, fosA4, floR, tet(B), dfrA12,*	*astA, iss, pic, iroN*	O45:H4	IncI2, IncFII, IncFIC(FII), IncFIB(AP001918)
B25	*E. coli*	2	*mcr-1.1*	ST162	*aph(4)-Ia, bla*$_{TEM-1C}$*, sul3, tet(B), dfrA5, mph(A)*	*gad, lpfA, iss*	O134:H19	IncX4, IncFII(29)
*B28	*E. coli*	>32	*mcr-1.1*	ST1727	*bla*$_{TEM-1B}$*, qnrS1, fosA4, floR, sul2, sul3, tet(A)*	*iss, lpfA, cma, gad, iroN*	:H7	IncI2, IncFIB(AP001918), IncFII(pCoo), p0111
*B42	*E. coli*	2	*mcr-1.1*	ST48	*strA,aph(3')-Ia, aph(6)-Id, aadA2, bla*$_{TEM-1B}$*, bla*$_{DHA-1}$*, qnrS1, qnrB4, fosA4, cmlA1, floR, sul1, sul3, dfrA1, dfrA15, dfrA17, mph(A)*	*gad*	:H11	IncX4, IncN
B47	*E. coli*	1	*mcr-1.1*	ST1140	*aph(4)-Ia, bla*$_{TEM-1C}$*, fosA4, floR, sul3, dfrA1, mph(A)*	*gad, gadB*	O38:H39	IncI1

*B52	*E. coli*	4	*mcr-1.1*	ST359	*aph(4)-Ia, aadA2, bla*$_{TEM-1B}$*, bla*$_{DHA-1}$*, qnrB4, cmlA1, floR, sul1, sul3, dfrA17, mph(A)*	*cba, iroN, gad, astA, cma, iss, lpfA, cvaB, tsh*	NT	IncFII, IncFIC(FII), IncI2, IncI1, IncX1, IncFIB(AP001918)
Farm C								
*C1	*E. coli*	1	*mcr-1.1*	ST7687	*aph(4)-Ia, aph(3')-Ia, aac(3)-Iid, bla*$_{TEM-1B}$*, floR, sul3*	*eila, air, gad, gadB*	O17/77:H31	IncX4, IncHI2
*C19	*E. coli*	2	*mcr-1.1*	ST10	*aph(3")-Ib, aadA2, aph(6)-Id, aph(3')-Ia, bla*$_{TEM-1B}$*, qnrS1, fosA4 , floR, catA2, sul1, sul2, sul3, dfrA5, dfrA12, mph(A)*	*gad, astA, gadB*	:H32	IncQ1, IncHI2, IncFII(29), IncI2, IncI1
C22	*E. coli*	2	*mcr-1.8*	ST101	*aph(4)-Ia, aadA1, aac(3)-Iva, aph(3')-Ic, bla*$_{TEM-1B}$*, bla*$_{CTX-M-65}$*, qnrS1, fosA4, floR, cmlA1, sul3, tet(A), tet(M), dfrA15, dfrA14*	*astA, cma, lpfA, iss, eilA, gad, iha, lpfA, mchF*	O88:H31	IncHI1A, IncHIB(R27), IncFIA(HI1), IncFII, IncY, IncI2, IncFIC(FII), IncFIB(AP001918) IncI1, IncX1

C28	*E. coli*	1	*mcr-1.1*	ST2372	*strA, aph(3')-Ia, aph(4)-Ia, strB, bla*$_{TEM-1B}$*, oqxA, oqxB, fosA4, floR, cmlA1, catA2, catA1, sul3, tet(A), tet(M), mph(A)*	*astA, cma, gad, iroN*	O37:H25	ColpVC, IncI2, IncFII, IncFIB(AP001918), Col156, IncB/O/K/Z, ColpVC, IncFII
C45	*E. coli*	2	*mcr-1.1*	ST1642	*aph(4)-Ia, bla*$_{TEM-1C}$*, qnrS1, fosA4, floR, sul3, tet(A),*	*lpfA, astA*	O8:H7	IncFII, IncY, IncFIB(AP001918), IncFIB(pIF82), IncI2, IncX1, IncFII(29)
C55	*E. coli*	2	*mcr-1.1*	ST155	*strA, aph(4)-Ia, aph(3')-Ia, strB, bla*$_{TEM-1B}$*, fosA4, floR, sul3, dfrA1, dfrA5*	*mchF, gad, iroN, tsh, lpfA, iss*	O38:H21	IncI2, IncX4, IncFII(29)
Humans								
1	*E. coli*	4	*mcr-1.1*	ST224	*aadA2, aadA1, cmlA1, floR, sul3, tet(A), mph(A)*	*cma, iss, iroN, gad, lpfA*	O102:H23	IncX4, IncFIB(AP001918), IncFII
8	*E. coli*	4	*mcr-1.1*	ST206	*aadA1, aadA2, aph(3'')-Ib, aph(4)-Ia, aph(6)-Id, bla*$_{TEM-1B}$*, fosA4, cmlA1,*	*astA*	O96:H5	IncHI1A, IncFII(29), IncX4,

					floR, sul2, sul3, dfrA12, dfrA17			IncHI1B(R27), IncFIA(HI1)
25	*E. coli*	2	*mcr-1.1*	ST58	*aadA1, bla*$_{TEM-1B}$ *QnrS1, cmlA1, sul3, tet(A), dfrA15*	*astA, cma, iss, gad, lpfA*	O8:H25	IncFIC(FII), IncFIB(AP001918), IncI2
*46	*E. coli*	4	*mcr-1.1*	ST8379	*aadA1, aadA2, bla*$_{TEM-1A}$*, cmlA1, floR, sul3, tet(A), dfrA12, mef(B)*	*gad*	O45:H26	IncN, Col440I, IncFIA(HI1), IncFIB(K)
49	*E. coli*	2	*mcr-1.1*	ST746	*aph(3')-Ia, aadA1, bla*$_{DHA-1}$*, qnrS1, qnrB4, floR, sul1, sul2, sul3, tet(A), dfrA17, mph(A)*	*gad*	O8:H37	IncX4, IncX2
50	*E. coli*	4	*mcr-1.1*	ST69	*aadA1, qnrS1, floR, sul3, tet(B)*	*eilA, gad, lpfA, air*	O25:H18	IncFIA(HI1), IncHI1A, IncHI1B(R27)
53	*E. coli*	4	*mcr-1.1*	ST93	*aph(3'')-Ib, aph(6)-Id, aph(3')-Ia, aadA1, bla*$_{TEM-1B}$*, fosA4, sul3, sul2, tet(A)*	*astA, cba, cma, iha, iss*	O51:H52	IncHI1A, IncHI1B(R27), IncFIA(HI1), p0111, IncI2

54	*E. coli*	2	*mcr-1.1*	ST1642	*aph(3')-Ia, bla*$_{TEM-176}$*, QnrS1, floR, tet(A), dfrA14*	*iss, astA, lpfA, gad*	O48:H7	IncFIA, IncFIB(AP001918), IncI2, IncFIB(pLF82), IncX1
59	*E. coli*	2	*mcr-1.1*	ST38	*aph(4)-Ia, aadA1, aadA2, bla*$_{TEM-1B}$*, cmlA1, floR, sul3, tet(B), dfrA17*	*iss, senB, eilA, iha, iatB, gadB, AfaB*	O86:H45	IncFIA(HI1), IncHI1A, IncHI1B(R27), IncN
60	*E. coli*	2	*mcr-1.1*	ST1638	*aadA1, bla*$_{TEM-176}$*, qnrS1, floR, tet(A), dfrA1*	*iss, iroN, gadB*	O124:H12	IncX4, IncX1, InFII, IncI2, IncFIB (AP001918), Col (MG828)
61	*E. coli*	2	*mcr-1.1*	ST1011	*aph(4)-Ia, aadA2, bla*$_{TEM-1B}$*, floR, catA1, sul1, sul3, dfrA5, dfrA12, mph(A)*	*iss, eilA, iatB*	O157:H16	IncFIB(AP001918), IncFII, IncI2, Col156
63	*E. coli*	>32	*mcr-1.1*	ST1011	*aph(4)-Ia, aadA2, aac(3)-Iva, aac(3)-Iid, aadA1, bla*$_{TEM-1B}$*, fosA4, floR, catA1, sul1, sul3,*	*iss, astA*	:H16	IncX4, IncFIB(K), IncX1

					tet(A), dfrA12, mph(A)			
*64	*E. coli*	4	*mcr-1.1*	ST 8380	*aadA1, QnrS1, floR, sul3, dfrA14, mph(A),*	*gad, eilA, lpfA, iatB*	:H31	IncHI1A, IncHI1B(R27), IncFIA(HI1)
67	*E. coli*	4	*mcr-1.1*	ST372	*aph(6)-Id, bla*$_{\text{TEM-1B}}$*, catA1, sul2, tet(B), mph(A)*	*iss, vat*	:H31	IncFII(pRSB107), IncX4
70	*E. coli*	4	*mcr-1.1*	ST46	*aadA1, bla*$_{\text{TEM-1B}}$*, qnrS1, fosA4, floR, sul3, tet(A)*	*gad*	O9:H4	IncHI1B(R27), IncFIA(HI1), IncHI1A
*84	*E. coli*	2	*mcr-1.1*	ST8168	*vanC1XY, mdf(A)*	*astA*	:H29	IncX4
86	*E. coli*	4	*mcr-1.1*	ST1121	*aph(6)-Id, aph(3'')-Ib, bla*$_{\text{TEM-1B}}$*, QnrS1, floR, sul3, tet(A), dfrA14,*	*gad, lpfA, lambda bor*	:H38	IncX4, p0111
92	*E. coli*	2	*mcr-1.1*	ST1201	*tet(A)*	*lambda bor, etpD*	O124:H38	IncI2, IncFIB(AP001918), IncFII(pCoo)
96	*E. coli*	4	*mcr-1.1*	ST48	*aadA1, bla*$_{\text{TEM-176}}$*, qnrS1, floR, sul3, tet(A), dfrA5*	*gad, iss, etpD*	O8:H11	IncX4, IncX1, p0111

106	*E. coli*	>32	*mcr-1.1*	ST359	*aph(4)-Ia, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, aadA2, bla*$_{DHA-1}$*, bla*$_{TEM-1B}$*, qnrB4, cmlA1, floR, catA2, sul1, sul2, sul3, tet(A), tet(D), dfrA17, mph(A)*	*iroN, cba, astA, cma, iss, lpfA, mchF*	O35:	IncFII, IncFIC(FII), IncI1, IncFIB(AP001918), IncI2, IncFII
109	*E. coli*	>32	*mcr-1.8*	ST93	*aph(4)-Ia, aph(3'')-Ib, aph(6)-Id, bla*$_{TEM-1B}$*, qnrS1, qnrD, fosA4, floR, catA2, sul3,*	*iss, iha, astA*	O51:H52	IncHI1B(R27), IncFIA(HI1),IncHI1A
111	*E. coli*	4	*mcr-1.1*	ST115	*aph(3')-Ia, aph(3'')-Ib, aph(6)-Id, aadA1, bla*$_{TEM-1B}$*, qnrS1, fosA4, cmlA1, floR, sul2, sul3, tet(B), mph(A)*	*iss, astA, celB*	O21:H16	Col8282, Col156, IncY, IncI2, IncFIB(AP001918), IncFII, IncX1, IncB/O/K/Z
112	*E. coli*	4	*mcr-1.8*	ST117	*bla*$_{TEM-1B}$*, tet(A)*	*iss, lpfA, ireA, hlyE*	O2:H4	IncFII(pHN7A8), IncFII(pSE11), IncFII, IncI2, Col156, IncFIB(AP001918)

113	*E. coli*	2	*mcr-1.1*	ST1011	*aph(3')-Ia, aadA1, bla*$_{TEM-176}$*, qnrS1, cmlA1, floR, sul3, tet(A), dfrA14*	*astA, cma, eilA, iss, lpfA, iatB*	O86:H4	IncI2, IncFII**,** ColpVC, IncFIC(FII), IncFIB(pLF82), IncFIB(AP001918), IncX1, IncY
*121	*E. coli*	2	*mcr-1.1*	ST8376	*aph(3')-Ia, aadA17, bla*$_{TEM-1B}$*, qnrS1, fosA4, floR, tet(A), dfrA12, lnu(F), mph(A)*	*gad*	O8:	IncX4, IncFII, IncX1
124	*E. coli*	2	*mcr-1.8*	ST1011	*aph(4)-Ia, aadA1, aadA2, aac(3)-Iva, bla*$_{TEM-1B}$*, qnrS1, fosA4, cmlA1, floR, catA1, sul2, sul3, tet(A), mph(A)*	*eilA, iatB*	O157:H9	IncI2, IncU, p0111, Col(MG828)

Table 13. Characteristics of 52 colistin-resistant *E. coli* strains isolated from chicken cloacal and human faecal clinical samples. *- ST were determined by EnteroBase database. The letters (A, B and C) represents individual farms where the chicken cloacal samples were collected. NT- Non typeable by SerotypeFinder 2.0, Centre for Genomic Epidemiology (CGE).

E. coli Isolates	IncI MLST	IncI Allele ardA, pilL, repI1, sogS, trbA	IncHI1 MLST	IncHI1 Allele HCM1_043, HCM1_064, HCM1_099, HCM1_116, HCM1_178ac, HCM1_259	IncN MLST	IncN Allele korA, repN, traJ
Chicken						
A8	NT	NT	Unknown, nearest ST 3,1,10	1,1,1,1,-,2	NT	NT
A12	Unknown	-,-,-,-,-	NT	NT	NT	NT
A13	NT	NT	Unknown, nearest ST10,3,1	1,1,1,1,-,2	NT	NT
A25	Unknown	-,-,-,-,-	NT	NT	NT	NT
A28	ST26 (CC-2)	4,1,1,2,13	NT	NT	NT	NT
A31	NT	NT	NT	NT	NT	NT

A33	NT	NT	Unknown	-,-,-,-,-,-	NT	NT
A41	NT	NT	Unknown, nearest ST1,3,10	1,1,1,1,1,2	NT	NT
A53	Unknown, nearest ST 8,208,67,1 14,174	2,3,5,10,6	Unknown	-,-,-,-,-,-	Unknown, nearest ST12,2,3,1,17	-,1,-
B1	Unknown, nearest ST1	9,1,1,1,1	Unknown, nearest ST 1,3,10	1,1,1,1,1,2	ST1	1,1,1
B5	ST13	1,1,1,1,5	NT	NT	Unknown, nearest ST1,3,17,12,2	-,1,-
B7	Unknown	-,-,-,-,-	Unknown	-,-,-,-,-,-	Unknown, nearest ST12,2,17,3,1	-,1,-

B11	NT	NT	Unknown, nearest ST 1,10,3	1,1,1,1,-,2	NT	NT
B12	Unknown	-,-,-,-,-	Unknown	-,-,-,-,-,-	Unknown, nearest ST12,1,2,17,3	-,1,-
B23	Unknown	-,-,-,-,-	NT	NT	NT	NT
B25	NT		NT	NT	NT	NT
B28	Unknown	-,-,-,-,-	NT	NT	NT	NT
B42	NT	NT	NT	NT	ST1	1,1,1
B47	Unknown, nearest ST1	9,1,1,1,1,	NT	NT	NT	NT
B52	Unknown, nearest ST121	4,6,1,2,22	NT	NT	NT	NT
C1	NT		Unknown	-,-,-,-,-,-	NT	NT
C19	ST80 (CC-31)	4,2,1,4,5	Unknown	-,-,-,-,-,-	NT	NT

C22	Unknown, nearest ST295	48,-,-,-,-	NT	NT	NT	NT
C28	ST71 (CC-7)	11,10,4,14,8	NT	NT	NT	NT
C45	Unknown	-,-,-,-,-	NT	NT	NT	NT
C55	Unknown, nearest ST3,220,101	1,2,2,-,4	NT	NT	NT	NT
Human						
1	NT	NT	NT	NT	NT	NT
8	NT	NT	Unknown, nearest ST1,10,3	1,1,1,1,1,2	NT	NT
25	Unknown	-,-,-,-,-	NT	NT	NT	NT
46	NT	NT	NT	NT	Unknown, nearest ST17,12,2,3,1	-,1,-
49	NT	NT	NT	NT	NT	NT

50	NT	NT	Unknown, nearest ST10,1,3	1,1,1,1,-,2	NT	NT
53	Unknown, nearest ST 214, 276	37,-,-,-,-	Unknown, nearest ST3,1,10	1,1,1,1,-,2	NT	NT
54	Unknown	-,-,-,-,-	NT	NT	NT	NT
59	Unknown	-,-,-,-,-	Unknown, nearest ST1,10,3	1,1,1,1,1,2	NT	NT
60	Unknown	-,-,-,-,-	NT	NT	NT	NT
61	Unknown	-,-,-,-,-	NT	NT	NT	NT
63	NT	NT	NT	NT	NT	NT
64	NT	NT	Unknown, nearest ST10,3,1	1,1,1,1,-,2	NT	NT
67	NT	NT	NT	NT	NT	NT
70	NT	NT	Unknown, nearest ST1,3,10	1,1,1,1,-,2	NT	NT

84	NT	NT	NT	NT	NT	NT
86	NT	NT	NT	NT	NT	NT
92	Unknown	-,-,-,-,-	NT	NT	NT	NT
96	NT	NT	NT	NT	NT	NT
106	Unknown, nearest ST284,18,100,121,164,47,26	4,6,1,2,35	NT	NT	NT	NT
109	NT	NT	Unknown, nearest ST10, 3, 1	1,1,1,-,2	NT	NT
111	Unknown, nearest ST221	39,-,-,-,-	NT	NT	NT	NT
112	Unknown	-,-,-,-,-	NT	NT	NT	NT
113	Unknown	-,-,-,-,-	NT	NT	NT	NT
121	NT	NT	NT	NT	NT	NT

124	ST71 (CC-7)	11, 10, 4, 14, 8	NT	NT	NT	NT

Table 14. pMLST of colistin-resistant *E. coli* isolated from chicken. Numbering of alleles with the respective plasmid scheme are in order. *CC-clonal complex, NT- Non typeable, - missing alleles

Sequence Type, ST	**Chicken Farm A**	**Chicken Farm B**	**Chicken Farm C**	**Human Clinical Samples**
ST48	-	*E. coli* B42	-	*E. coli* 96
ST115	*E. coli* A8	-	-	*E. coli* 111
ST117	*E. coli* A41	*E. coli* B5, *E. coli* B23	-	*E. coli* 112
ST359	-	*E. coli* B52	-	*E. coli* 106
ST1011	-	*E. coli* B7, *E. coli* B12	-	*E. coli* 61, *E. coli* 63, *E. coli* 113
ST1642	-	-	*E. coli* C45	*E. coli* 54

Table 15. Colistin-resistant *E. coli* clones identified from 3 chicken farms versus human clinical samples. The six common STs found in chicken and human faecal samples.

Colistin-resistant *E. coli* Isolates	Clermontyping	Triplex PCR
Chickens		
A8	D	D
A12	A	D*
A13	B1	B2*
A25	A	D*
A28	A	D*
A31	D	D
A33	D	D
A41	E	NT
A53	Unknown/novel	NT
B1	B1	B1
B5	F	D*
B7	D	D
B11	B1	A*
B12	D	A*
B23	F	B1*
B25	B1	B1
B28	B1	B2*
B42	A	B2*
B47	E	NT
B52	B1	NT
C1	B2	B2
C19	A	A
C22	D	D
C28	B1	D*
C45	B1	NT
C55	B1	NT
Humans		
1	B1	NT
8	A	NT
25	B1	NT
46	A	NT
49	A	NT
50	D	NT
53	A	NT
54	B1	NT
59	D	NT
60	A	NT
61	D	NT
63	D	NT

64	D	NT
67	B2	NT
70	A	NT
84	B1	NT
86	A	NT
92	A	NT
96	A	NT
106	Unknown/novel	NT
109	Unknown/novel	NT
111	D	NT
112	F	NT
113	D	NT
121	A	NT
124	D	NT

Table 16. *In silico* ClermoTyping and triplex PCR phylogroup of 52 colistin-resistant *E. coli* strains. *Discrepancies observed, NT- Not Tested

Part 3: A novel PMPR determinant recovered from poultry farms in Brunei Darussalam

4.7 Methods

4.7.1 Bacterial isolate

E. coli C22 from the chicken cloacal sample were investigated.

4.7.2 Antibiotic Susceptibility Testing (AST) and Minimum Inhibitory Concentration (MIC)

AST and MICs were performed as mentioned in 4.5.6

Apart from colistin, additional MICs were tested against *E. coli* C22 that includes polymyxin B, cephalothin, cefotaxime, ceftazidime, ceftaroline, ciprofloxacin, florfenicol and fosfomycin were determined by agar dilution (mentioned in section 4.4.3.5) and/or the E-test method (bioMérieux, Marcy l'Etoile, France) according to CLSI and EUCAST (CLSI, 2018; EUCAST, 2018).

Overproduction of chromosomal AmpC β-lactamase by C22 was assessed in a modified Hodge test using a cefoxitin (30 µg) disc and *E. coli* ATCC 25922 as the reporter strain on MH II agar supplemented with and without cloxacillin (200 mg/L) (Mammeri et al., 2008). Effects of AmpC inhibitors were assessed using a Total ESBL + AmpC Confirm kit (Rosco Diagnostics, Denmark).

4.7.3 Amplification of *mcr* genes by PCR

The *mcr* genes were determined by PCR and cycling conditions as mentioned in section 4.5.7 and 4.5.7.1 respectively.

The region flanking the *mcr* sequence was investigated by PCR mapping using primers targeting the insertion element IS*ApI1* and the *nikB* gene using combinations of IS*ApI1*-F (5'-ATCCAACCATTTGAACGACCGTCC-3'),
IS*ApI1*-R (5'-ACCCAGTGCTTCTGTTACCAAACG-3'),
Nik B-F (5'-ATGAAATTGAGAAAGCACAGGGACGGGATT-3') and
NikB-R (5'-GTGAATTGTCACGCTGTTGCTG-3') specific primers.

PCR amplicons were cleaned using MinElute® PCR purification Kit (Qiagen, Crawley, UK) as mentioned in Chapter 2, section 2.3.8

4.7.4 Replicon Typing

Plasmid replicon typing was performed on *E. coli* C22 using commercial PCR-based replicon typing kit (DIATHEVA, Cartoceto, Italy) as mentioned in Chapter 3, section 3.3.7.5.1

4.7.5 DNA cloning

Positive amplicons were purified using Qiaquick PCR purification kits (Qiagen, Crawley, UK) and ligated to the pCR-Blunt II TOPO vector (Invitrogen, Paisley, UK). See Chapter 3, section 3.3.7.4.3 for detailed PCR cloning description.

4.7.6 Conjugation

Transfer of plasmid mediated resistance genes by conjugation was investigated using sodium azide (AZ) resistant *E. coli* J53 as the recipient. *E. coli* C22 and J53 grown to mid-log phase in Luria-Bertani (LB) broth were mixed 1:1 in 4.5 ml fresh LB and incubated at 37°C for a further 4 h without shaking. Transconjugants (ECJ53/C22) were selected by plating 200 µl of the conjugation onto MH II agar supplemented with colistin (4 mg/L) and sodium azide (150 mg/L). The efficiency of conjugation was calculated as the number of transconjugants per donor cell (t/d). Donor and transconjugant cultures were serially diluted (10^{-1} – 10^{-7}) in phosphate buffered saline (PBS) and 20 µl of each dilution plated onto MH II agar supplemented with colistin (donor) and colistin/sodium azide (transconjugant) using the Miles and Misra Method (Miles et al., 1938). Successful transfer of *mcr* to transconjugants was confirmed by PCR.

Antimicrobial susceptibility of transformants and transconjugants was performed by Etest and by broth microtitre dilution MIC determination for colistin and polymyxin B.

The presence of IncI2-like in *E. coli* C22 was further confirmed with IncI2-like plasmid to transconjugants (ECJ53/C22) by PCR using primers IncI2_F (5'-CTGTCGGCATGTCTGTCTC-3') and IncI2_R (5'-CTGGCTACCAGTTGCTCTAA-3') with the resulting amplicons sequenced using the Sanger method as mentioned in Chapter 2, section 2.3.9

4.7.7 Whole Genome Sequencing (WGS)

4.7.7.1 Sample preparation and packaging and transportation of samples

See Chapter 3, section 3.3.9.1and

3.3.9.2

4.7.7.2 Sequencing of isolates and *in silico* analysis of WGS data

The entire genome of *E. coli* C22 isolate and accessory plasmids were sequenced using Illumina HiSeq technology. Genomic DNA was extracted and purified by Solid Phase Reverse Immobilisation (Agencourt, SPRI, Beckman Coulter) and quantified using the Quantit dsDNA HS assay (Thermo-Fisher, East Grinstead, UK) and an Eppendorf AF2200 plate reader (Eppendorf UK Limited, Stevenage). Genomic DNA libraries were prepared using a Nextera XT Library Prep Kit (Illumina, San Diego, USA) with the following modifications: Two nanograms of DNA were used as the input, and PCR elongation time was increased to 1 min from 30 seconds. DNA quantification and library preparation were then carried out on a Hamilton Microlab STAR automated liquid handling system (Hamilto, Nevada, USA). The library was quantified using the Kapa Biosystems Library Quantification Kit for Illumina on a Roche light cycler 96 qPCR machine (Roche Diagnostics Ltd, Burgess Hill, UK). Libraries were sequenced on the Illumina HiSeq using a 250 bp paired end protocol.

Raw reads were adapter trimmed using Trimmomatic 0.30 with a sliding window

quality cutoff of Q15 (Bolger et al., 2014). De novo assembly was performed on samples using SPAdes version 3.7, (Bankevich et al., 2012) and contigs annotated using Prokka 1.11 (Seemann, 2014).

Contigs and the draft genome assembly of C22 were anlaysed *in silico* using online tools (http://www.genomicepidemiology.org/) available at the Centre for Genomic Epidemiology (Lygby, Denmark). Genes encoding genotypic and phenotypic resistance (ResFinder 3.1, KmerResistance 2.2), virulence determinants (Virulence Finder 2.0), serotype (SerotypeFinder 2.0) chromosomal (MLST 2.0) and plasmidic (PlasmidFinder 2.0, pMLST 2.0) epidemiological markers were compared to reference sequences in Pubmed (NCBI). Sequences with < 100 % nucleotide identity were analysed by blastx (NCBI) to identify potential new alleles or non-synonymous mutations. Mutations in the *lpxCAD, pmrA/B, phoP/Q* operons and insertions within *mgrB* previously found to be associated with polymyxin resistance were sought by pairwise alignment with sequences from *E. coli* str. K-12 substrain MG1655 (Accession number: NC_000913.3).

Further characterization of the plasmid pEC-MCR1.8 and images was performed by our collaborators in Leiden University, Netherlands.

4.7.7.3 Comparative analysis of *mcr* alleles

The amino acid sequences of MCR alleles deposited in GenBank (last accessed 17/04/2018) known to confer reduced susceptibility to polymyxins were aligned using ClustalW algorithm.

4.7.8 NG-Test MCR-1 lateral flow assay

During the characterization of our novel variant, NG-Test MCR-1 lateral flow assay was developed and provided to us by NG Biotech, France for evaluation. This was tested against our colistin-resistant *E. coli* isolates including *E. coli* C22. However, there was only limited number of kits available for testing.

NG-Test MCR-1 (NG Biotech, France) is a rapid qualitative immunoassay for the detection of the MCR-1 enzyme using a cultured bacterial colony. The sample loaded onto lateral flow cassette well will migrate by capillarity through nitrocellulose membrane towards the conjugate pad, and if MCR-1 positive, the enzyme will react with labelled anti-MCR-1 mouse monoclonal antibodies immobilized on the test zone (T) producing a positive red line as well as the control zone (C). A negative result will only produce red line with the control zone (C). Notably, due to varying intensity of the red line test (T) depending on the MCR-1 enzyme level in the sample, a weak line is considered as a positive result.

The control line (C) is made of labelled streptavidin and labelled monoclonal antibodies reacting with biotin BSA and goat anti-mouse polyclonal antibodies immobilized on the membrane.

4.7.8.1 Bacterial Isolates

Thirty-six positive MCR-1 producing *Escherichia coli* obtained from chicken cloacal and humans faecal swabs were collected from Brunei were tested. Eight clinical isolates from the Royal London Hospital (*Aeromonas* sp. AH10(2018), n=1; *Escherchia coli* EC5(2018) and EC60, n=2; *Klebsiella pneumoniae* KP25(2018), KP27(2018) and KP28(2018) n=3; *Salmonella* sp. SAL1 and SAL2 n=2) and 9 types strains (*Proteus mirabilis* NCTCC13376, *Serratia marcescens* NCTCC10211, *Enterococcus faecalis* ATCC29212, *Enterococcus gallinarum* ATCC49573, *Candida albicans* ATCC10231, *Enterobacter cloacae* NCTC10005, *Stenotrophomonas maltophilia* NCTC10258, *Pseudomonas aeruginosa* ATCC27853, *Escherichia coli* ATCC25922) were also used for evaluation in this study. Bacterial identification was performed by MALDI-ToF (matrix-assisted laser desorption ionisation-time of fight). Mutliplex *mcr* PCR and/or Illumina MiSeq were used for *mcr* gene detection.

Additionally, human faecal swabs that were found to be positive for having Enterobacteriaceae with MCR-1 (n=14) and swabs that were negative (n=2) were used to further assess the potential of the MCR-1 immunoassay to detect MCR-1 from direct swabs.

4.7.8.2 Sample preparation

Thirty-six *E. coli* MCR-1, 8 clinical isolates from Royal London Hospital and 9 control strain type, overnight bacterial culture was grown on MH 2 agar. Five drops (150 µl) of extraction buffer were dispensed in an Eppendorf tube provided. A colony was taken from MH 2 agar using 5 µl sterile loop and suspended in the tube with the extraction buffer. The tube lid was then closed and vortex to homogenise the mixture before use.

100 µl of the mixture were pipetted and dispensed on the lateral flow device. Results were read after 15 minutes.

As with the swabs, all the 16 swabs were inoculated in 3ml TSB (Trypticase soy broth) with colistin (final concentration 1 mg/L) and incubated at 37^{o}C shaking for 2hr. Positive swabs that became turbid (n= 14) were centrifuged and their supernatant was used for the lateral flow test. Similarly, the same process with the negative swabs that grew turbid (n= 1).

4. 8 Results

4.8.1 Susceptibility testing, typing and virulence-associated genes in *E. coli* C22

Isolate *E. coli* C22 was recovered in pure culture on CHROMagar COL-*APSE* media, confirmed as *E. coli* by colonial morphology and by MALDI-TOF MS analysis. Susceptibility testing confirmed resistance to colistin (MIC 8 mg/L) and polymyxin B (12 mg/L) and also an MDR phenotype. *E. coli* C22 was resistant to all penicillins, cephalosporins (except ceftazidime), aztreonam, quinolones, aminoglycosides (except amikacin), fosfomycin, chloramphenicol and tetracyclines (Table 17). The isolate was susceptible to carbapenems (imipenem, meropenem) the combination of ceftazidime / avibactam (MIC = 0.032 mg/L) and to tigecycline (MIC = 0.38 mg/L). No AmpC-like activity was detected in either the modified Hodge or using the ROSCO combined disc test. Amongst the veterinary antimicrobials tested, resistance to apramycin, florfenicol, pefloxacin, enrofloxacin and ceftiofur was inferred, as there was complete growth of C22 up to the margins of the discs (Table 17).

Antimicrobial agent	Disc susceptibility		MIC (µg/ml)	Interpretation
	Concentration (µg)	Zone diameter (mm)		
Aminoglycosides				
Amikacin	30	19	≤ 8	S
Apramycin•	15	6	NA	R
Gentamicin	10	11	> 4	R
Kanamycin	30	16	NA	R
Neomycin	30	9	NA	R
Netilmicin	30	13	3	R
Spectinomycin	100	22	NA	R
Streptomycin	10	6	NA	R
Tobramycin	10	6	> 4	R
β-lactams: Carbapenems				
Ertapenem	10	22	> 1	I
Imipenem	10	25	≤ 2	S
Meropenem	10	25	≤ 2	S
β-lactams: Cephalosporins				
Cephalothin	30	6	NA	R
Cefoxitin	10 / 30	20 / 25	NA	R
Cefuroxime	30	6	> 8	R
Cefpodoxime	10	6	NA	R
Cefotaxime	30	12	> 2	R
Ceftriaxone	30	12	> 2	R
Ceftiofur•	30	6	NA	R
Cefepime	30	17	> 4	R
Cefpirome	30	6	NA	R
β-lactams: Monobactams & Penicillins				
Aztreonam	30	22	3	R
Ampicillin	2	6	> 8	R
β-lactam / β-lactamase inhibitor combinations				
Amoxicillin/Clavulanate	30	6	> 8	R
Ceftazidime/Avibactam	50	27	≤ 8	S
Ceftolozane/Tazobactam	40	22	> 1	R
Piperacillin/Tazobactam	85	25	≤ 8	S
Folate pathway inhibitors				
Sulfamethoxazole	100	6	NA	R
Trimethoprim	5	6	> 4	R
Fluoroquinolones / Quinolones				
Ciprofloxacin	1 / 5	6 / 11	> 0.5	R
Enrofloxacin•	5	8	NA	R
Levofloxacin	5	12	> 1	R

Moxifloxacin	5	10	NA	R
Norfloxacin	10	10	> 1	R
Pefloxacin•	5	6	NA	R
Phenicols				
Chloramphenicol	30	6	> 8	R
Florfenicol•	30	6	NA	R
Lipopeptides				
Colistin	25	12	> 2	R
Tetracyclines				
Doxycycline	30	10	NA	R
Minocycline	30	11	NA	R
Tetracycline	10 / 30	6 / 6	NA	R
Tigecycline	5	21	≤ 1	S

Table 17. Antimicrobial susceptibility of *E. coli* C22. • Antimicrobial used in veterinary medicine; NA – no zone diameter breakpoint available, S- susceptible, R – resistant, I - intermediate

There was no reaction with any of the O specific antisera contained in the Statens Serum pools used and a serotype could therefore not be assigned phenotypically.

Analysis of the *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA* gene sequences used in the *E. coli* multi-locus sequence typing scheme (MLST) (Zhou et al., 2018), identified alleles 43, 41, 15, 18, 11, 7 and 6, consistent with sequence type (ST) 101, a globally disseminated clone, belonging to phylogenetic lineage B (Fernandes et al., 2016). PCR-based replicon typing identified multiple plasmid replicons in C22 belonging to FIA, FIB, FIC, FII, HI1A, HI1B, I1, I2, X1 and Y types. The presence of *lpfA* (fimbrial adhesin), *iroN* (enterobactin siderophore receptor protein), *iss* (serum resistance), *gad* (acid resistance) and *cma* (inhibition of lysis) virulence associated genes were also documented with *iroN*, *iss* and *cma* co-localised to an IncFIB plasmid, whereas *lpfA*, *gad* and an additional copy of *iss* were mapped to the chromosome.

4.8.2 Polymyxin Resistance Determinants

Multiplex PCR confirmed the presence of a *mcr-1*-like gene and Sanger sequencing, revealed a variant with a single non-synonymous nucleotide difference (A8G) from *mcr-1.1* (Table 18) designated *mcr-1.8* by NCBI (Accession number KY683842) and resulting in a glutamine to arginine substitution (Q3R) in the transmembrane portion of the protein, physically distant from the extracellular catalytic domain. The previously identified MCR-1.2 and MCR-1.12 also contain respectively the amino acid substitutions Q3L and Q3H with no attributable loss of catalytic function (Di Pilato et al., 2016). PCR mapping

confirmed the *nikB* ABC transporter 220 bp upstream of the *mcr* coding region but absence of the IS*ApI1* sequence.

The entire *mcr* gene along with the upstream element and promoter was inserted into pCR-Blunt II TOPO and transformed in *E. coli* TOP10. Colistin and polymyxin B MICs were increased by 4- to 16- fold (Table 19).

Polymyxin resistance was transferable from *E. coli* C22 to J53. All transconjugants recovered displayed colistin and polymyxin B MICs of 4 mg/L (Table 19) and were also positive for the *mcr-1.8* gene. Conjugation was estimated to occur at a frequency of 2.6×10^{-4} t/d.

PEtN	**Species**	1	2/1	3	6/7	12	24	39	62	156	216	235	279	355	413	441	444	453	537
MCR-1.1	*E. coli*	M	M	Q	V	R	A	I	M	M	A	M	T	A	V	D	A	H	R
MCR-1.2	*K. pneumoniae*	.	.	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
MCR-1.3	*E. coli*	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.
MCR-1.4	*E. coli*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.
MCR-1.5	*E. coli*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Y	.
MCR-1.6	*Salmonella enterica*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	H
MCR-1.7	*E. coli*	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.
MCR-1.8	***E. coli***	**.**	**.**	**R**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**
MCR-1.9	*E. coli*	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.
MCR-1.10	*Moraxella* sp	.	V	.	.	C	S	.	.	V	.	T	.	T	.	.	T	.	.
MCR-1.11	*E. coli*	.	.	.	V*	.	.	.	.	.	.	.	.	.	.	.	.	.	.
MCR-1.12	*E. coli*	.	.	H	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
MCR-1.13	*E. coli*	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.
MCR-1.14	*E. coli*	.	.	.	.	.	.	.	L	.	.	.	.	.	.	.	.	.	.
MCR-1.15	*K. pneumoniae*	_	.	.	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.

Table 18. Amino acid substitutions in MCR1.2 – 1.15 relative to the MCR-1 peptide sequence. GeneBank MCR protein accession numbers: 1.1 (WP_049589868.1), 1.2 (WP_065274078.1), 1.3 (WP_077064885.1), 1.4 (WP_076611062.1), 1.5 (WP_076611061.1), 1.6 (WP_077248208.1), 1.7 (WP_085562392.1), 1.8 (WP_085562407.1), 1.9 (WP_099982800.1), 1.10 (WP_096807442.1), 1.11 (WP_099982815.1), 1.12 (WP_104009850.1),

1.13 (WP_109545056.1), 1.14 (SPQ84451.1), 1.15 (WP_116786830.1). Amino acid numbering based on MCR 1.1 peptide sequence. Identical amino acids represented as . Deletion in MCR-1.15 represented as _ Insertion in MCR-1.11 represented as V*

Strain	CEF	CTX	CAZ	CPT	CST	PMB	*FOF	TET	CIP	CHL	KAN	AZM	TGC	CZA
E. coli C22	>256	32	0.5	>32	8	12	>1024	256	12	>256	6	24	0.38	0.032
E. coli J53 AZR	8	0.047	0.094	0.06	0.38	0.75	0.38	2	0.016	6	2	3	NT	NT
E. coli J53 AZR:pEC-MCR1.8	6	0.047	0.125	0.06	**4**	**4**	0.19	2	0.012	6	3	3	NT	NT
E. coli TOP10	NT	NT	NT	NT	0.125	0.5	NT	NT	NT	NT	NT	NT	NT	NT
E. coli TOP10:*mcr-1.8*	NT	NT	NT	NT	**2**	**2**	NT	NT	NT	NT	NT	NT	NT	NT

Table 19. MICs (mg/L) by Etest for antibiotics against wild-type strain, recipient and transconjugant strains of *E. coli.* CEF, cephalothin; CTX, cefotaxime; CAZ, ceftazidime, CPT, ceftaroline; CST, colistin; PMB, polymyxin B; *FOF, fosfomycin (MH II media supplemented with Glucose 6-

phospate); TET, tetracycline; CIP, ciprofloxacin; CHL, chloramphenicol; KAN, kanamycin; AZM, Azithromycin; TGC, Tigecycline; CZA, ceftazidime/avibactam, NT; Not tested.

4.8.2.1 Characterisation of plasmid, pEC-MCR1.8

Whole-genome sequencing of *E. coli* C22 with the Illumina HiSeq platform (Illumina, Inc., San Diego, CA) followed by *de novo* assembly and annotation identified the *mcr-1.8* gene as the only antimicrobial resistance gene, located between the *topB* (encoding a DNA topoisomerase III) and *nikB* (relaxase) genes, on a 63,056 bp IncI2 plasmid (pEC-MCR1.8) (performed with the assistance of our collaborator from Leiden University, Netherlands). Nucleotide sequence analysis of pEC-MCR1.8 (KY792081) confirmed a similar position of *mcr-1.8* and absence of the IS*Apl1* locus as in other IncI2 plasmids encoding *mcr-1*-like genes (Figure 32). The graphical sequence comparison was generated using Easyfig software (Figure 32). The presence of shufflon segments present in raw reads were assembled using the pMRY16-002_4 as an IncI2 reference plasmid and the assembled shufflon region was confirmed by Sanger sequencing (performed with the assistance from our collaborators from Federation University, Australia and Leiden University, Netherlands) (Brouwer et al., 2015; Sekizuka et al., 2017).

Apart from *mcr-1.8* gene, pEC-MCR1.8 predicted to encode 85 open reading frames in total, including genes for replication, maintenance, partitioning and stability, as well as conjugal transfer/ formation of type IV pilus. Immediately downstream of *mcr-1.8* was the *pap2* gene predicted to encode a membrane-associated phosphatase enzyme (Fan et al., 2014).

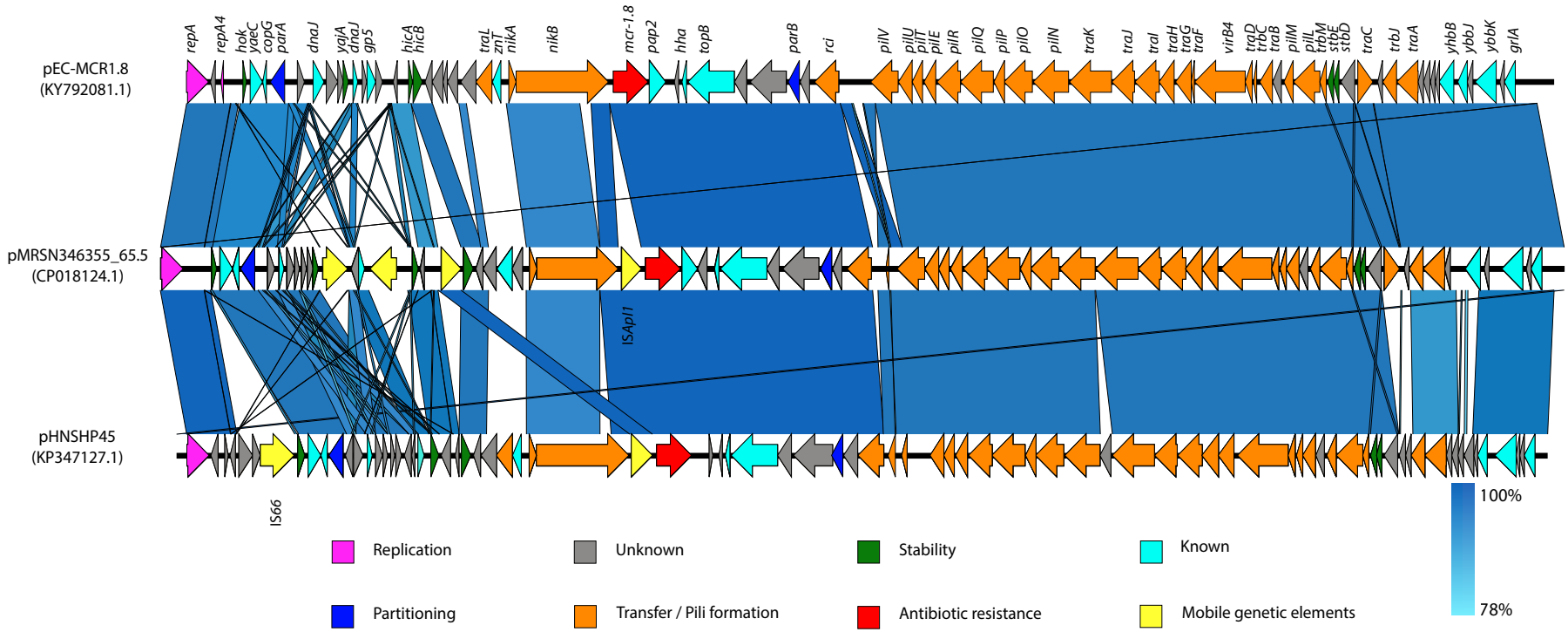

pEC-MCR1.8
(KY792081.1)
pMRSN346355_65.5
(CP018124.1)
pHNSHP45
(KP347127.1)
repA
repA4
hok
yaeC
copG
parA
dnaJ
yajA
dnaJ
gp5
hicA
hicB
traL
znT
nikA
nikB
mcr-1.8
pap2
hha
topB
parB
rci
pilV
pilU
pilT
pilE
pilR
pilQ
pilP
pilO
pilN
traK
traJ
traI
traH
traG
traF
virB4
traD
traC
traB
pilM
pilL
trbM
stbE
stbD
traC
trbJ
traA
yhbB
ybbJ
ybbK
grlA
ISApl1
IS66
Replication
Unknown
Stability
Known
Partitioning
Transfer / Pili formation
Antibiotic resistance
Mobile genetic elements
100%
78%

Figure 32. Comparison of pEC-MCR1.8 with selected IncI2 plasmids carrying *mcr-1.1.* Open reading frames are represented with arrows and the direction of transcription by arrowheads. Open reading frames encoding proteins involved in replication, partitioning, stability, transfer/ type IV pilus formation, antibiotic resistance and other known or unknown functions are colour-coded. The shufflon region is indicated with black rectangles. Areas shaded in blue indicate nucleotide identity. Figure was generated by our collaborators in Lieden University, Netherlands.

4.8.2.2 Resistome of *E. coli* C22

Comparative analysis of the WGS data *in silico* and the phenotypic susceptibility data was used to define and characterise the entire resistome of *E. coli* C22 confirms the presence of multiple resistance genes to aminoglycosides (*aph(4)-Ia*, *aadA1*, *aac(3)-IVa*, *aph(3')-Ic*), β-lactams (bla_{TEM-1B}, $bla_{CTX-M-65}$), fluoroquinolones (*qnrS1*), fosfomycin (*fosA4*), phenicols (*cmI1, floR*), trimethoprim (*dfrA15, dfrA14*), sulphonamides (*sul3*) and tetracyclines (*tetA, tetM*).

No mutations in the *lpxCAD* or *mgrB* genes were identified in C22 but polymorphisms were present in *phoP/Q* and *pmrA/B* predicted to encode the amino acid changes I44L (*phoP)*, I165F (*phoQ),* S29G (*pmrA)*, D282G and Y358N *(pmrB)* relative to the K12 sequence (Sun et al., 2009).

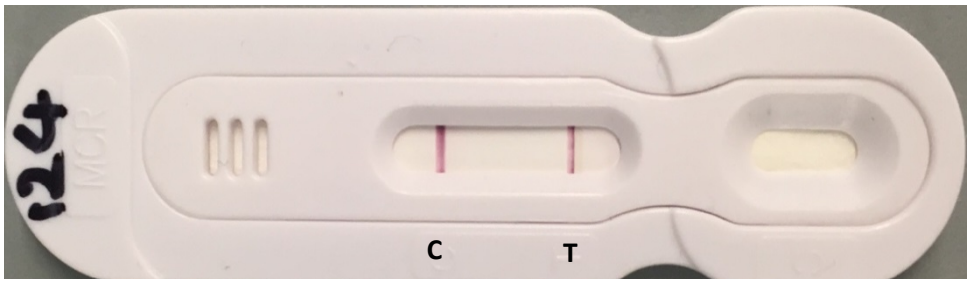
Of note β-lactam- ($bla_{CTX-M-65}$), tetracycline- (*tetA*), phenicol- (*floR*) and aminoglycoside-resistance (*aph(4)-Ia*, *aac(3)-IVa*,) were associated with an I1 plasmid whereas fosfomycin- (*fosA4*) and sulfonamide-resistance (*sul3*) were localised to a HI mulitreplicon (HIA, HIB and FIA) plasmid.

In silico WGS analysis with SerotypeFinder 2.0 predicted an O88:H31 serotype, which is also not detectable using the available antisera pools.

4.8.3 NG-Test MCR-1 rapid testing

All 36 *E. coli mcr-1* isolates tested positive (Figure 33A) and 9 type strain and 8 non *mcr-1* clinical isolates with different resistance mechanism were negative (Figure 33B) (Table 20).

A



B

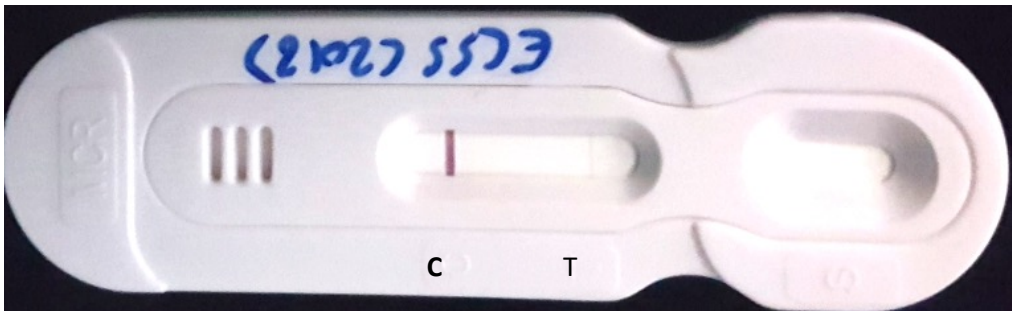


Figure 33. NG-Test MCR-1 (NG Biotech, France) cassette. A) Positive results showing red lines on both the Control (C) and Test (T) panels. B) Negative results showing red lines only with the Control (C).

Samples	**NG-Test MCR-1 Lateral flow**	
	Positive	**Negative**
E. coli MCR-1 isolates from chickens and humans (n= 36)	36	0
Clinical isolates from the Royal London Hospital (n= 8)	0	8
Control type strains (n= 9)	0	9
Positive human swabs (n= 15)	14	1
Negative human swabs (n= 2)	0	2

Table 20. NG-Test MCR-1 lateral flow evaluated against 70 samples.

4.9 Summary

- CHROMagar COL-*APSE* was developed and evaluated for the isolation and differentiation of colistin-resistant Gram-negative pathogens
- Both CHROMagar COL-*APSE* and SuperPolymxin media were able to support the growth of COL resistant Gram-negative bacteria while suppressing COL resistant Gram-positive pathogens.
- CHROMagar COL-*APSE* supported the growth of all COL resistant strains down to an inoculum as low as 10^1 CFU in clinical isolates.
- Similarly, 10^1 CFU inocula were also supported by SuperPolymyxin, but only for 50/58 (86%) of the *E. coli* isolates with COL resistance accompanied by *mcr-1*, suggesting it may have slightly lower sensitivity in the detection of these strains.
- CHROMagar COL-*APSE* was slightly more sensitive in the detection of *Enterobacteriaceae* producing MCR-1, it also provides the benefit of presumptive chromogenic identification.
- Period prevalence studies were undertaken with 300 chicken cloacal swabs from three different farms and 254 human faecal samples from Microbiology Laboratory, RIPAS Hospital, Brunei Darussalam.
- All swabs were screened using CHROMagar COL-*APSE* media. Growth of *E. coli* on media were further screened for *mcr*-related genes.
- High prevalence rates of *E. coli mcr* gene were observed in chicken (58%) and human samples (41.7%).
- Co-resistance was highest with aminoglycosides, quinolones, phenicols, β-

lactams antibiotics in both chicken and human colistin-resistant *E. coli* isolates.

- RAPD PCR showed clonal diversity and no avian (APEC) or human enterotoxigenic (ETEC) serogroups were found.
- Tripex *PCR* phylogroup and *in silico* ClermonTyping showed the most prevalent phylotypes were mostly commensals. However, there were discrpancies observed in 50% of the colistin-resistant *E. coli* isolates in chickens while performing both methods.
- 52 colistin-resistant *E. coli* isolates were selected for WGS analysis based on their distinguishable RAPD profiles.
- Colistin MICs ranges from 1->32 mg/L
- Diverse sequence type (STs) were found from both chicken and humans. However, there were overlapping clones observed (ST48, ST115, ST117, ST359, ST1011 and ST1642).
- Four novel *mcr-1.8* variants were observed from chicken and human faecal samples.
- *In silico* serotyping by WGS analysis showed no APEC or ETEC serogroups.
- The *fosA4* gene was observed more in colistin-resistant *E. coli* isolates isolated in chickens than from humans.
- A novel *mcr-1.8* variant was first found from chicken cloacal samples during surveillance and this isolate was *E. coli* C22.
- *E. coli* C22 MICs to colistin and polymyxin B were MIC 8 mg/L and 12 mg/L, respectively and was resistant to all penicillins, cephalopsorins (except ceftazidime), aztreonam, quinolones, aminoglycosides (except amikacin),

fosfomycin, chloramphenicol and tetracyclines.

- PCR mapping confirmed the *nikB* ABC transporter but absence of the IS*ApI1* sequence.
- PCR-based replicon typing identified multiple plasmid replicons in C22 belonging to FIA, FIB, FIC, FII, HI1A, HI1B, I1, I2, X1 and Y types.
- Virulence associated genes were observed which included *lpfA* (fimbrial adhesin), *iroN* (enterobactin siderophore receptor protein), *iss* (serum resistance), *gad* (acid resistance) and *cma* (inhibition of lysis). And with *iroN*, *iss* and *cma* co-localised to an IncFIB plasmid, whereas *lpfA*, *gad* and an additional copy of *iss* were mapped to the chromosome.
- The *mcr-1*-like gene in *E. coli* C22 carried a non-synonymous mutation at position 8 (A→G) resulting in a glutamine to arginine (Q→ R).
- *E. coli* C22 belonged to sequence type (ST) 101, a globally disseminated clone and SerotypeFinder 2.0 predicted an O88:H31 serotype.
- The *mcr-1.8* gene was the only resistance gene located on IncI2 plasmid (pEC-MCR1.8) by PCR mapping, replicon typing and analysis of the entire WGS data.
- Multiple resistance genes are also observed *in silico* in *E. coli* C22 to aminoglycosides (*aph(4)-Ia*, *aadA1*, *aac(3)-IVa*, *aph(3')-Ic*), β-lactams ($bla_{\text{TEM-1B}}$, $bla_{\text{CTX-M-65}}$), fluoroquinolones (*qnrS1*), fosfomycin (*fosA4*), phenicols (*cmI1, floR*), trimethoprim (*dfrA15, dfrA14*), sulphonamides (*sul3*) and tetracyclines (*tetA, tetM*).

- The NG-Test MCR-1 (NG Biotech, France) lateral flow assay showed high sensitivity and specificity. A robust diagnostic tool for the determination of *mcr-1* genes.

Chapter 5

Comparative virulence of polymyxin resistant *E. coli* of human and avian origin

5.1 Introduction

In Chapter 4, the prevalence and surveillance of plasmid-mediated polymyxin resistance in poultry farms and human faecal samples in Brunei Darussalam was reported. In this chapter, the pathogenicity and fitness costs of acquiring plasmid-mediated polymyxin resistance in *Galleria mellonella* infection model are investigated.

G. mellonella is an insect from the order *Lepidoptera* which comes from the family *Pyralidae* (snout moths) (Tsai et al., 2016). *G. mellonella* larvae (greater wax moth or honeycomb moth) have been extensively studied for virulence as well as for drug testing (Tsai et al., 2016). More importantly, the impact of acquiring antibiotic resistance gene on bacterial fitness (Zhang et al., 2017). The range of microorganism that has been studied in *G. mellonella* model includes *Acinetobacter baumannii*, (Peleg et al., 2009; Gaddy et al., 2012) *Pseudomonas aeruginosa*, (Jander et al., 2000; Miyata et al., 2003) *Staphylococcus aureus*, (Desbois and Coote, 2011) and *Candida albicans* (Fuchs et al., 2010). *G. mellonella* larvae are inexpensive, easily obtained in large numbers, no ethical approval required, does not require special lab equipment and their short life cycle makes them ideal for large-scale studies (Ramarao et al., 2012; Tsai et al., 2016). Although lacking in adaptive immune response, their innate immune response shows a similar immune response in vertebrates (Tsai et al., 2016). The innate immune response consists of the cellular and humoral immune response (Tsai et al., 2016).

Cellular response involves phagocytic cells known as hemocytes, found within the hemolymph which functions analogously to mammalian blood (Tsai et al., 2016). Other than phagocytosis, these hemocytes also functions in encapsulation and clotting (Tsai et al., 2016). While, the humoral response involves soluble effector molecules that immobilize and kill the pathogen and includes complement-like proteins, melanin and antimicrobial peptides (Tsai et al., 2016).

Pathogenicity in *E. coli* are not only dependent on their phylotypes or serotypes but also from acquiring virulence factors. Genetic virulence factors include flagella, curli, fimbriae, adhesion, biofilm (Bekal et al., 2003; Gomes et al., 2011; Kao et al., 2014; Faraji et al., 2016) or biochemical factors such as host cell surface modifying enzymes, toxins and antibiotics which contributes to the physical attributes of bacteria and provide a competitive advantage (Cossart et al., 1989; Burnside et al., 2010; Kim et al., 2015; Schroeder et al., 2017).

MCR-1 dissemination has now been seen in over 40 countries since its first discovery in late 2015, in China (Skov and Monnet, 2016). The main bacterial host of *mcr-1* has been identified mainly in *Escherichia coli* and has been reported on both broad-host-range and narrow-host-range of plasmid replicon types, including IncI2, IncX4, IncP, IncHI1 and IncHI2 (Liu et al., 2016; Sonnevend et al., 2016; Webb et al., 2016; Zhang et al., 2016). The successful dissemination of resistance plasmids largely depends on the fitness cost imposed on hosts (Andersson and

Hughes, 2010). Therefore, there is a need to understand the biological cost of resistance which is critical for controlling the dissemination of multidrug-resistant strains.

Studies on the biological effects of colistin resistance have been reported mainly in *Acinetobacter baumannii* with extremely limited data on *Enterobacteriaceae* (Hraiech et al., 2013; Beceiro et al., 2014; Wand et al., 2015). Additionally, there are limited studies on the effect of *mcr-1* on fitness and virulence in *Enterobacteriaceae*. However, there have been studies in *G. mellonella* model looking at virulence of extraintestinal pathogenic *E. coli* isolates which causes a range of serious infection such as bacteremia, meningitis pneumonia and more commonly associated with uncomplicated urinary tract infection (Alghoribi et al., 2014; Williamson et al., 2014; Ciesielczuk et al., 2015).

The presence of plasmid-mediated polymyxin resistance, *mcr* genes in animals and humans has raised the question whether the expression of *mcr-1* genes would modulate pathogenicity. Hence, the impact of *mcr- 1* gene on *in vivo* fitness and virulence is further warranted.

5.2 Objectives

- To investigate the impact of acquiring plasmid-mediated polymyxin resistance (PMPR) in *E. coli* isolated from chickens and humans on the survival, virulence and fitness in *G. mellonella* infection model.

5.3 Methods

5.3.1 *Galleria mellonella* assay

Forty colistin-resistant *E. coli* strains (humans, n= 20 and chickens, n= 20) (Table 21) were tested for virulence in *G. mellonella* larvae. Characteristics of all colistin-resistant *E. coli* strains used in this study are mentioned in Chapter 4, section 4.6.2, Table 12A 5 µl loop full *E. coli mcr-1* colony picked up from overnight MH 2 agar was inoculated into 3 ml of LB broth in 25 ml sterile universal tube. Serial dilution was performed (100 µl pellet suspension in 900 µl PBS) at a final dilution of 1 x 10^7 CFU/ml (1 x 10^5 CFU/larvae) which is the optimal bacterial inoculum (10^5 CFU/larvae) prepared using a published method (Betts et al., 2014; Ciesielczuk et al., 2015).

Colistin-resistant *E. coli* strains	
Chicken	**Human**
A8	H1
A12	H8
A13	H25
A28	H49
A33	H53
A41	H54
A53	H59
B1	H60
B7	H61
B11	H64
B25	H67
B28	H84
B47	H86
B52	H92
C1	H96
C19	H106
C22	H109
C28	H112

C45	H113
C55	H124

Table 21. Colistin-resistant *E. coli* strains from chickens and humans. Human colistin-resistant *E. coli* strains are given a letter 'H' (Human) in front of the number due to the statistical program used.

Ten *G. mellonella* larvae (TruLarv™, BioSystems Technology, Devon, UK) per test isolate were infected with 10 µl aliquot of the serially diluted bacterial suspension (10^7 CFU/ml) in the first left proleg (Figure 34, Figure 35 and Figure 36) using a 22-gauge gastight syringe (Hamilton, Bonaduz, Switzerland). Another 10 sets of *G. mellonella* larvae were injected with PBS as a stress control. Larvae were incubated aerobically at 37° C on Grade 1 WhatmanTM filter paper (Whatman plc, Maidstone, UK) in a 90 mm petri dish sterile polystyrene clear (Fisher Scientific, Loughborough, UK) for 72 hours.

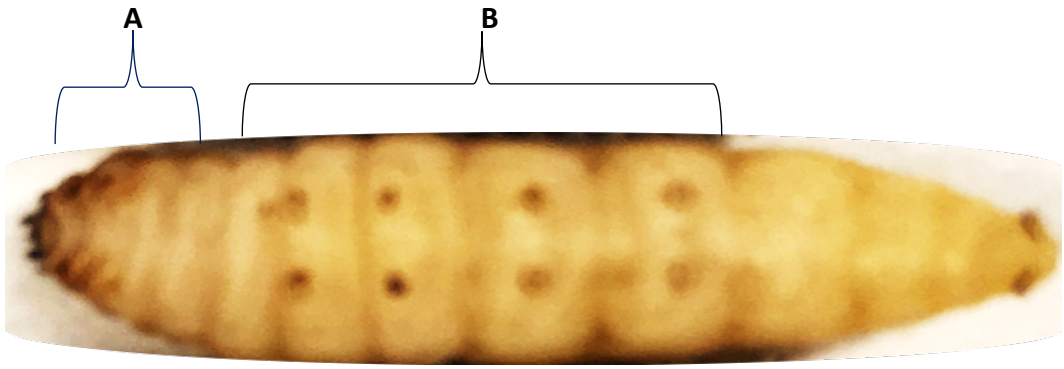


Figure 34 (Not to scale). External morphology of *G. mellonella* larva in supine position. A. Mesothoracic legs B. Prothoracic/Pro-legs. (Adapted from Smith et al.) (Smith, 1965b)

Figure 35. Ten larvae per plate were used for the virulence studies.

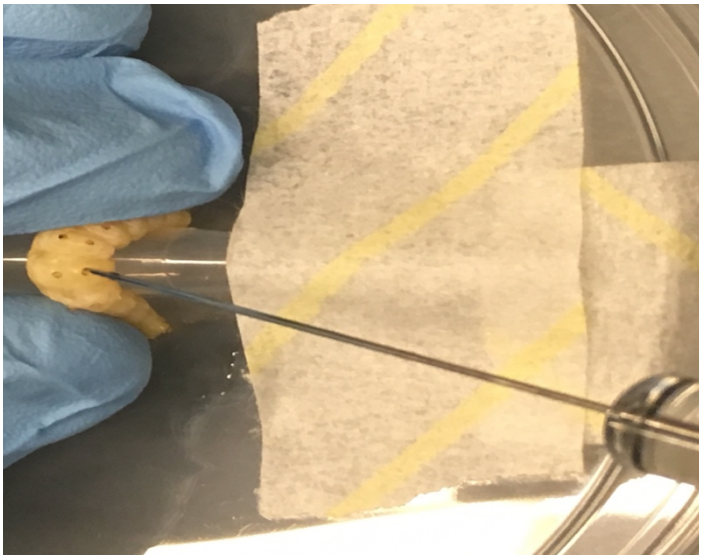


Figure 36. Injection of 10^7 CFU/ml (10^5 CFU/larva) of the test isolates in the first proleg.

Larvae were considered dead if they did not respond to touch. The virulence assay was performed in triplicates on three separate occasions with three different batches of larvae.

Petri dishes were incubated at 37° C and monitored daily for 3 days (24 h, 48 h and 72 h) post-injection to record morbidity and mortality. *G. mellonella* larvae were considered infected if they expressed dark pigmentation (melanisation) after inoculation. Larvae were scored as dead if they did not respond to touch stimuli by blunt sterile forceps.

5.3.2 *Galleria mellonella* assay - transconjugants

Bacterial conjugation was performed as mentioned in Chapter 4, Part 3, section 4.7.6. Transconjugants were selected by growth on LB agar supplemented with colistin (4 mg/L) using *E. coli* J53 (EC J53) as the recipient.

Two colistin-resistant *E. coli* transconjugants (*E. coli* A53/ECJ53 and *E. coli* B28/ECJ53) were provided with assistance from our MSc student (Sadia Miah). *E. coli* C22 conjugation was performed successfully as mentioned in Chapter 4, section 4.8.2. Overall, there were only three successful transconjugants observed (*E. coli* B28/EC J53, *E. coli* A53/EC J53 and *E. coli* C22/EC J53).

All the transconjugants and EC J53 (control) were infected with differing bacterium inoculums in order to elicit appropriate live and dead larvae populations. An inoculum of 10^9 CFU/ml (10^7 CFU/larvae) was then selected for the infection. The virulence and fitness cost of acquiring the plasmid MCR-1 were then assessed. The susceptibility testing was performed for all three transconjugants and the recipient ECJ53. These transconjugants were characterized by WGS (WGS analysis methods mentioned in Chapter 3, section 3.3.9).

5.3.3 Survival analysis

Survival analysis was performed using Kaplan-Meier survival curves. Kaplan-Meier is used to measure the fraction of subjects living for certain amount of time after treatment, (Goel et al., 2010) however in this study; it is the measure of mortality (number of *G. mellonella* larvae death) after being infected. This analysis was already performed in a previously published study (Ciesielczuk et al., 2015). Cox proportional hazards model was used to make a pairwise comparison between isolate (Ciesielczuk et al., 2015). In addition, this model was also use to investigate the impact of individual *E. coli* sequence types (ST) and previously published virulence determinants (Ciesielczuk et al., 2015) on *G. mellonella* larval survival. Survival curve analysis was then performed with assistance from our collaborator Dr Lynette M Phee.

5.3.4 Statistical analysis

Statistical analysis performed using StataCorp. 2013. Stata Statistical Software: Release 13. College Station, TX: StataCorp LP.

Statistical analysis was performed with assistance from our collaborator Dr Lynette M Phee.

5.3.5 Growth curve assay

Fitness cost was performed by investigating the growth kinetics of three successful *E. coli mcr-1-like* transconjugants and the recipient (*E. coli* B28/EC J53, *E. coli* A53/EC J53 and *E. coli* C22/EC J53 and ECJ53)

A 5 µl of overnight culture were inoculated in 3 ml LB broth and incubated on an innova 2100 platform shaker (New Brunswick Scientific; Eppendorf, Stevenage UK) at 37° C for 24 h. After 24 hours, the bacterial suspension was further diluted, 3 µl into 3 ml of LB broth. 100 µl of the diluted suspension was dispensed into 96-well round bottom microtiter plate in triplicates incubated and analysed for 19 hours using CLARIOstar (BMGLABTECH, Aylesbury, UK) microplate reader, grew at 37°C with vigorous aeration (200 rpm). The culture cell density was determined every 30 minutes by measuring the OD_{540}.

The curves were generated by CLARIOstar Data analysis. The curves with error bar were produced using GraphPad Prism version 8.0 and statistical analysis were calculated using Mann-Whitney U test.

5.3.6 Endotoxin detection

ToxinSensor™ Chromogenic LAL Endotoxin Assay kit (antibodies-online GmbH, Aachen, Germany) was used in this study. This test utilizes a modified Limulus

Amebocyte Lysate and a synthetic color producing substrate to detect endotoxin chromogenically in a broad range of 0.005 - 1 EU/ml.

Twelve *E. coli mcr-1-like* isolates were investigated for endotoxin activity. Six isolated from chickens (*E. coli* C22, *E. coli* C28, *E. coli* B52, *E. coli* B28, *E. coli* C55, *E. coli* B47) and 6 from humans (*E. coli* 1, *E. coli* 8, *E. coli* 49, *E. coli* 59, *E. coli* 60, *E. coli* 61). All these isolates were selected based on the differences in their STs. Characteristics of these isolates are mentioned in Chapter 4, section 4.6.2, Table 12Table 12

A 5 µl loop of test isolate was inoculated in 3 ml LB broth in a sterile universal tube incubated at 37° C for 24 hours on innova 2100 platform shaker (New Brunswick Scientific; Eppendorf, Stevenage UK). 1 ml of the bacterial mixture from overnight were pipetted out into sterile 1.5 ml sterile Eppendorf tube and centrifudged on Heraeus Biofuge Pico centrifuge (DJB Labcare, Buckinghamshire, UK) at 13,000 rpm for 1 minute to pellet the cells. The supernatant of the test isolates was removed and placed into a new sterile Eppendorf tube.

100 µl of standards, samples (supernatant) and LAL reagent water were dispensed into different endotoxin-free vials. 100 µl of reconstituted LAL was added to each vial. The capped vial was then vortexed for 3 seconds. All vials were then incubated at 37°C on a heat block for 45 minutes. Final endotoxin concentration was serially diluted to produce a concentration of 1, 0.5, 0.25 and 0.125 EU/ml. Briefly, 0.2 ml

of 5 EU/ml endotoxin stock solution was added with 0.8 ml of LAL Reagent Water to make the 1 EU/ml solution. Serial dilution with concentration was then made 0.5, 0.25, 0.125 EU/ml (500 µl of 1 EU/ml solution was transferred into a vial containing 500 µl LAL Reagent water). The endotoxin concentration was then incubated for 10 minutes at 37°C.

After incubation, 100 µl of reconstituted chromogenic substrate solution was added to each vial. The mixtures were mixed gently by swirling and incubated for 6 minutes at 37°C. 500 µl of reconstituted stop solution (color-stabilizer #1), color-stabilizer #2, color-stabilizer #3 were added to each vial and mixed gently by swirling. 100 µl of the solution was then dispensed into flat bottom well plate (nunc™ 96, SLS, Nottingham, UK) in triplicates.

The absorbance of each reaction was read at 545 nm with distilled water as blank to adjust the photometer to zero absorbance. CLARIOstar (BMGLABTECH, Aylesbury, UK) microplate reader was used to read the absorbance. Standards (linear regression) and the test results were plotted on a graph generated by the CLARIOstar Data analysis programme.

5.4 Results

5.4.1. *G. mellonella* analysis in *E. coli mcr-1-like* wildtype strains

G. mellonella killing showed differences in larval survival rates for all the fourty *E. coli mcr-1-like* strains (wild-type) as shown in Figure 37, Figure 38, Figure 39 and Figure 40.

Pairwise comparison of hazard ratio was performed for all these strains (human versus avian origin) and ranked from the most virulent to the least virulent shown in Table 22.

Virulence factors (VF) were ranked based on their Hazard Ratio and Kaplan-Meier curve analysis shown in Table 23 and Figure 41. A strain with *cvaB* (colivin V secretion/processing ATP-binding protein) showed the most heightened virulence according to the calculated Hazard Ratio. Similarly, other VF such as *vat*, *hylE*, *AfaB*, *senB*, *tsh*, *eptD*, *cba*, *pic*, *iatB*, *iss* and *cma* were observed to be virulent (Table 23 and Figure 41 A to L). Interestingly, carriage of *lpfA*, *gadB*, *gad*, *invasin*, *air*, *celB* and *gad* are associated with lower virulence scores and low mortality rates (Table 23 and Figure 41 M to S).

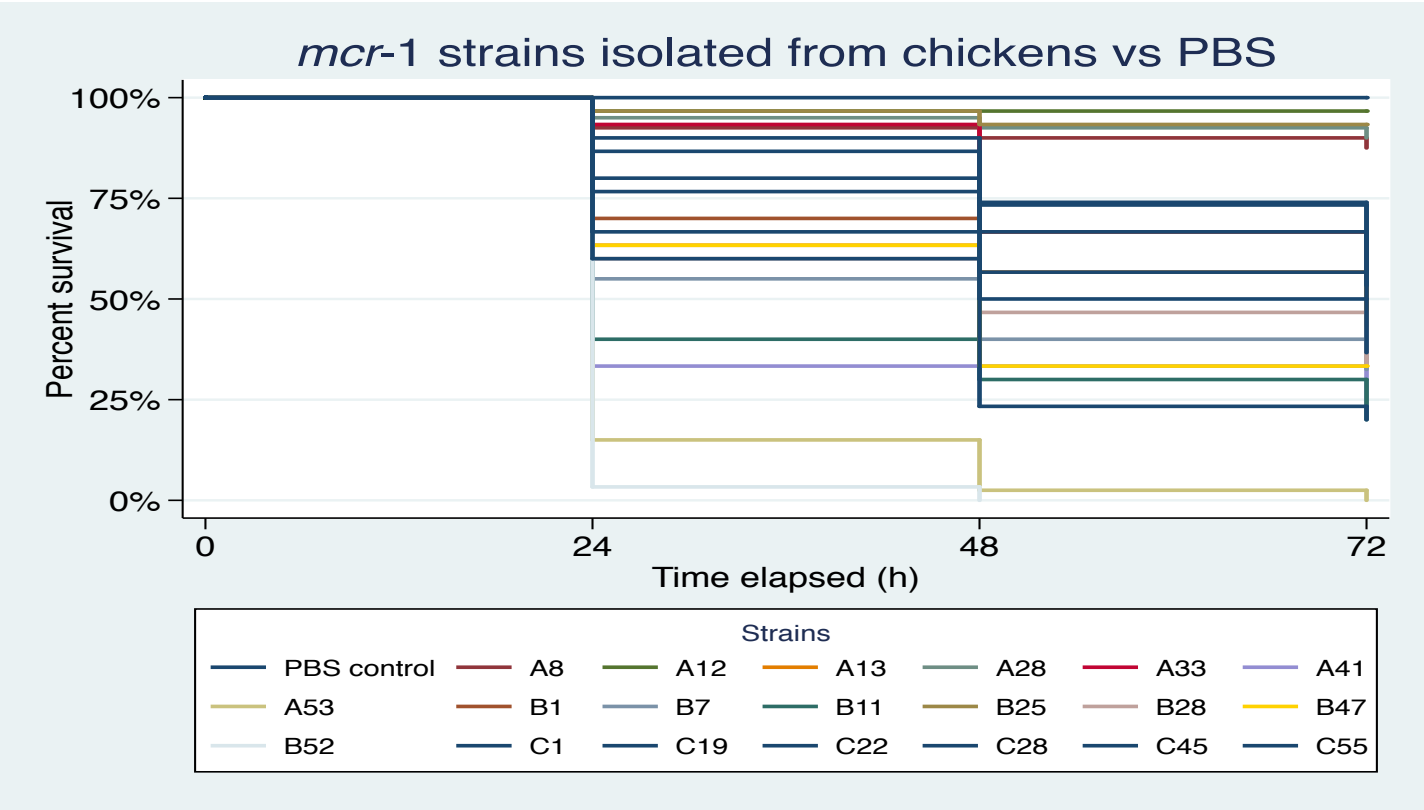
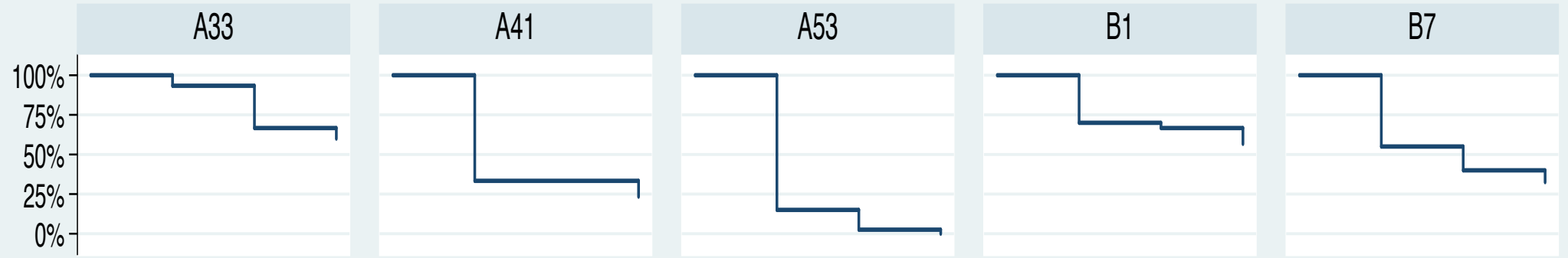
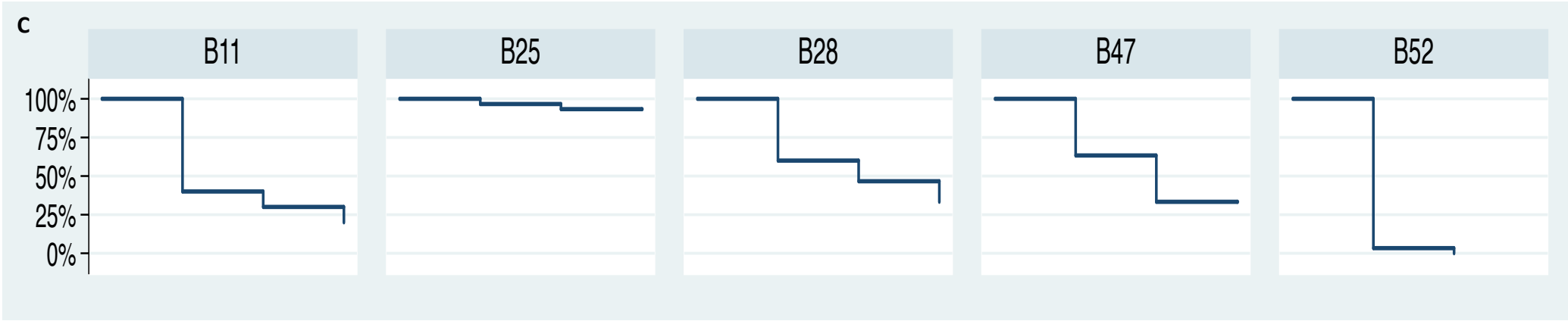


Figure 37. Kaplan-Meier survival curves for all 20 colistin-resistant *E. coli* strains isolated from chickens versus the PBS control over 72h.


A
PBS control
A8
A12
A13
A28
100%
75%
50%
25%
0%

B




c
B11
B25
B28
B47
B52
100%
75%
50%
25%
0%

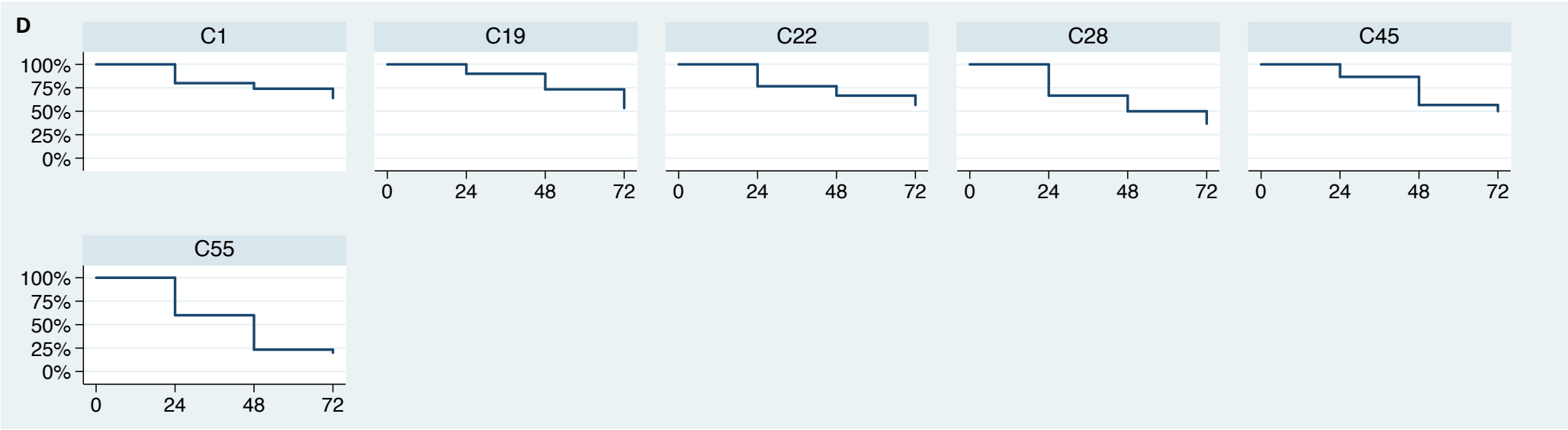


Figure 38. A to D represent Kaplen-Meier survival estimates (Percent survival versus Time elapsed (h)) of individual strains from chicken.

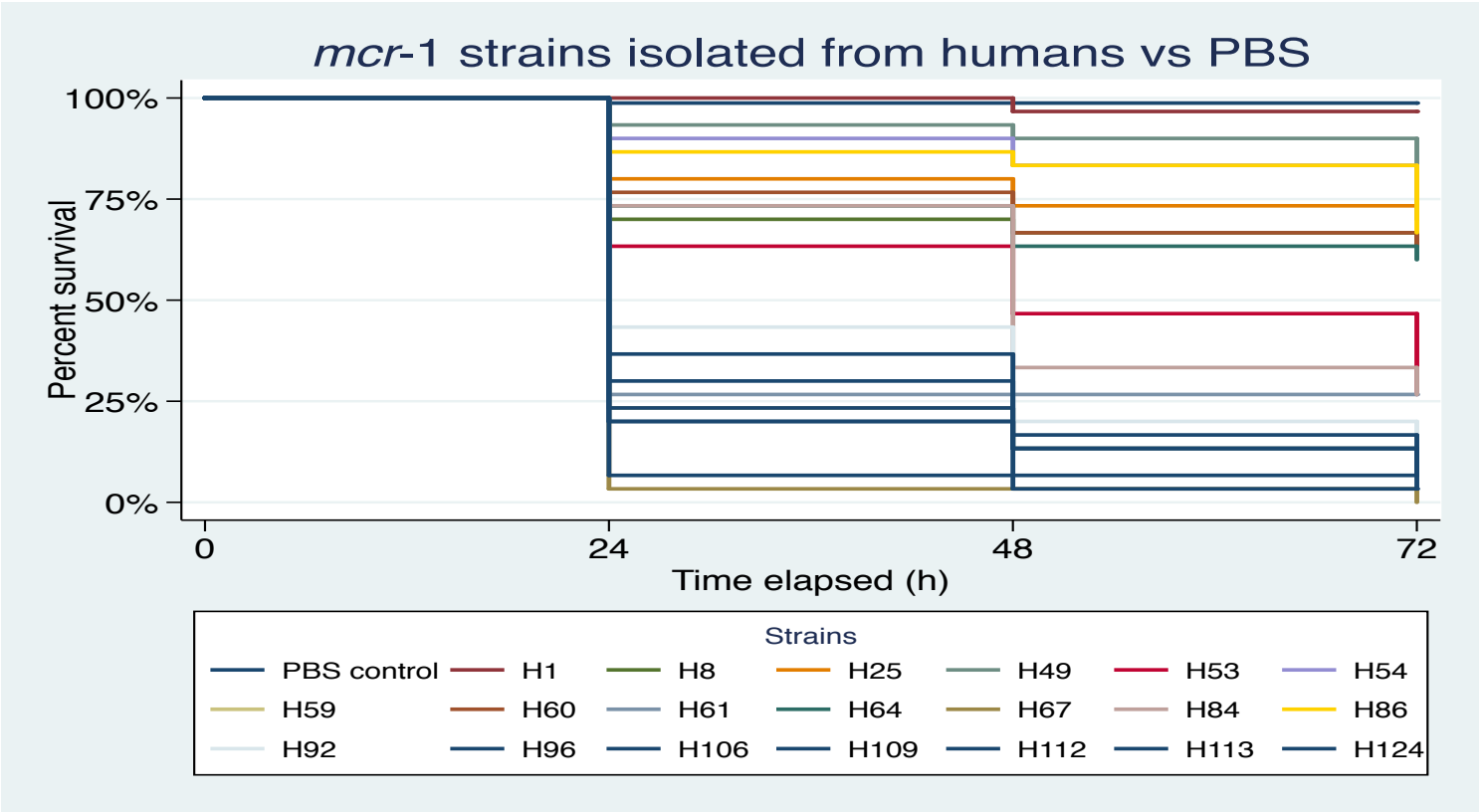
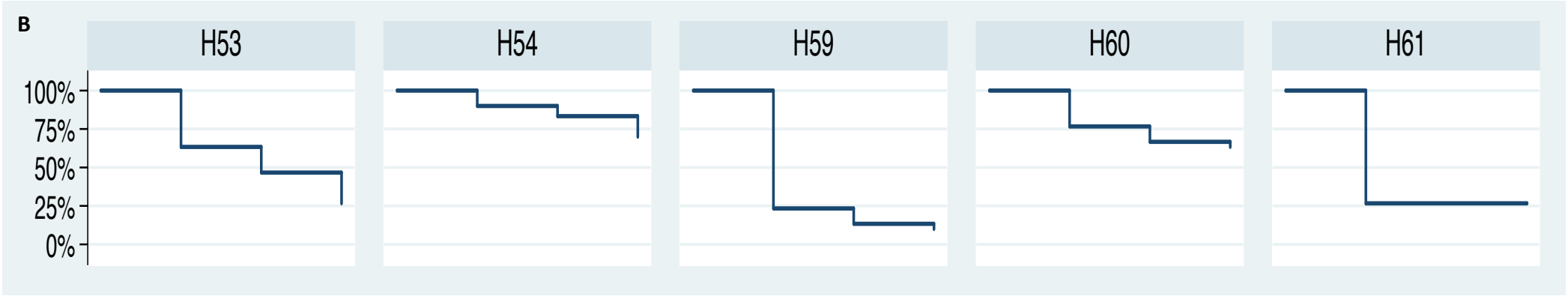
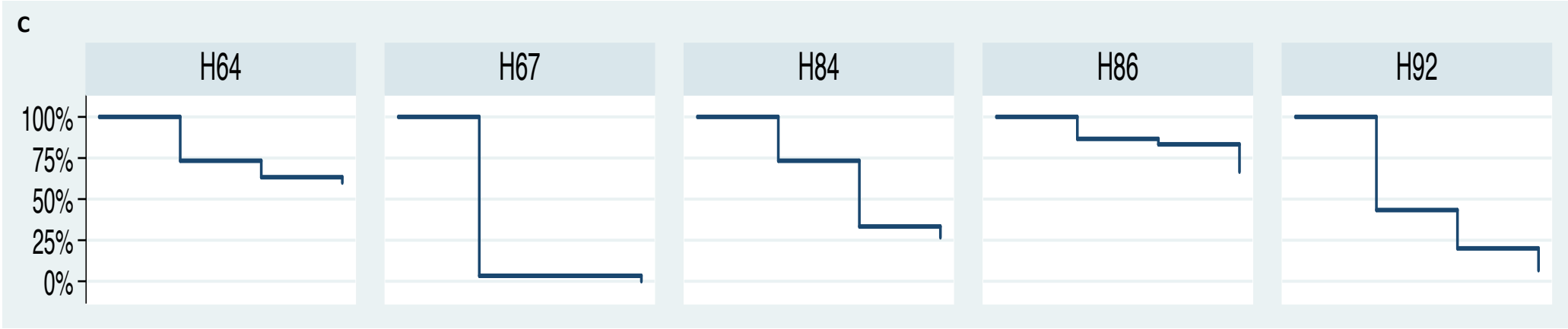


Figure 39. Kaplan-Meier survival curves for all 20 colistin-resistant *E. coli* strains isolated from humans versus PBS control over 72h.


A
PBS control
H1
H8
H25
H49
100%
75%
50%
25%
0%


B
H53
H54
H59
H60
H61
100%
75%
50%
25%
0%


c
H64
H67
H84
H86
H92
100%
75%
50%
25%
0%

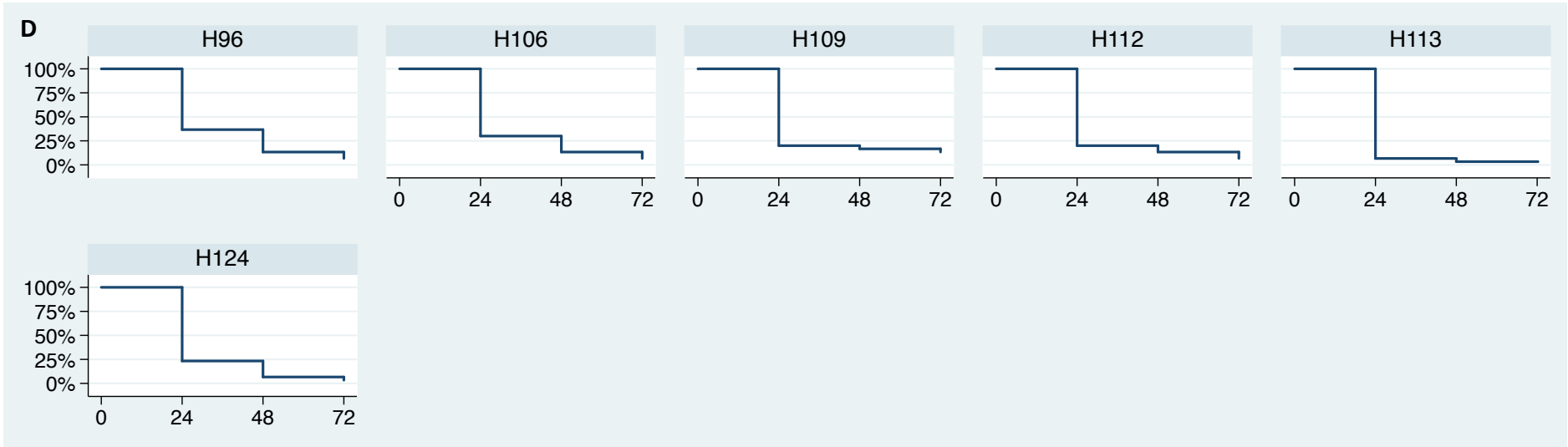


Figure 40. A to D represent Kaplen-Meier survival estimates (Percent survival versus Time elapsed (h)) of individual strains from humans.

Strains	Origin	Sequence type (ST)	Plasmid replicon	Virulence factor	Serotype	Rank
B52	Avian	ST359	IncFII, IncFIC(FII), IncI2, IncI1, IncX1, IncFIB(AP001918)	*cba, iroN, gad, astA, cma, iss, lpfA, cvaB, tsh*	NT	1
H67	Human	ST372	IncFII(pRSB107), IncX4	*iss, vat*	:H31	2
H113	Human	ST1011	IncI2, IncFII**,** ColpVC, IncFIC(FII), IncFIB(pLF82), IncFIB(AP001918), IncX1, IncY	*astA, cma, eilA, iss, lpfA, iatB*	O86:H4	3
A53	Avian	ST2223	IncI2, IncN, IncX4, IncX1, p0111, IncI, IncHI2A, IncHI2, IncFII(pSE11), InFII, IncFIB(AP001918), IncB/O/K/Z, ColpVC, Col156	*iroN, gad, astA, cma*	O75:H42	4
H124	Human	ST1011	IncI2, IncU, p0111, Col(MG828)	*eilA, iatB*	O157:H9	5
H112	Human	ST117	IncFII(pHN7A8), IncFII(pSE11), IncFII, IncI2, Col156, IncFIB(AP001918)	*iss, lpfA, ireA, hlyE*	O2:H4	6
H59	Human	ST38	IncFIA(HI1), IncHI1A, IncHI1B(R27), IncN	*iss, senB, eilA, iha, iatB, gadB, AfaB*	O86:H45	7

H106	Human	ST359	IncFII, IncFIC(FII), IncI1, IncFIB(AP001918), IncI2, IncFII	*iroN, cba, astA, cma, iss, lpfA, mchF*	O35:	8
H109	Human	ST93	IncHI1B(R27), IncFIA(HI1),IncHI1A	*iss, iha, astA*	O51:H52	9
H96	Human	ST48	IncX4, IncX1, p0111	*gad, iss, etpD*	O8:H11	10
H92	Human	ST1201	IncI2, IncFIB(AP001918), IncFII(pCoo)	*lambda bor, etpD*	O124:H38	11
B11	Avian	ST4684	IncHI1B(R27), IncHI1A, IncX4, IncFIA(HI1)	*lpfA*	O9:H7	12
H61	Human	ST1011	IncFIB(AP001918), IncFII, IncI2, Col156	*iss, eilA, iatB*	O157:H16	13
A41	Avian	ST117	col(BS512), IncHI1B(R27), IncI1, IncHI1A, IncFIA(HI1)	*iss, iroN, cma, astA, pic, eilA, gad, iha, lpfA, gadB, ireA, invasin*	O8:H19	14
C55	Avian	ST155	IncI2, IncX4, IncFII(29	*mchF, gad, iroN, tsh, lpfA, iss*	O38:H21	15
H84	Human	ST8168	IncX4	*astA*	:H29	16
H53	Human	ST93	IncHI1A, IncHI1B(R27), IncFIA(HI1), p0111, IncI2	*astA, cba, cma, iha, iss*	O51:H52	17
B7	Avian	ST1011	IncN, IncHI2, IncI2	*iss, iatB*	:H25	18
B47	Avian	ST1140	IncI1	*gad, gadB*	O38:H39	19

B28	Avian	ST1727	IncI2, IncFIB(AP001918), IncFII(pCoo), p0111	*iss, lpfA, cma, gad, iroN*	:H7	20
C28	Avian	ST2372	ColpVC, IncI2, IncFII, IncFIB(AP001918), Col156, IncB/O/K/Z, ColpVC, IncFII	*astA, cma, gad, iroN*	O37:H25	21
A13	Avian	ST2473	IncFIA(HI1), IncHI1A, IncHI1B(R27)	*iss, lpfA, gad*	O168:H28	22
C45	Avian	ST1642	IncFII, IncY, IncFIB(AP001918), IncFIB(plF82), IncI2, IncX1, IncFII(29)	*lpfA, astA*	O8:H7	23
B1	Avian	ST711	IncI1, IncX4, IncHI1B(R27), IncN, IncHI1A, IncFII, IncFIA(HI1)	*iroN, lpfA, cma*	O8:H10	24
C19	Avian	ST10	IncQ1, IncHI2, IncFII(29), IncI2, IncI1	*gad, astA, gadB*	:H32	25
C22	Avian	ST101	IncHI1A, IncHIB(R27), IncFIA(HI1), IncFII, IncY, IncI2, IncFIC(FII), IncFIB(AP001918) IncI1, IncX1	*astA, cma, lpfA, iss, eilA, gad, iha, lpfA, mchF*	O88:H31	26
H64	Human	ST8380	IncHI1A, IncHI1B(R27), IncFIA(HI1)	*gad, eilA, lpfA, iatB*	:H31	27
A33	Avian	ST2732	IncHI2, IncFII	*astA, cma, eilA, gad, iha, lpfA, iatB, mchF*	O33:H28	28
H8	Human	ST206	IncHI1A, IncFII(29), IncX4, IncHI1B(R27), IncFIA(HI1)	*astA*	O96:H5	29

H60	Human	ST1638	IncX4, IncX1, InFII, IncI2, IncFIB (AP001918), Col (MG828)	*iss, iroN, gadB*	O124:H12	30
C1	Avian	ST7687	IncX4, IncHI2	*eila, air, gad, gadB*	O17/77:H31	31
H86	Human	ST1121	IncX4, p0111	*gad, lpfA, lambda bor*	:H38	32
H25	Human	ST58	IncFIC(FII), IncFIB(AP001918), IncI2	*astA, cma, iss, gad, lpfA*	O8:H25	33
H54	Human	ST1642	IncFIA, IncFIB(AP001918), IncI2, IncFIB(pLF82), IncX1	*iss, astA, lpfA, gad*	O48:H7	34
H49	Human	ST746	IncX4, IncX2	*gad*	O8:H37	35
A8	Avian	ST115	IncFI, ColpVC, Col8282, IncHI1A, IncFIA(HI1)	*iss, celb, astA, iroN, iha, lpfA, gadA, eilA, invasin*	O21:H15	36
A28	Avian	ST226	IncI1	*gad, ireA*	O18:H10	37
B25	Avian	ST162	IncX4, IncFII(29)	*gad, lpfA, iss*	O134:H19	38
A12	Avian	ST2705	p0111, IncI2, IncX1	*gadB*	:H10	39
H1	Human	ST224	IncX4, IncFIB(AP001918), IncFII	*cma, iss, iroN, gad, lpfA*	O102:H23	40

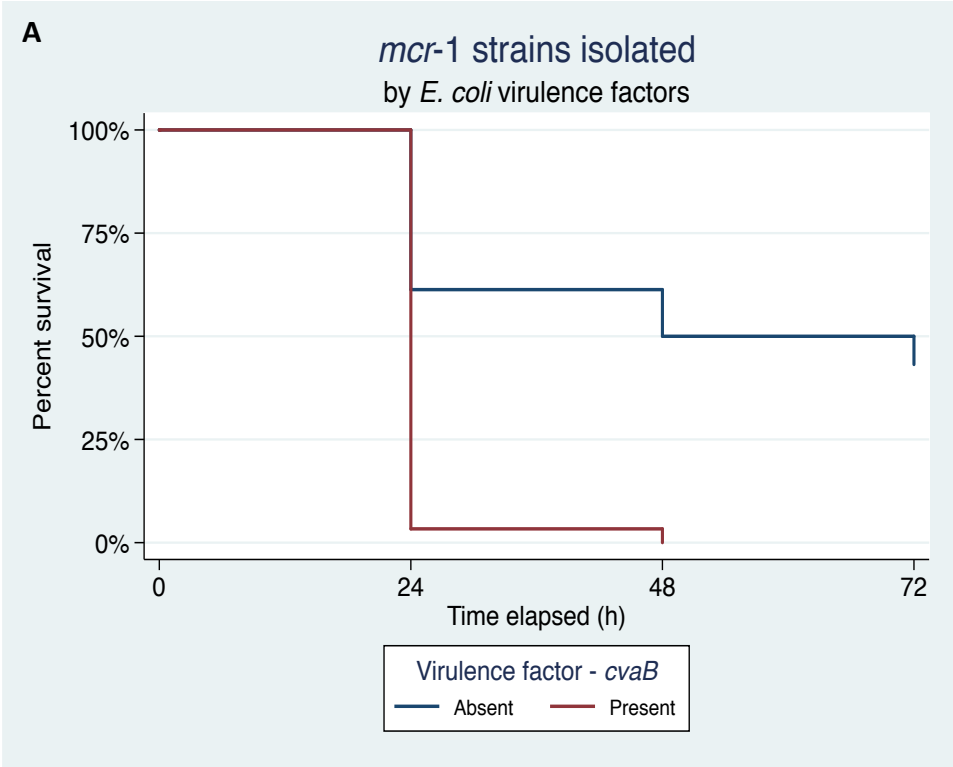
Table 22. Virulence ranking of colistin-resistant *E. coli* strains ranking from most virulent (1) to least virulent (40) based on their Hazard Ratio.

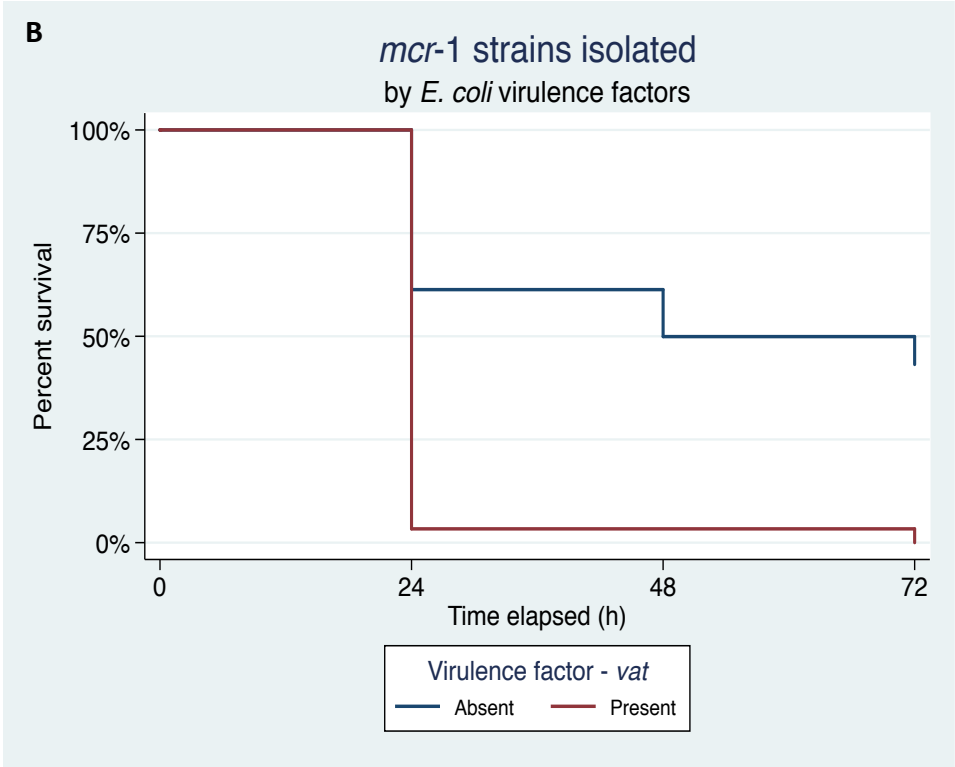
NT- Non Typeable.

Virulence factors	*p*-value	Hazard Ratio (HR)	95% Confidence Interval, CI
cvaB	<0.001	2.546	1.761, 3.681
vat	<0.001	2.515	1.740, 3.636
hlyE	<0.001	2.095	1.434, 3.061
AfaB	<0.001	1.989	1.353, 2.924
senB	<0.001	1.989	1.353, 2.924
tsh	<0.001	1.973	1.494, 2.607
eptD	<0.001	1.953	1.486, 2.567
cba	<0.001	1.932	1.530, 2.440
pic	0.046	1.526	1.007, 2.312
iatB	<0.001	1.472	1.234, 1.757
iss	<0.001	1.353	1.167, 1.569
cma	0.004	1.254	1.077, 1.459
mchF	0.346	1.120	0.885, 1.419
astA	0.131	1.120	0.967, 1.298
iroN	0.166	1.119	0.954, 1.313
lambdabor	0.669	1.074	0.775, 1.489
eilA	0.854	1.016	0.861, 1.198
Iha	0.935	1.008	0.833,1.220
ireA	0.777	0.961	0.730, 1.265
lpfA	0.006	0.815	0.704, 0.943
gadB	0.019	0.787	0.644, 0.961
gad	<0.001	0.653	0.564, 0.756

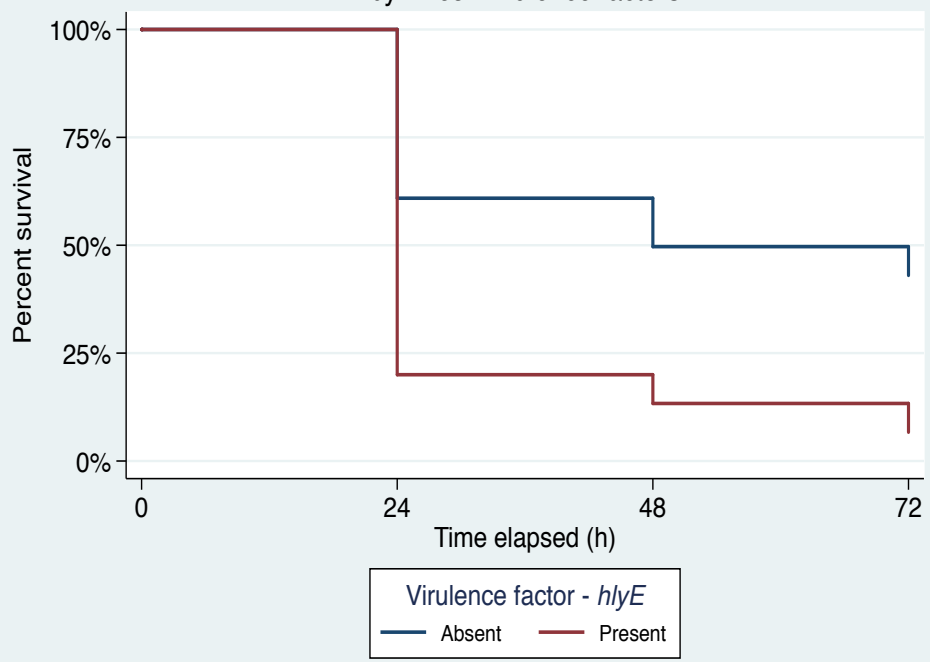
invasin	0.019	0.636	0.436, 0.928
air	0.01	0.542	0.339, 0.865
celB	<0.001	0.174	0.072, 0.419
gadA	<0.001	0.174	0.072, 0.419

Table 23. Virulence factors present in colistin-resistant *E. coli* strains isolated from chicken and humans ranked according to their Hazard Ratio (HR). *cvaB*- colicin V secretion/processing ATP-binding protein, *vat*- vacuolating autotransporter toxin, *hlyE*- avian *E. coli* haemolysin, *AfaB*- chaperone AfaB, *senB*- plasmid-encoded enterotoxin, *tsh*- temperature sensitive hemagglutinin, *eptD*- type II secretion protein, *cba*- colicin B, *pic*- serine protease autotransporters of Enterobacteriaceae (SPATE), *iatB*- inverse autotransporter beta-barrel domain-containing protein, *iss*- Increased serum survival, *cma*- colicin M, *mchF*- ABC transporter MchF, *astA*- EAST1 heat-stable toxin, *iroN*- enterobactin siderophore receptor protein, *lambdabor*- serum resistance lipoprotein bor, *eilA*- *salmonella* HilA homolog*, iha*- adherence protein*, ireA*- siderophore receptor*, lpfA*- long polar fimbriae*, gadB*- glutamate decarboxylase beta*, gad*- glutamate decarboxylase*, invasin*- invasin region at inverse autotransporter, beta-domain, *iatB, air*- enteroaggregative immunoglobulin repeat protein*, celB*- endonuclease colicin E2 and *gadA*- glutamate decarboxylase alpha.

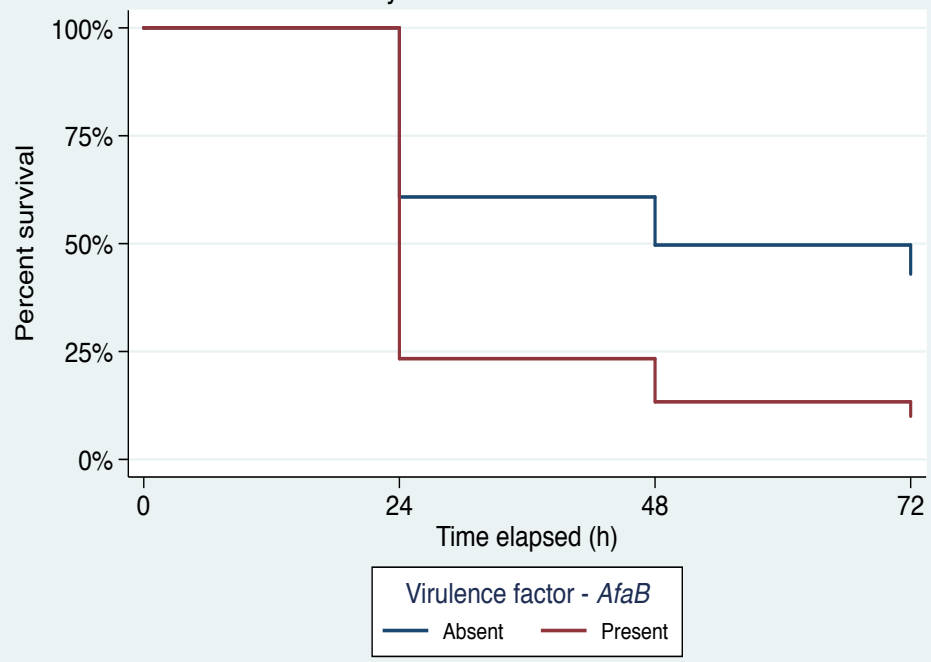

A
mcr-1 strains isolated
by *E. coli* virulence factors
100%
75%
50%
25%
0%
Percent survival
0
24
48
72
Time elapsed (h)
Virulence factor - *cvaB*
Absent
Present

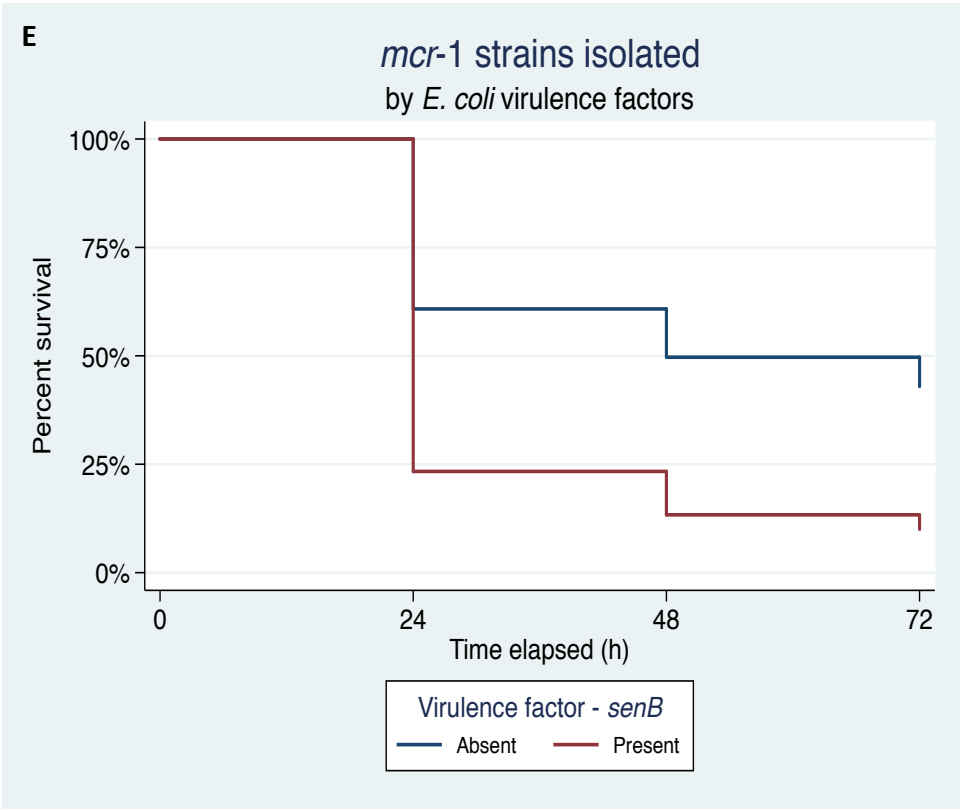

B
mcr-1 strains isolated
by *E. coli* virulence factors
100%
75%
50%
25%
0%
Percent survival
0
24
48
72
Time elapsed (h)
Virulence factor - *vat*
Absent
Present

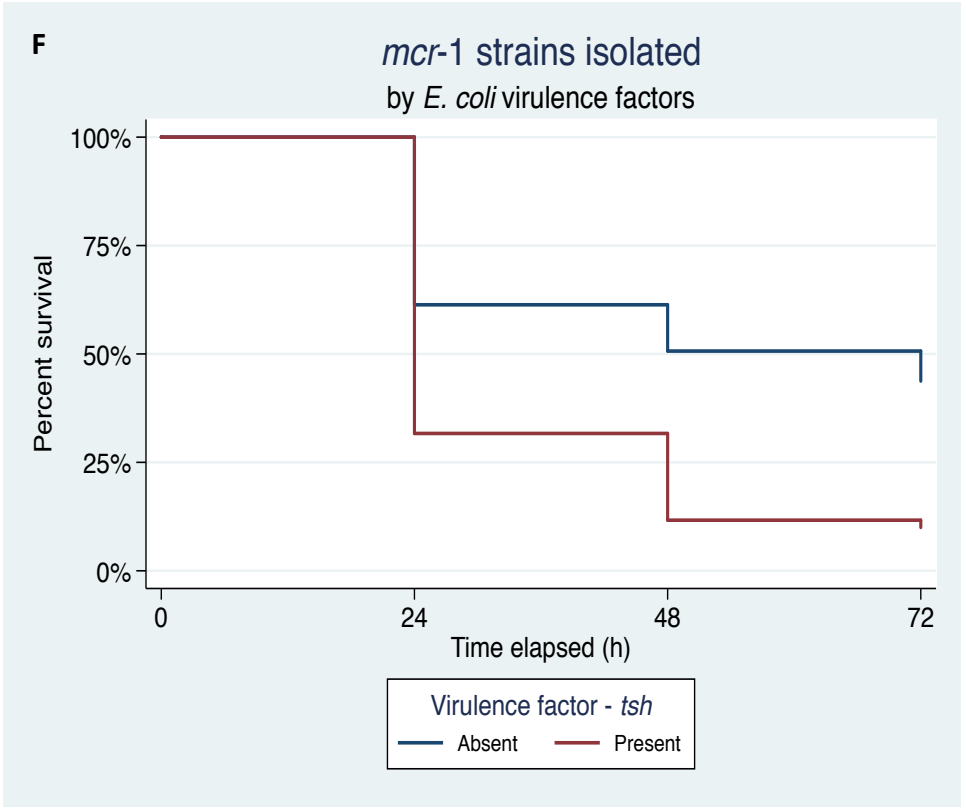
C


mcr-1 strains isolated
by *E. coli* virulence factors
Percent survival
100%
75%
50%
25%
0%
0
24
48
72
Time elapsed (h)
Virulence factor - *hlyE*
Absent
Present

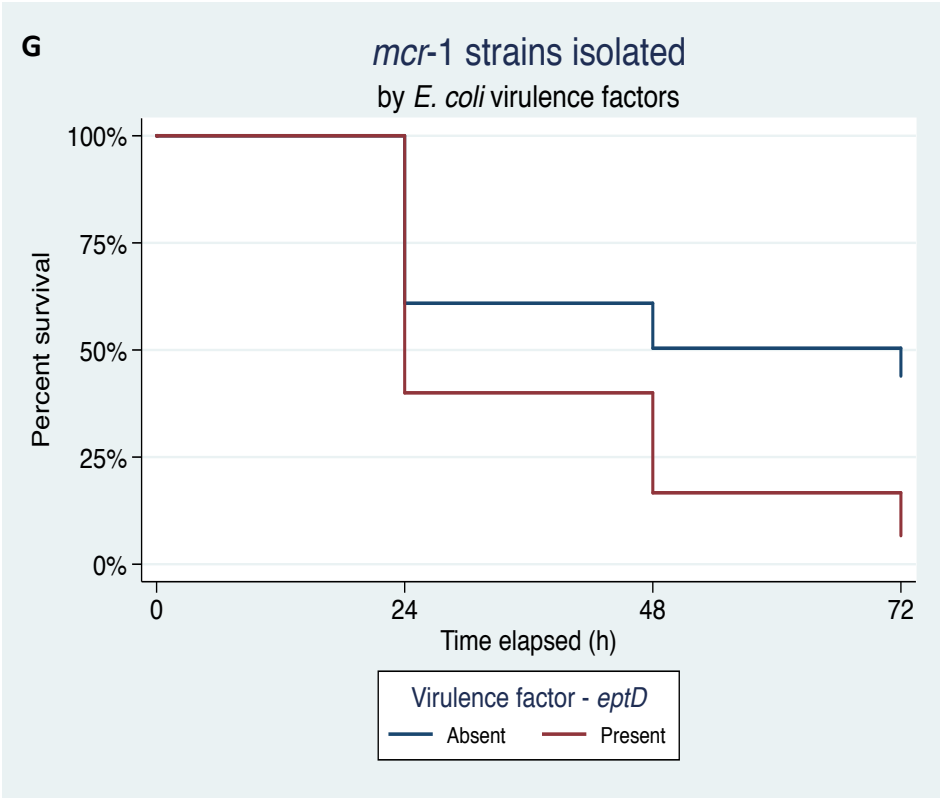
D


mcr-1 strains isolated
by *E. coli* virulence factors
Percent survival
100%
75%
50%
25%
0%
0
24
48
72
Time elapsed (h)
Virulence factor - *AfaB*
Absent
Present

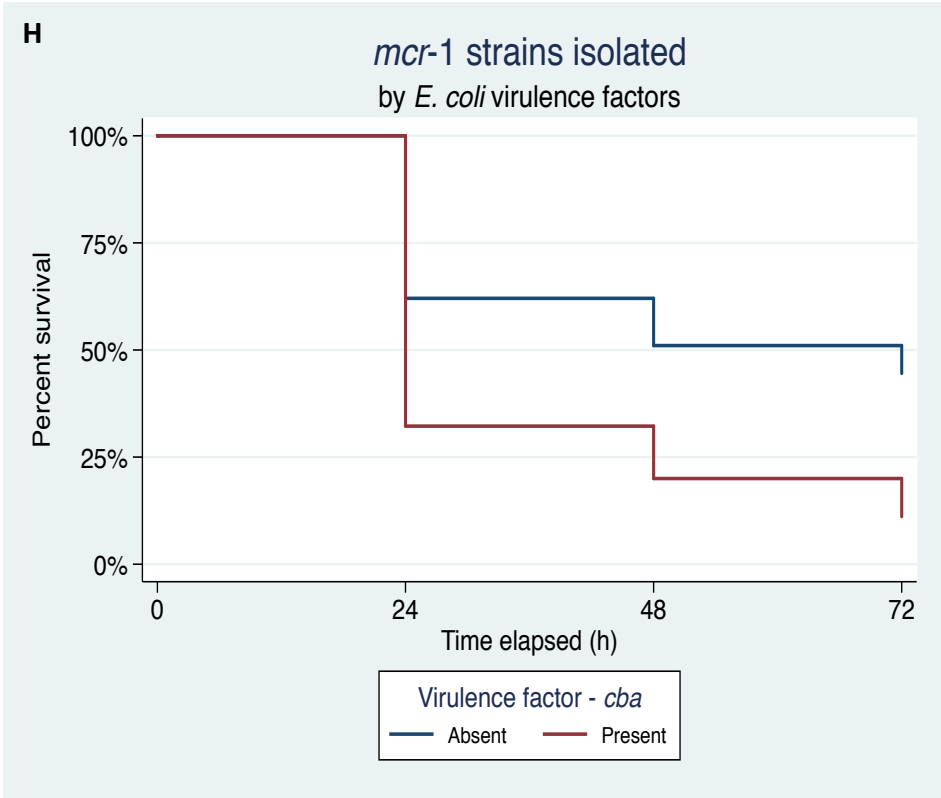

E
mcr-1 strains isolated
by E. coli virulence factors
100%
75%
50%
25%
0%
Percent survival
0
24
48
72
Time elapsed (h)
Virulence factor - senB
Absent
Present


F
mcr-1 strains isolated
by E. coli virulence factors
100%
75%
50%
25%
0%
Percent survival
0
24
48
72
Time elapsed (h)
Virulence factor - tsh
Absent
Present

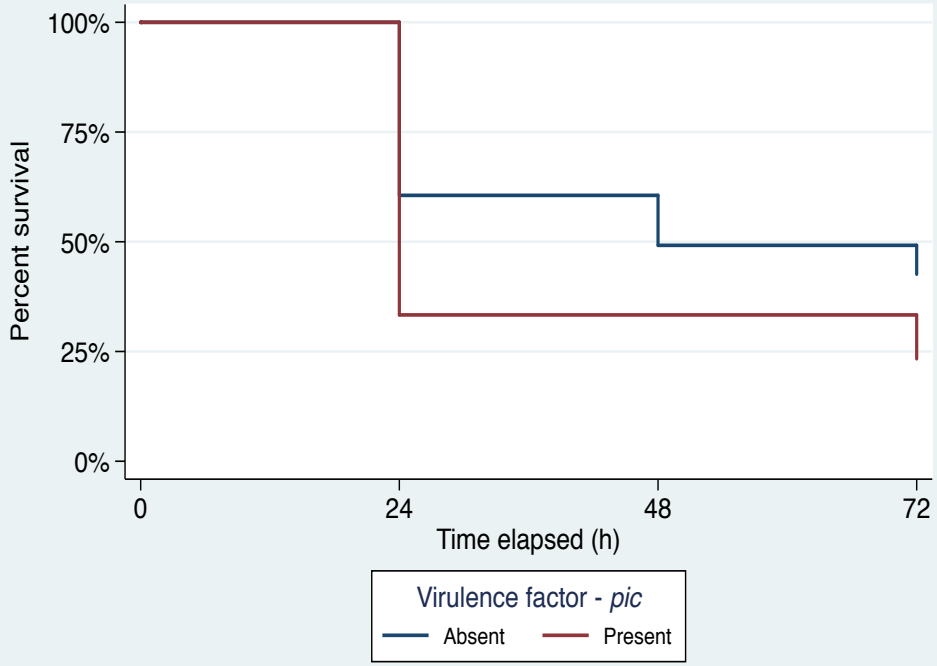
G


mcr-1 strains isolated
by *E. coli* virulence factors
100%
75%
50%
25%
0%
Percent survival
0
24
48
72
Time elapsed (h)
Virulence factor - *eptD*
Absent
Present

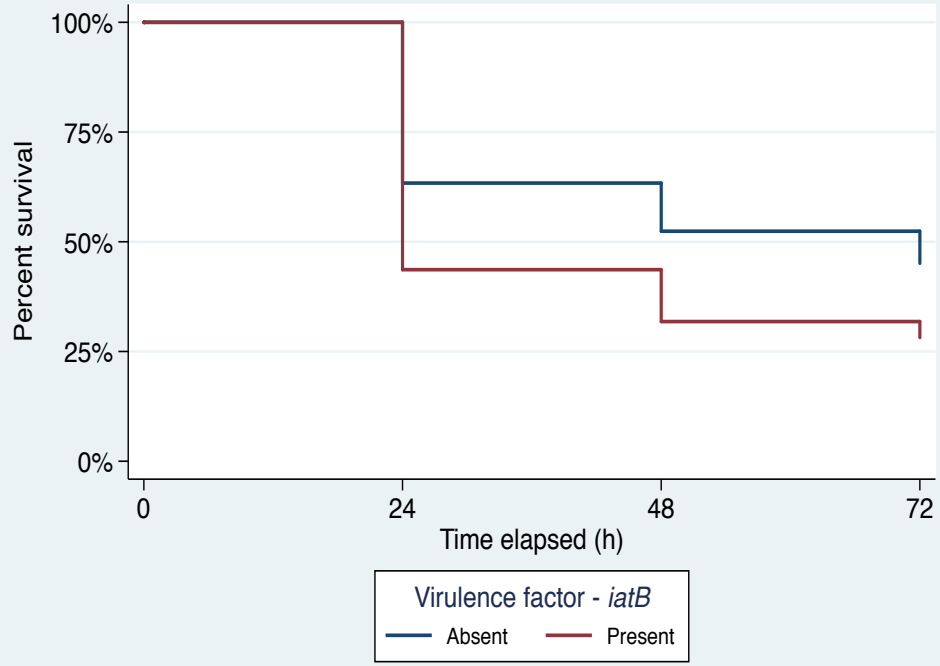
H


mcr-1 strains isolated
by *E. coli* virulence factors
100%
75%
50%
25%
0%
Percent survival
0
24
48
72
Time elapsed (h)
Virulence factor - *cba*
Absent
Present

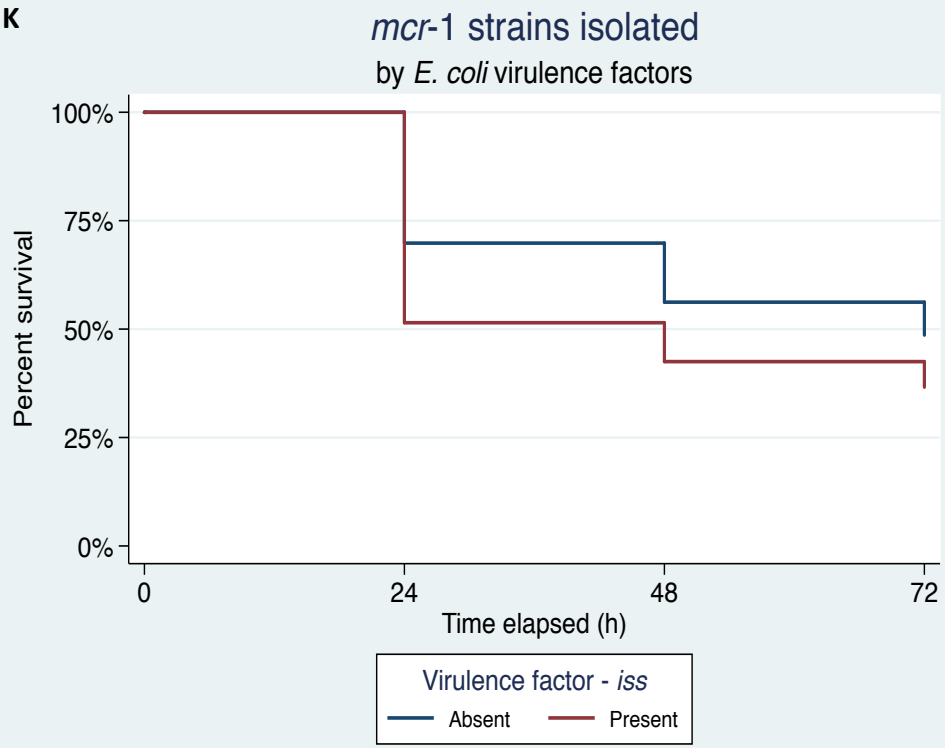
I


mcr-1 strains isolated
by *E. coli* virulence factors
Percent survival
100%
75%
50%
25%
0%
0
24
48
72
Time elapsed (h)
Virulence factor - *pic*
Absent
Present

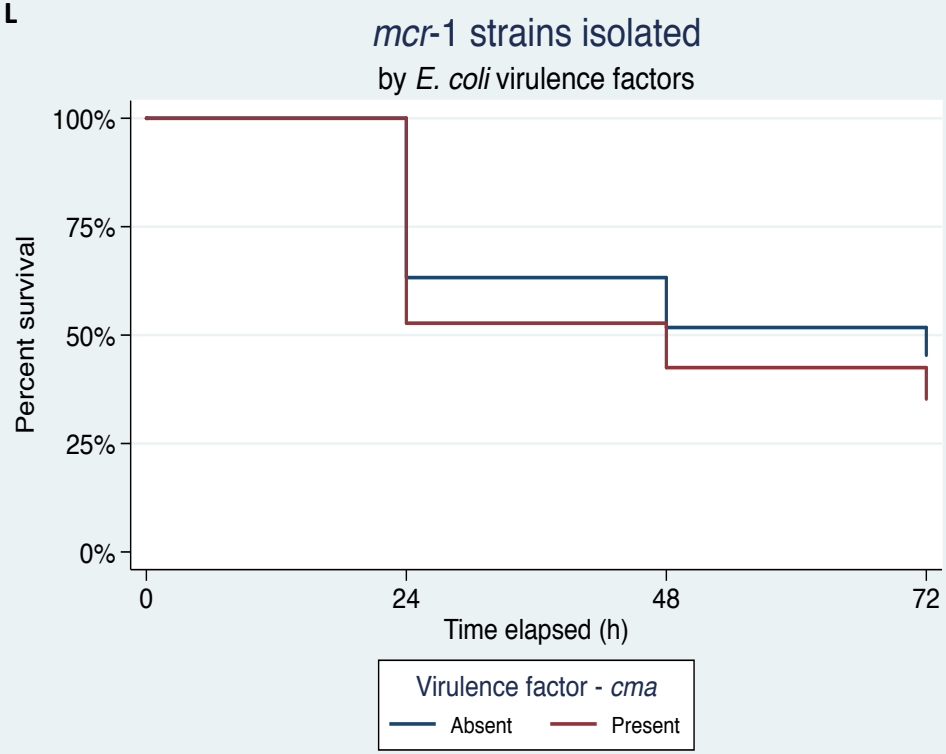
J


mcr-1 strains isolated
by *E. coli* virulence factors
Percent survival
100%
75%
50%
25%
0%
0
24
48
72
Time elapsed (h)
Virulence factor - *iatB*
Absent
Present

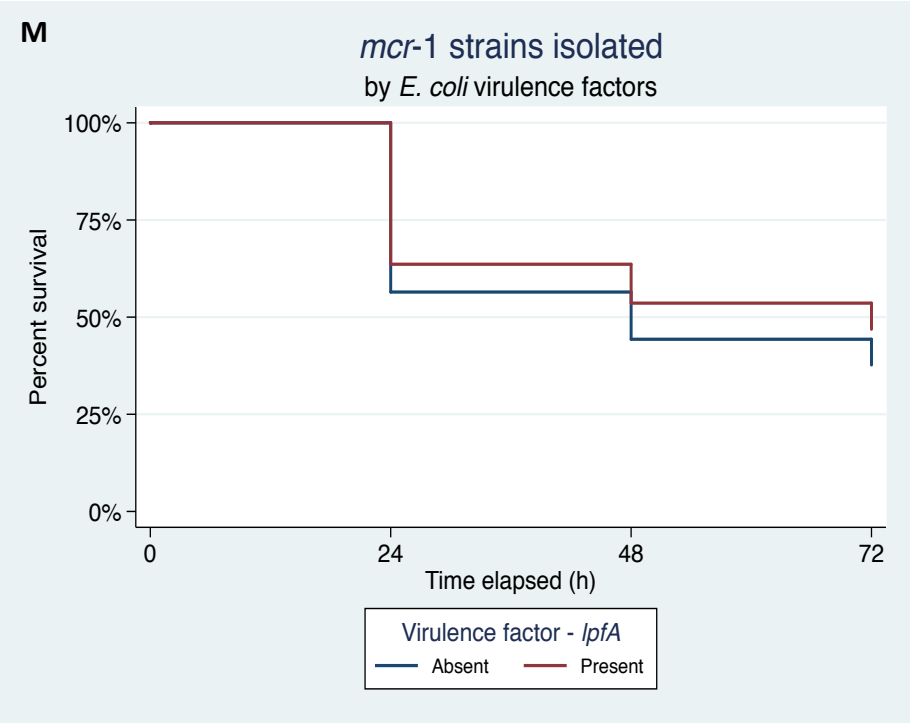
K


mcr-1 strains isolated
by *E. coli* virulence factors
100%
75%
50%
25%
0%
Percent survival
0
24
48
72
Time elapsed (h)
Virulence factor - *iss*
Absent
Present

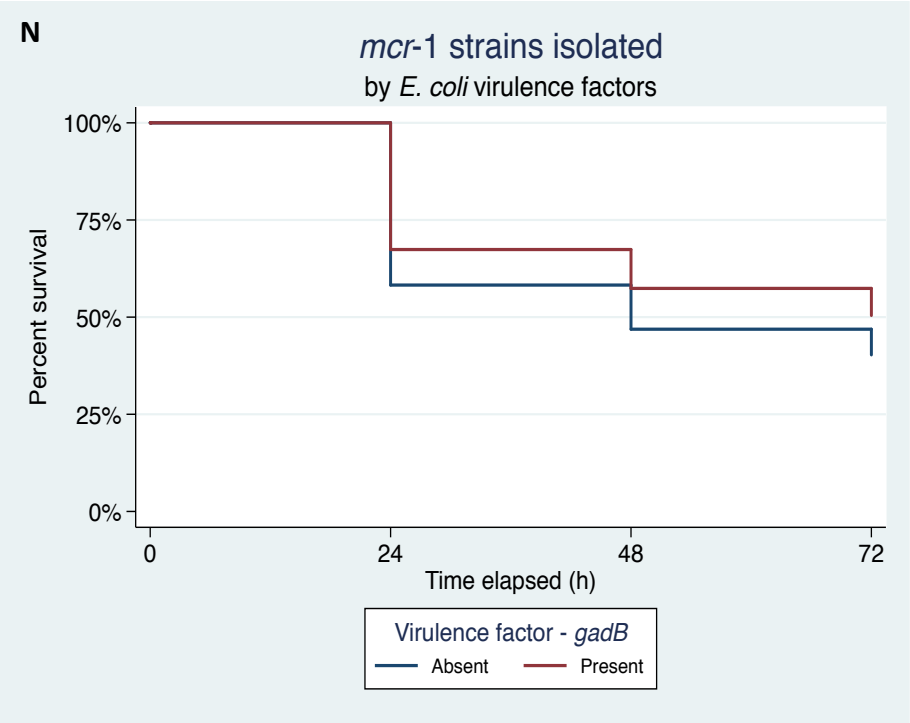
L


mcr-1 strains isolated
by *E. coli* virulence factors
100%
75%
50%
25%
0%
Percent survival
0
24
48
72
Time elapsed (h)
Virulence factor - *cma*
Absent
Present

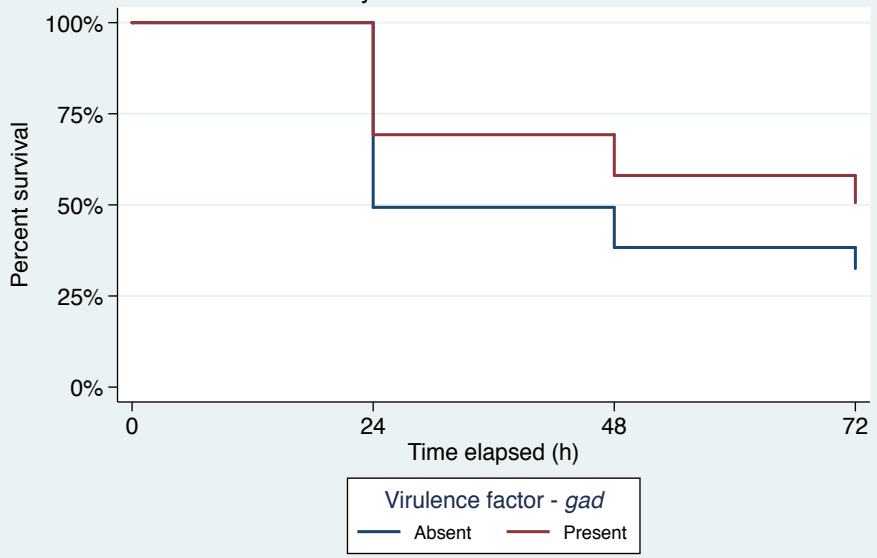
M


mcr-1 strains isolated
by *E. coli* virulence factors
100%
75%
50%
25%
0%
Percent survival
0
24
48
72
Time elapsed (h)
Virulence factor - *lpfA*
Absent
Present

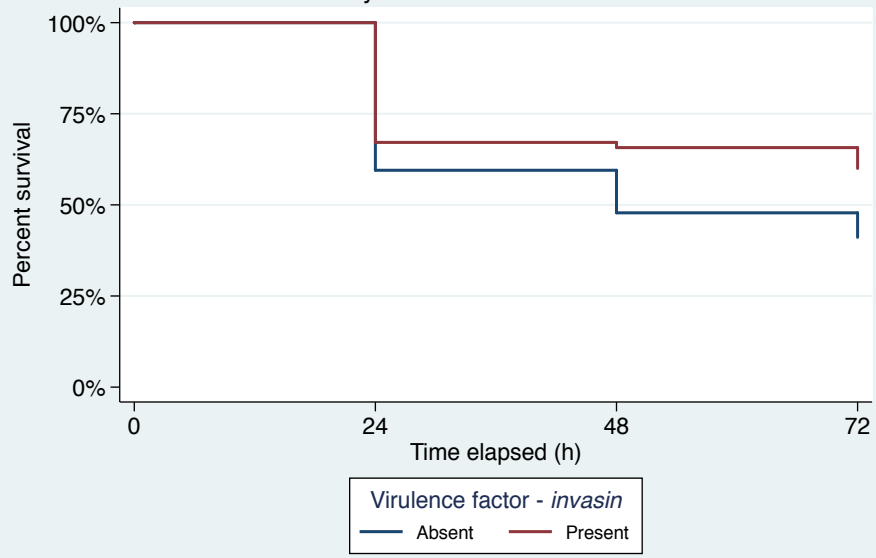
N


mcr-1 strains isolated
by *E. coli* virulence factors
100%
75%
50%
25%
0%
Percent survival
0
24
48
72
Time elapsed (h)
Virulence factor - *gadB*
Absent
Present

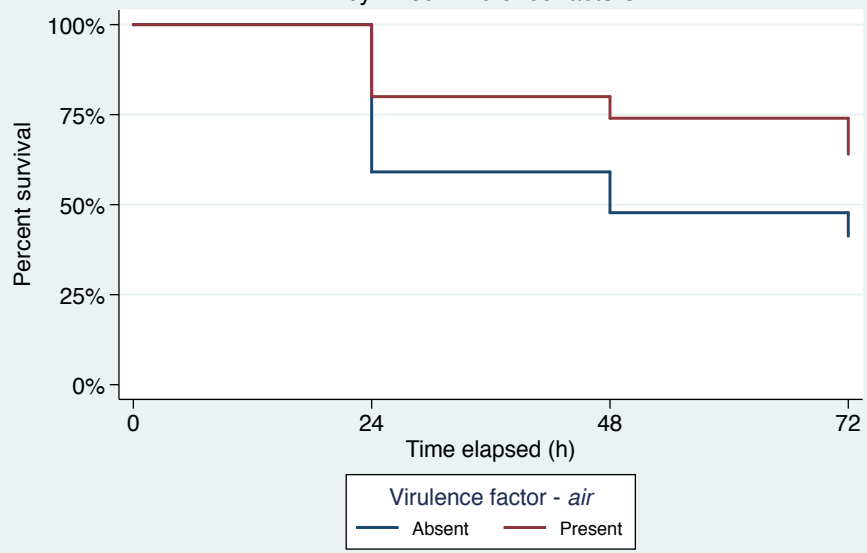
O


mcr-1 strains isolated
by *E. coli* virulence factors
100%
75%
50%
25%
0%
Percent survival
0
24
48
72
Time elapsed (h)
Virulence factor - *gad*
Absent
Present

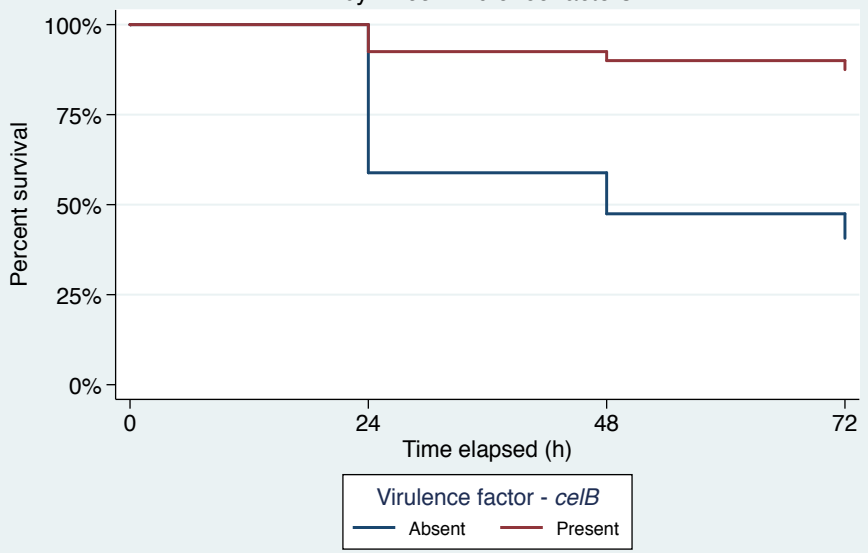
P


mcr-1 strains isolated
by *E. coli* virulence factors
100%
75%
50%
25%
0%
Percent survival
0
24
48
72
Time elapsed (h)
Virulence factor - *invasin*
Absent
Present

Q


mcr-1 strains isolated
by *E. coli* virulence factors
100%
75%
50%
25%
0%
Percent survival
0
24
48
72
Time elapsed (h)
Virulence factor - *air*
Absent
Present

R


mcr-1 strains isolated
by *E. coli* virulence factors
100%
75%
50%
25%
0%
Percent survival
0
24
48
72
Time elapsed (h)
Virulence factor - *celB*
Absent
Present

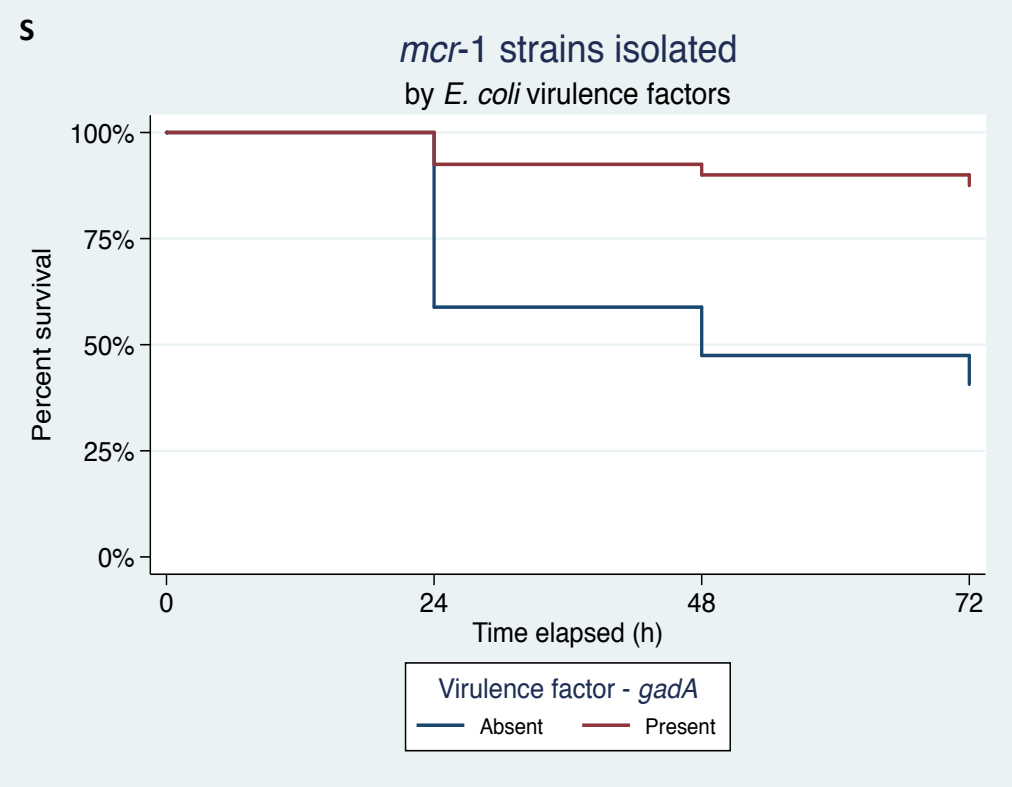


Figure 41. Kaplan Meier survival curves of individual virulence factor genes. A to L represents individual virulence factor genes with high Hazard Ratio scores showing high mortality rates. M to S shows lower ranked VF scores with low mortality rates.

All *E. coli mcr-1* isolates showed an endotoxin activity of greater than >1 EU/ml (Figure 42 and Table 24).

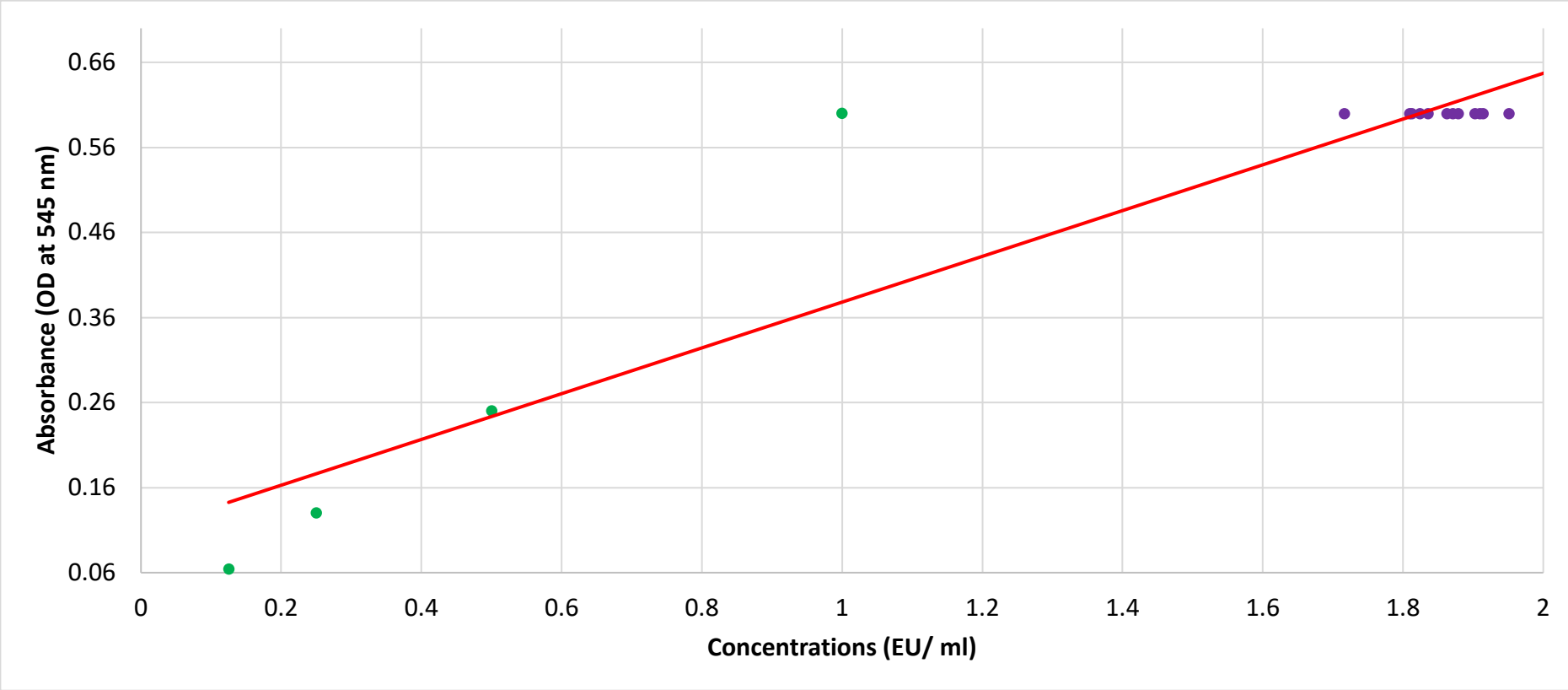


Figure 42. Endotoxin activity of colistin-resistant *E. coli* strains isolated from chickens and humans. Green dots – represents the standards (0.125, 0.25, 0.5 and 1 EU/ml). Purple dots – test isolates with endotoxin activity that clusters between 1.716 – 1.951 EU/ml.

Isolates	Endotoxin activity (EU/ml)	Sequence type, ST	Origin
E. coli 61	1.951	ST1011	Human
E. coli B28	1.914	ST1727	Avian
E. coli B52	1.909	ST359	Avian
E. coli 1	1.902	ST224	Human
E. coli B47	1.878	ST1140	Avian
E. coli 60	1.871	ST1638	Human
E. coli C22	1.862	ST101	Avian
E. coli 59	1.835	ST38	Human
E. coli 49	1.824	ST746	Human
E. coli C55	1.812	ST155	Avian
E. coli C28	1.809	ST2372	Avian
E. coli 8	1.716	ST206	Human

Table 24. Endotoxin activity (EU/ml) of colistin-resistant *E. coli* strains from chickens and humans.

5.4.2 *G. mellonella* analysis in *E. coli mcr-1-like* transconjugants

E. coli C22/EC J53 transconjugant showed enhanced virulence when compared to the background EC J53 with HR of 21.653 followed by *E. coli* B28/EC J53 and *E. coli* A53/EC J53 (Table 25, Figure 43 and Figure 44).

WGS analysis of all the transconjugants showed *E. coli* B28/EC J53 co-harboured other plasmids while *E. coli* A53/EC J53 and *E. coli* C22/EC J53 harboured only one plasmid replicon type, IncI2 (Table 26). In addition, *E. coli* B28/EC J53 transconjugant acquires more virulence factors. However, the most virulent genes were not observed according to the calculated Hazard Ratio. In addition, *E. coli* B28/EC J53 showed less *G. mellonella* killing compared to *E. coli* C22/EC J53 transconjugant. As with *E. coli* A53/EC J53 transconjugant is shown to be less virulent than *E. coli* C22/EC J53, although both transconjugant carried similar replicon type and virulence factor genes.

Susceptibility testing showed *E. coli* C22/EC J53 transconjugant was resistant to 3rd generation cephalosporin hence acquiring an ESBL phenotype (Table 27). Growth kinetics of the transconjugant and ECJ53 recipient strain did not show obvious difference after 20h assessment. Mann-Whitney U test showed all the three curves were not significant with *p*-value of 0.5887 (Figure 45), 0.3095 (Figure 46) and 0.5887 (Figure 47). Therefore, acquiring *mcr-1-like* genes does not affect the growth of the host.

Transconjugants (7log/larvae)	Hazard Ratio (HR)	*p*-value	95% Confidence Interval, CI
E. coli C22/ECJ53	21.653	<0.001	8.434, 55.592
E. coli B28/ECJ53	18.198	<0.001	7.447, 44.473
E. coli A53/ECJ53	4.253	0.007	1.476, 12.260

Table 25. Virulence ranking of colistin-resistant *E. coli* transconjugants according to their Hazard Ratio.

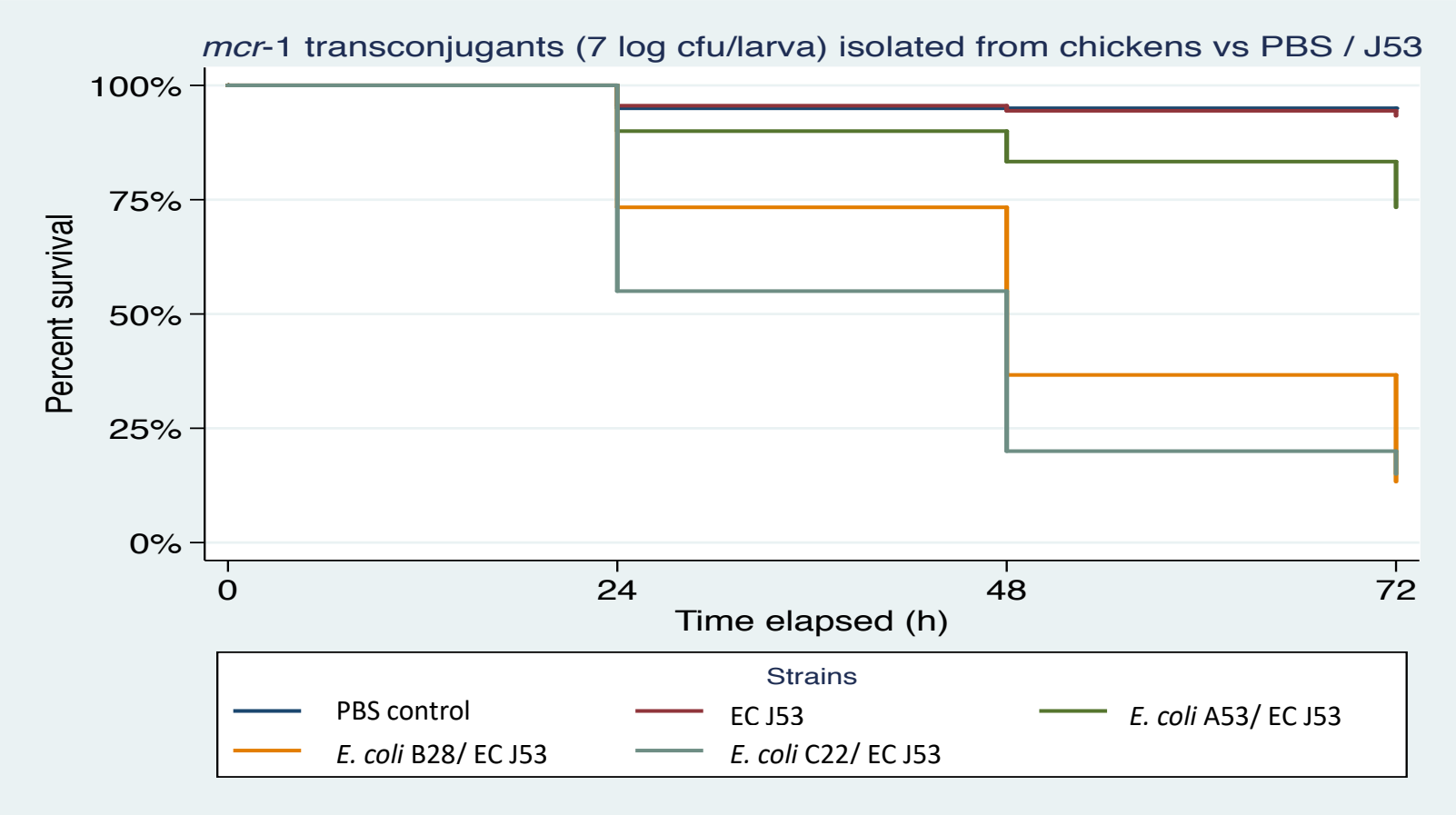
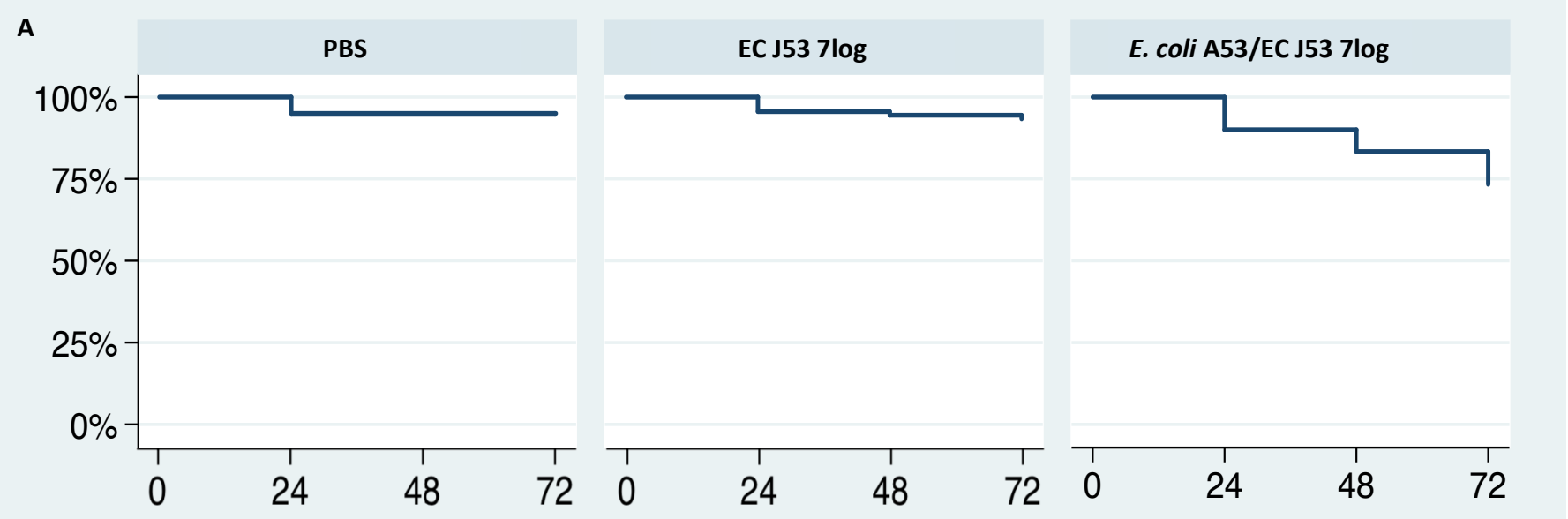


Figure 43. *E. coli mcr-1-like* transconjugants at 7 log cfu/larva isolated from chicken versus PBS/J53 control.


A
PBS
EC J53 7log
E. coli A53/EC J53 7log
100%
75%
50%
25%
0%
0
24
48
72
0
24
48
72
0
24
48
72

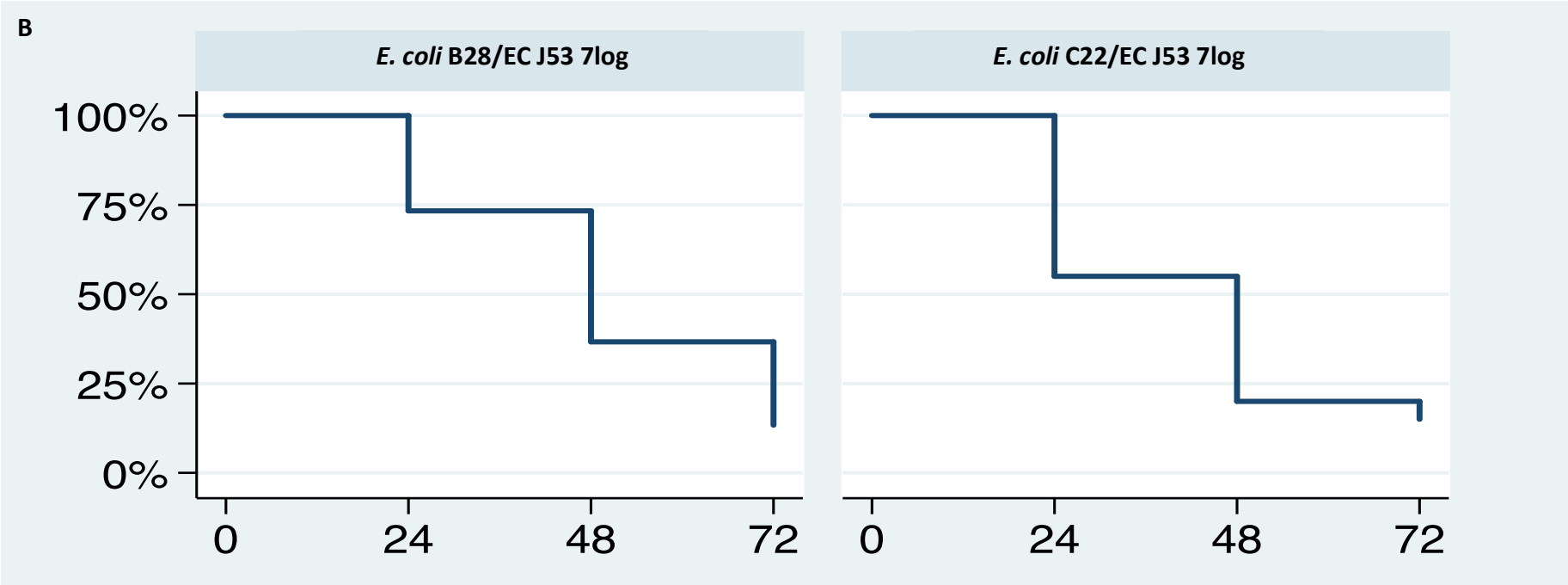


Figure 44. A &B Survival curves of transconjugants at 7 log cfu/larva, PBS and J53.

Characteristics	Donor			Recipient	Transconjugant		
	***E. coli* A53**	***E. coli* B28**	***E. coli* C22**	***E. coli* J53**	***E. coli* A53/EC J53**	***E. coli* B28/EC J53**	***E. coli* C22/EC J53**
Main resistance gene	*mcr-1.1*	*mcr-1.1*	*mcr-1.8*	-	*mcr-1.1*	*mcr-1.1*	*mcr-1.8*
Plasmid replicon type	IncI2, IncN, IncX4, IncX1, p0111, IncI, IncHI2A, IncHI2, IncFII(pSE11), InFII, IncFIB(AP001918), IncB/O/K/Z, ColpVC, Col156	IncI2, IncFIB(AP001918), IncFII(pCoo), p0111	IncHI1A, IncHIB(R27), IncFIA(HI1), IncFII, IncY, IncI2, IncFIC(FII), IncFIB(AP001918) IncI1, IncX1	-	IncI2	IncI2, IncFIB(AP001918), IncFII(pCoo)	IncI2
MLST	ST 2223	ST 1727	ST 101	ST 10	ST 10	ST 10	ST10
Virulence associated gene	*iroN, gad, astA, cma*	*iss, lpfA, cma, gad, iroN*	*astA, cma, lpfA, iss, eilA, gad, iha, lpfA, mchF*	*iss, gad*	*iss, gad*	*iss, gad, iroN, cma*	*iss, gad*

Table 26. Summary of the characteristics of the *E. coli mcr-1-like* wild-type donor strains, recipient and its transconjugants.

Isolate	MIC (mg/L) Etest CT	Zone Diameter (mm) AMC (30)	KF (30)	FOX (30)	CPD (10)	EFT (30)	FEP (30)	ETP (10)	IMP (10)	MEM (10)	ENR (5)	CIP (5)	PEF (30)	MH (30)	C (30)	FFC (30)	AK (30)	K (30)	APR (15)
***E. coli* A53**	>256	6	6	6	6	21	30	23	27	28	6	11	6	8	6	6	20	6	6
***E. coli* A53/EC J53**	6	19	17	27	25	25	33	28	28	30	27	28	26	22	24	22	19	19	16
***E. coli* J53**	2	21	18	26	25	22	35	31	29	32	29	30	32	22	24	24	19	20	16
***E. coli* B28**	>256	6	6	6	6	22	35	18	29	19	6	6	6	11	6	6	24	6	6
***E. coli* B28/EC J53**	4	16	16	28	26	26	34	32	30	32	20	24	18	20	6	6	19	19	16
***E. coli* J53**	2	21	18	26	25	22	35	31	29	32	29	30	32	22	24	24	19	20	16
***E. coli* C22**	8	6	6	25	6	6	17	22	25	25	8	11	6	11	6	6	19	6	6
***E. coli* C22/EC J53**	4	13	6	28	6	6	18	29	28	30	27	27	26	22	23	24	19	18	16
***E. coli* J53**	0.38	21	18	26	25	22	35	31	29	32	29	30	32	22	24	24	19	20	16

Table 27. Susceptibility testing of transconjugants and their respective donor and recipient (EC J53) according to EUCAST, CLSI and/or CLSI VET guidelines. (CLSI, 2018) (CLSIVET08, 2018) (EUCAST, 2018) CT= colistin, AMC= augmentin, KF= cephalothin, FOX= cefoxitin, CPD= cefpodixme, EFT= ceftiofur, FEP= cefepime, ETP= ertapenem, IMP= imipenem, MEM= meropenem, ENR= enrofloxacin, CIP= ciprofloxacin, FFC= florfenicol,

AK= amikacin, K= kanamycin, APR= apramycin. MIC breakpoint for colistin - susceptible ≤2 and resistant 2. Red colour- Resistance, Green colour – Sensitive, Yellow colour – Intermediate and Unshaded box- no reference breakpoint guidelines available.

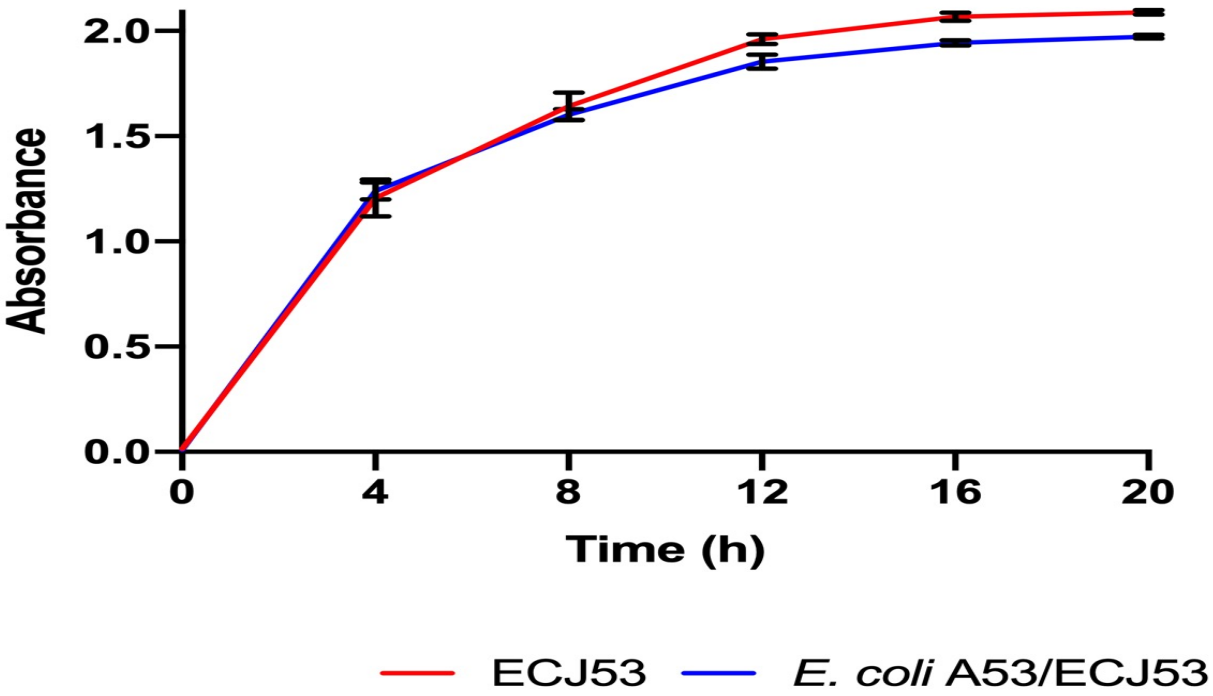


Figure 45. Growth kinetics between *E. coli* A53/ECJ53 transconjugant and EC J53 recipient.

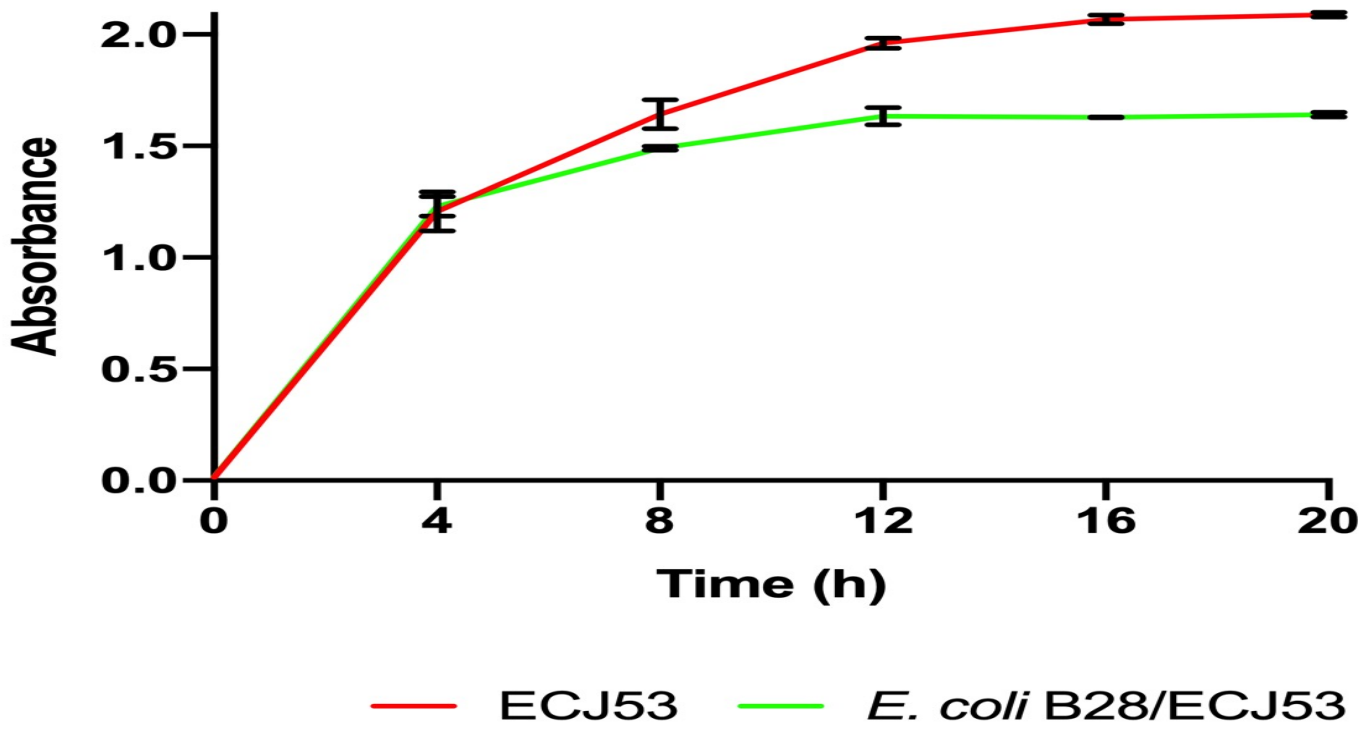


Figure 46. Growth kinetics between *E. coli* B28/ECJ53 transconjugant and EC J53 recipient ECJ53.

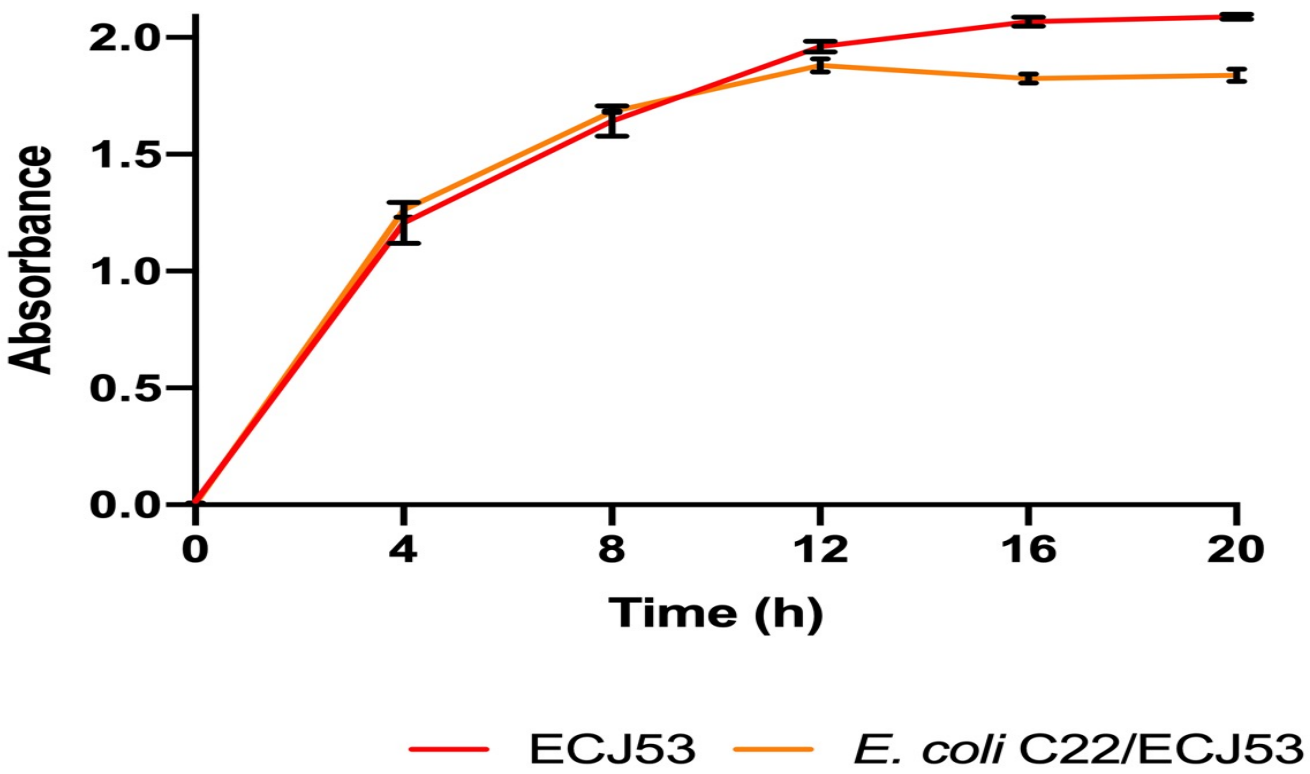


Figure 47. Growth kinetics between *E. coli* C22/ECJ53 transconjugant and EC J53 recipient.

5.5 Summary

- Forty *E. coli* colistin resistance strains isolated from chickens and humans were investigated for virulence in *G. mellonella* infection model.
- Differences in *G. mellonella* killing were observed in all strains isolated from chickens and humans. These data were analysed by using Kaplan-Meier survival curves and cox proportional hazards ratio.
- *cvaB* (colivin V secretion/processing ATP-binding protein) promoted 2.546 greater *G. mellonella* killing but was present in only one strain. Other VF were *vat*, *hylE*, *AfaB*, *senB*, *tsh*, *eptD*, *cba*, *pic*, *iatB*, *iss* and *cma* also showed virulence: Likewise, carriage of *lpfA*, *gadB*, *gad*, *invasin*, *air*, *celB* and *gad* were associated with reduced virulence.
- No difference in endotoxin activity in colistin-resistant *E. coli* strains was observed in LAL assay.
- Plasmids from three donor strains (*E coli* A53, *E. coli* B28 and *E. coli* C22) enhanced the virulence of EC J53 transconjugants.
- However, plasmids from *E. coli* C22 and *E. coli* A53 showed differences in *G. mellonella* killing, despite the transfer of similar virulence factors and plasmid replicon type. The only difference being the plasmid from *E. coli* C22 encoded MCR-1.8.
- The *E. coli* B28/EC J53 transconjugant carried additional plasmid replicon types and additional virulence factors.
- Growth assays did not demonstrate an obvious fitness cost on acquisition of *mcr-1-like* encoding plasmids.

Chapter 6

Discussion

6.1 Introduction

Antimicrobial resistance is a critical global problem, posing threats and challenges in human medicine and public health (WHO, 2018). The rise of AMR and drying-up of the antimicrobial development pipeline has impacted on the ability of antibiotics to save countless lives, limbs, surgical procedures and cancer (Spellberg et al., 2004). Economically AMR is costly whereby within the next 30 years, treating AMR could cost up to US$3.5 billion per year (Hofer, 2019). Aggravating this problem further is the emergence and spread of critical Gram-negative pathogens (carbapenem-resistant *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacteriaceae*) (WHO, 2017a) which further limits therapeutic options, especially when carbapenems have become last-line antibiotics for treatment (Papp-Wallace et al., 2011). Plasmid-mediated carbapenem resistance (IMP-like, VIM-like, KPC-like, NDM-like, OXA-48-like) acquired by these pathogens has disseminated globally (Nordmann et al., 2011a). Colistin, an old antibiotic has therefore been repurposed to treat multidrug-resistance clinical infections acquiring these carbapenemase genes. Unfortunately, with the recent emergence of plasmid-mediated colistin resistance, *mcr-1* reported China in 2015, the AMR crisis has escalated further (Liu et al., 2016). The *mcr-1* gene has been found in animals and humans which further threatens public health (Liu et al., 2016).

The complex public health issue has led to the 'One Health' approach to tackle the problem. This need to involve multiple government sectors (human health, animal health, agriculture, environment, policy makers) able to communicate and work together to achieve public health outcomes and strengthen AMR surveillance (WHO, 2017b). Furthermore, AMR surveillance is still underdeveloped in most developing countries especially in Southeast Asia (SEA) which includes Brunei Darussalam (Hsu et al., 2017). AMR surveillance is important in this region as it has been seen as a hotspot for wider global resistance dissemination (Hsu et al., 2017).

The WHO suggest other approaches apart from surveillance to tackle AMR which focus on improving awareness and the understanding of AMR, reducing the incidence of infection, optimizing the use of antimicrobial agents, and an increase in investment into new medicines, vaccines and diagnostic tools (WHO, 2015). Such guidelines would help resource-poor countries tackle and contain AMR and prevent dissemination locally and internationally.

6.2 Phenotypic and genotypic analysis of carbapenem-resistant Gram-negative Non-fermenters from Brunei Darussalam

6.2.1 Molecular analysis of carbapenem-resistant *A. baumannii* (CRAB) clinical isolates in Brunei and Southeast Asia

Carbapenem-resistant *Acinetobacter baumannii* (CRAB) is an important opportunistic pathogen, usually associated with hospital-acquired infections. In *A. baumannii,* $bla_{\text{OXA-23}}$ is the most prevalent carbapenemase-encoding gene worldwide (Mugnier et al., 2010; Hammami et al., 2011; Bakour et al., 2014).

Additionally, one of the most successful clonal lineages of *A. baumannii* is international clone II (IC II), which clusters in clonal complex 92 (CC92) according to the multilocus sequence typing (MLST) Oxford scheme developed by Bartual et al. (Bartual et al., 2005) and sequence type 2 (ST2) using the alternative Pasteur MLST scheme (Matsui et al., 2014). *A. baumannii* IC 2 has been associated with nosocomial outbreaks and multidrug resistance (Diancourt et al., 2010) and has spread globally (Karah et al., 2012). In Japan, *A. baumannii* IC 2 is also reported to be strongly associated with multidrug or carbapenem resistance (Endo et al., 2012; Kouyama et al., 2012; Matsui et al., 2014) and has caused nosocomial outbreaks (Asai et al., 2014; Alshahni et al., 2015; Ushizawa et al., 2016).

In this study, all the CRAB isolates carried an OXA-23-like gene and produced the intrinsic OXA-51-like carbapenemase enzymes. Most of the isolates from Brunei belonged to either Global Clone I/GC1/IC1 and II/GC2/IC2, which are again the typical clones circulating in SEA. Co-resistance to other antibiotics was also identified including ampicillin, ceftazidime, cefepime, ciprofloxacin, netilmicin, piperacillin, piperacillin/tazobactam, ampicillin/sulbactam, gentamicin, co-trimoxazole and ticarcillin/clavulanic acid hence, exhibiting an MDR phenotype.

There are a wide range of mobile genetic elements related to carbapenem resistance in *A. baumannii* which includes IS*Aba1*, IS*Aba2*, IS*Aba3*, IS*Aba4* and IS18 (Pagano et al., 2016). It is also well known that the presence of the IS*Aba1* insertion element upstream of $bla_{\text{OXA-23}}$ gene acts as a promoter increasing the level of expression of $bla_{\text{OXA-23}}$ required to cause carbapenem resistance (Poirel et al., 2011a; Turton et al., 2006). In study done Villalón et al., IS*Aba1* was the common insertion sequences with the highest prevalence (93.2%) in epidemic clones of multidrug-resistant *A. baumannii* investigated from Spain (Villalon et al., 2013).

Other mobile genetic elements include three transposons related to bla_{OXA-23}: Tn*2006*, Tn*2007* and Tn*2008* (Pagano et al., 2016). Tn*2006* in relations to bla_{OXA-23} gene is flanked by two copies of the insertion sequence IS*Aba1*, which is located in opposite directions (Pagano et al., 2016). As with Tn*2008*, it is similar to Tn*2006* but lacks the second copy of IS*Aba1* (Pagano et al., 2016). In Tn*2007*, the bla_{OXA-23} gene is associated with one copy of IS*Aba4* located upstream to this gene (Mugnier et al., 2010).

Three important classes of integron 1, 2 and 3 that have an important role in the dissemination of antimicrobial resistance genes (Mazel, 2006; Gillings, 2014). The highest prevalence was identified with class 1 integron in *A. baumannii* isolates in Europe, Asia and United States (Lee et al., 2009).

Additionally, most acquired MBL genes in *A. baumannii* have been found within class 1 integrons, often containing an array of resistance gene cassettes (Peleg et al., 2008; Potron et al., 2015). While class 2 integrons are included in the Tn*7* family of transposons, containing a transposition module formed by five transposition genes, *tns*A, *tns*B, *tns*C, *tns*D, and *tns*E, rather than the one or two seen in many other transposable elements and consist of an integrase gene followed by gene cassettes (Mazel, 2006; Pagano et al., 2016). However, with class 3 integrons, it is less prevalent than class 2 but there are also located in transposons (Cambray et al., 2010).

CRAB clinical isolates from Brunei also harboured *armA* and a class 1 integron, 2 of which contained gene cassettes similar to the ones reported by Pai et al. (Pai et al., 2003) *aacA4'-8, catB8, aadA1a, dfrA1b, aacA3* and *bla*P2 (also named as $bla_{\text{CARB-1/2}}$ subgroup) (carbenicillin-hydrolysing penicillinase) (Ito and Hirano, 1997) cassette which further support resistance to most of the aminoglycoside antibiotics tested in this study. Of note, it is known that when multiple genes are present in a cassette, the position of the cassette in the array influences the level of antibiotic resistance expressed and is highest when the AMR gene is first in the cassette (Collis and Hall, 1995).

Although, not much is known with the MGE in clinical CRAB isolates in Brunei, hence, this will impact on the ability to define the $bla_{\text{OXA-23}}$ epidemiology in this study.

The earliest *A. baumannii* isolates shown to carry the *armA* gene were recovered in 2003 in Korea (Lee et al., 2006). Since then, this gene has been reported in strains from North America (Doi et al., 2007a), Japan (Tada et al., 2014), China (Huang et al., 2012), Malaysia (Lean et al., 2015), Nepal (Shrestha et al., 2015), India (Saranathan et al., 2014) and Italy (Milan et al., 2016). It was also found in isolates from Norway recovered from patients that had previously been hospitalized in Asia (Karah et al., 2012).

In this study, the *armA* 16S methyltransferase that mediates pan-aminoglycoside resistance was also identified and correlated to the phenotypic resistance tested against the aminoglycoside antibiotics (gentamicin and netilmicin).

Fluoroquinolone resistance (ciprofloxacin) was also seen in our CRAB clinical isolates. However, in a study done by Peleg et al., plasmid-mediated quinolone resistance genes (PMQR) (*qnrA*, *qnrB* and *qnrS*) have yet to be demonstrated in *A. baumannii* (Peleg et al., 2008). It is likely that fluoroquinolone resistance in our isolates was due to a chromosomal mutation in the quinolone resistance-determining regions (QRDRs) (*gyrA*, *gyrB*, *ParC* and *ParE*). However, in a study done by Chopra et al. (Chopra and Galande, 2011) mutants generated from *A. baumannii* (ATCC 17978) with high-level fluoroquinolone resistance (ciprofloxacin gatifloxacin, moxifloxacin and norfloxacin MIC of > 16 mg/L) did not exhibit any mutations in *gyrA*, *gyrB*, *parC* and *parE* when compared with the parental strain. The first report of fluoroquinolone resistance in *A. baumannii* that is independent of mutations in QRDRs and the absence of PMQR (Chopra and Galande, 2011).

In relation to Southeast Asia, there are growing reports of OXA-23-like producing *A. baumannii* (Hsu et al., 2017). For example, in Singapore, the prevalence of CRAB in Singaporean public sector hospitals increased from 48% (2006-2008) to 62% in 2010 (Hsu et al., 2010). OXA carbapenemases were the dominant type and again belonged to global clones I/1 and II/2 (Koh et al., 2007; Koh et al., 2012; Kim et al.,

2013). Similarly in Malaysia, OXA-23 is also the major carbapenemase gene acquired by CRAB (Biglari et al., 2015 46), with a small collection of CRAB from a regional study identifying them also as members of global clone II/2 (Kim et al., 2013). Interestingly in Vietnam, a small fraction of OXA-23-like producing CRAB isolates have been shown to co-produce the NDM-1 carbapenemase gene (Tada et al., 2015a). There are no publicly available data on CRAB in Myanmar, Laos and East Timor, although it is likely that the organism is present in these countries and not reported (Hsu et al., 2017).

In summary, OXA-23 producing CRAB is the dominant carbapenemase circulating in Brunei's hospital similar to those reported in other SEA. Co-resistance to other antibiotics was also observed alongside acquired aminoglycoside resistance, hence limiting therapeutic options. In this study, GC 1/IC 1 and GC 2/IC 2 clones were reported in CRAB clinical isolates. Infection control remains the critical intervention for containment of this pathogen to prevent further dissemination.

6.2.2 Carbapenemase detection in carbapenem-resistant *Pseudomonas aeruginosa* (CRPA)

Multiple methods are available for the detection of carbapenemase production. These include phenotypic tests such as the Modified Hodge Test (MHT), disc-inhibitors synergy tests, chromogenic and nonchromogenic screening media, biochemical tests, spectrophotometry and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) (Osei Sekyere et al., 2015). Molecular detection of carbapenemase genes can be performed by PCR (conventional, multiplex, real-time, GeneXpert system, Check-Direct CPE), microarray, loop-mediated isothermal amplification and next generation sequencing (NGS) (Osei Sekyere et al., 2015). Most of the phenotypic methods perform well for the detection of carbapenemases in Enterobacteriaceae; however, their performance in Gram-negative non-fermenters (CRNF) warrants further investigation (Osei Sekyere et al., 2015).

To assess whether carbapenemases were likely to be produced by *P. aeruginosa* isolates (n= 22) collected in Brunei, we performed Blue-Carba test. Blue-Carba test has been reported to demonstrate 100% sensitivity and specificity, and this could not be confirmed here. All CRPA isolates were negative for carbapenemase production (n=22).

In Portugal, the Blue-Carba method was evaluated against 101 Enterobacteriaceae (n= 44), *Acinetobacter* (n= 43) and *Pseudomonas* (n= 19) strains acquiring Ambler Class A, B, and D carbapenemases (KPC, NDM, IMP, SPM and OXA) and 49 noncarbapenemase producers (susceptible or resistance to carbapenems) (Pires et al., 2013). Interestingly, with the *Pseudomonas* species., 12 *P. aeruginosa* and 1 *P. pseudoalcaligenes* harboring VIM-2 (Imipenem and meropenem MIC 1- >32 mg/L) were reported positive for carbapenemase production and 5 were negative (4 *P. aeruginosa* isolates with imipenem and meropenem MIC > 32 m/L and 1 *P. aeruginosa* with imipenem and meropenem MIC= 0.25 mg/L) (Pires et al., 2013).

P. aeruginosa clinical isolates from Brunei, although resistant to carbapenems (MIC ≥ 8 mg/L), no carbapenemases were detectable with the Blue-Carba assay. Hence, resistance is likely due to other non-enzymatic mechanisms such as porin loss due to mutations in the *OprD* gene leading to imipenem resistance or overexpression of efflux pumps in MexAB-OprM (Multidrug efflux system AB-Outer membrane protein M) (Meletis et al., 2012).

In Southeast Asia, a country such as Thailand, the prevalence of Multidrug-resistant (MDR) *P. aeruginosa* clinical isolates ranges from 20% and 30% as reported from 28 hospitals participating in The National Antimicrobial Resistance Thailand (NARST) program from 2000-2005 (Dejsirilert et al., 2009; Suwantarat and Carroll, 2016). Khuntayaporn et al. (Khuntayaporn et al., 2012) reported out of 261 MDR *P. aeruginosa* clinical isolates (collected during 2007-2009 from eight

tertiary hospitals across Thailand), the most common mechanism of imipenem resistance was the loss of OprD porin protein (98%). In Singapore, the prevalence of carbapenem-resistant *Pseudomonas aeruginosa*, CRPA (resistant to meropenem) reported by Tan et al. (Tan et al., 2008) was 11.2% of 188 isolates collected during 2006-2007. The carbapenem resistance mechanism reported in *P. aeruginosa* isolates was mostly multifactorial (Suwantarat and Carroll, 2016). While the acquired MBL genes (bla_{IMP-1}, bla_{IMP-7} and bla_{VIM-6}) represented only 1.7% of all *P. aeruginosa* isolates collected in 2001 at Singapore General Hospital (Suwantarat and Carroll, 2016). These MBL genes were previously reported in Japan, Canada and Malaysia (Koh, 2008). While in Vietnam, there is limited data on CRPA prevalence, although a novel bla_{IMP-51} has been reported (Tada et al., 2015b). In Malaysia, CRPA prevalence was 21% (Lim et al., 2009) while 32 out of 90 isolates of imipenem-resistant *P. aeruginosa* clinical isolates collected during 2005-2008 from the University of Malaysia Medical Center were positive for MBL genes identified by multiplex PCR; bla_{IMP-7} (n= 12), bla_{IMP-4} (n= 2), bla_{VIM-2} (n= 17) and bla_{VIM-11} (n= 1) (Khosravi et al., 2010).

Further evaluation for carbapenemase production was done by Simner et al. (Simner et al., 2017) in sixty-seven CRPA using a battery of assays; Rapid CarbaNP, Neo-Rapid Carb screen, Manual CarbaNP CLSI, Manual Blue Carba, Modified Carba NP, Boronic acid synergy test, Metallo-ß-lactamase Etest, Modified Hodge Test (MHT), Carbapenem inactivation method (CIM), Modified carbapenem inactivation method. From this evaluation, 14 CRPA isolates producing KPC, VIM,

IMP and SPM and 53 were non-carbapenemase producers could be differentiated (Simner et al., 2017). In addition, Rapidec Carba NP, Neo-Rapid Carb screen, Rapid Carba Blue Screen, Manual Blue Carba, Modified Carba NP and Modified carbapenem inactivation method assays showed 100% sensitivity with the 14 carbapenemase producing CRPA (Simner et al., 2017). However, only 100% specificity with Carbapenem inactivation method and Manual Carba NP CLSI in 53 non-carbapenemase producing CRPA (Simner et al., 2017).

In a large study done by Pasteran et al. (Pasteran et al., 2015) 300 clinical isolates (188 carrying known carbapenemase-encoding gene and 112 without carbapenemase production) were evaluated for the performance of the Blue-Carba test. Clinical isolates belonged to the National and Regional Reference Lab and submission from diverse locations in Latin America (Schmunis and Salvatierra-Gonzalez, 2006). Among the clinical isolates evaluated 22 *P. aeruginosa* harbors Class A (GES-5) and Class B (IMP-13, IMP-16, IMP-18, VIM-2, VIM-11, SPM-1) carbapenemase gene while 44 *P. aeruginosa* harboured ESBLs gene, AmpCs and other non-enzymatic resistance mechanisms (efflux overproduction, porin loss or both) (Pasteran et al., 2015). Positive carbapenemase production was observed with those *P. aeruginosa* isolates harboring Class A and B gene and negative with the 44 carbapenemases non-producers (Pasteran et al., 2015).

In another study done Sun et al. (Sun et al., 2017a) 256 Gram-negative isolates (139 carbapenemase producers and 117 carbapenemase non-producers) and among them were 8 *P. aeruginosa* isolates were evaluated using six phenotypic methods for carbapenemase detection (modified Hodge test (MHT), Triton Hodge test (THT), Carba NP test (CNPt), simplified Carba NP test (CNPt-direct), Blue-Carba NP test (BCT) and carbapenem inactivation method (CIM). All *P. aeruginosa* isolates did not harbor beta-lactamase gene and their MICs of imipenem and meropenem 4-16 mg/L and 2-8 mg/L, respectively. All showed no carbapenemase production with six phenotypic methods tested including the Blue-Carba test (Sun et al., 2017a).

From all these evaluation studies, the performance of the Blue-Carba test is best when Gram-negative non-fermenters harbour carbapenemase genes. With the low prevalence of CRPA acquiring MBL genes in Southeast Asia, it raises the question of whether Blue-Carba testing would be ideal in Brunei's Laboratory setting. In our study, the number of CRPA clinical isolates tested is low; hence, this warrant a more robust evaluation with a large number of samples.

On the contrary, a carbapenemase detection test is not required or encouraged to be performed in clinical microbiology laboratories for clinical management (Jimenez et al., 2017). The test is recommended for epidemiological reasons such as infection control purposes to avoid horizontal transmission and cohorting patients (Jimenez et al., 2017). It is also important to note that not all these phenotypic methods are readily available in developing countries, this is partly

due to the costs of these test and the laboratory capacity for testing these pathogens.

6.3 Characterisation of carbapenem-resistant *Klebsiella pneumoniae* (CRKP) from Brunei Darussalam

In an AMR surveillance studies, the rates of Extended Spectrum Beta-lactamase (ESBL) and carbapenem-resistant Enterobacteriaceae (CRE) in the Asia-Pacific region is much higher than in Europe and North America (Suwantarat and Carroll, 2016). Brunei Darussalam is in the Asia-Pacific region, more associated with the Southeast Asia region and in close proximity to countries such as Malaysia, the Philippines, Singapore and Thailand. In addition, the Study for Monitoring Antimicrobial Resistance Trends (SMART) programme has identified CTX-M-14/15 and NDM-like as the most prevalent ESBL and carbapenemase enzymes circulating here (Jean et al., 2017).

Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) is an important hospital-acquired pathogen (Bocanegra-Ibarias et al., 2017). CRKP have been found to carry diverse carbapenemases such as KPC-like, OXA-48-like, NDM-like and VIM-like (Bocanegra-Ibarias et al., 2017). In this study, we identified an outbreak of OXA-232 producing MDR *K. pneumoniae* of ST 231 that co-produced the class A β-lactamases $bla_{\text{TEM-1b}}$, $bla_{\text{SHV-11}}$ and $bla_{\text{CTX-M-15}}$ in Brunei's hospitals. The identification of OXA-232 producing MDR *K. pneumoniae* in our study was performed phenotypically by the OXA-48 K-SeT lateral flow assay which showed 100% sensitivity and specificity and also by molecular methods.

The OXA-232 carbapenemase is a class D β-lactamase with carbapenem hydrolyzing activity (CHDL) related to the OXA-48 enzyme from which it differs by five amino acids (Potron et al., 2013). It has been reported that OXA-232 producing *K. pneumoniae* isolates typically display carbapenem MICs of ≥ 32 mg/L (Potron et al., 2013; Al-Marzooq et al., 2015; Bousquet et al., 2014). Despite the raised MIC for carbapenems, kinetic studies show that OXA-232 actually hydrolyses carbapenems less efficiently compared to other OXA-48-like variants (Oueslati et al., 2015). The presence of a truncated Δ*ISEcp1* insertion element upstream of the $bla_{\text{OXA-232}}$ gene that provides a strong -35 and -10 promoter sequence on a high copy number plasmid (Poirel et al., 2003) and is required to mediate phenotypic resistance as seen in our isolates.

OXA-48 producing CRE are most prevalent in Europe, the Mediterranean and North Africa (van Duin and Doi, 2017). Multiple reports of nosocomial outbreaks have seen OXA-48 *K. pneumoniae* of various sequence types reported in European hospitals, a problem that is often exceptionally difficult to control (Semin-Pelletier et al., 2015). In terms of pathogenicity, the carriage of a number of virulence factors has been associated with more invasive and severe infections (Rodrigues et al., 2016). In a study done by Holt et al., ST 231 MDR *K. pneumoniae* clone acquiring yersiniabactin (*ybt*) and aerobactin (*iutA*) genes was associated with lethal cases of community-acquired pneumonia (Rodrigues et al., 2016). Additionally, in our preliminary studies using isolate OXA-232 producing *K. pneumoniae*, KP41 (2015) ST 231 clone in the *G. mellonella* infection model showed marked virulence when compared to PBS control (data unpublished).

In Southeast Asia, OXA-48 case has been reported only in Vietnam (Al-Marzooq et al., 2015). However, cases of OXA-232 have been found in Singapore (ST 231) (Teo et al., 2013), Malaysia (ST 14) (Al-Marzooq et al., 2015) and South Korea (Jeong et al., 2015; Kwon et al., 2016). A number of these strains also carried the New Delhi Metallo-β-lactamase (NDM-1) often from patients with a history of travel and medical treatment in India (Al-Marzooq et al., 2015). A retrospective study in Thailand between March 2012 and September 2016 identified CRKP isolates that harbour OXA-232 and a few co-harbouring OXA-232 and NDM-1 (Laolerd et al., 2018). However, as with the OXA-232 *K. pneumoniae* case recovered in Brunei, patients were hospitalized locally, and no history of recent travel could be obtained.

There are several reports of OXA-232 case outside of Southeast Asia; *K. pneumoniae* isolates producing both OXA-232 and NDM-1 have been recovered in the USA (Doi et al., 2014) and France (Potron et al., 2013; Bousquet et al., 2014). Most belonged to ST 14, a sequence type common amongst NDM-1 producing *K. pneumoniae* recovered in Europe (Teo et al., 2013). In Germany, an OXA-232 and NDM-1 was reported in an *E. coli* strain (Rodrigues et al., 2016) with the same patient subsequently developing an infection with OXA-232 producing *K. pneumoniae* months later, suggesting *in-vivo* plasmid transfer between species (Rodrigues et al., 2016). In Mexico City, Enterobacteriaceae producing OXA-232 in combination with CTX-M-15 ESBLs were reported during surveillance studies in tertiary care hospitals (Torres-Gonzalez et al., 2015). However, the molecular

analysis did not identify evidence of clonal spread in this setting, again suggesting inter and intra-species transmission of plasmid-mediated resistance (Torres-Gonzalez et al., 2015). In Mexico City, seven consecutive cases non-clonal Enterobacteriaceae isolates causing different clinical infections in a third-level paediatric hospital over four months period during April–July 2016 were reported (Aquino-Andrade et al., 2018). The OXA-232 was recovered from *E. coli* co-harbouring CTX-M-15 (ESBL) (Aquino-Andrade et al., 2018).

In Tunisia, Lahloui et al. recovered 11 CRKP clinical isolates in 2015, 8 were harbouring OXA-48, and 3 produced OXA-232 (Lahlaoui et al., 2017). All three OXA-232 isolates co-harbour other resistance determinants including CTX-M-15 (ESBL), SHV-11 (penicillinase), *aadA2* (aminoglycoside), *qnrB1* (quinolone), *fosA-like* (Fosfomycin), *sul1* (sulphonamide) and *dfrA12* (trimethroprim) and belonged to sequence type, ST147 (Lahlaoui et al., 2017). *K. pneumoniae* ST147 has been identified previously from Tunisia associated with NDM-1 and OXA-204 carbapenemase genes (Izdebski et al., 2015; Grami et al., 2016). Additionally, ST147 has also been identified in Egypt associated carrying the NDM-1, USA (NDM-5) and South Korea (NDM-7) (Lee et al., 2014; Shin et al., 2016).

An isolate similar to the clone found in our study, was reported in Switzerland by Mancini et al. (Mancini et al., 2018) where 6 CRKP clinical isolates were recovered from February to April 2017 from five different patients. Four CRKP clinical isolates

produced OXA-232 with *bla*$_{CTX-M-15}$ and *bla*$_{TEM-1b}$ resistance gene and only two had *bla*$_{CTX-M-15}$ and *bla*$_{TEM-1b}$ resistance genes (Mancini et al., 2018). However, all six CRKP clinical isolates belonged to sequence type, ST231 (Mancini et al., 2018). Sequencing of 16S rRNA aminoglycoside resistance genes revealed that all isolates possessed *rmtF* 16S rRNA methyltransferase gene (Mancini et al., 2018) but remained susceptible to ceftazidime/avibactam (Mancini et al., 2018). This is the first occurrence of the MDR ST231 clone in Europe (Mancini et al., 2018). A Similar ST231 clone has since been observed in a Polish hospital, from a patient previously hospitalized in Oman (Izdebski et al., 2018).

With regards to the mobility of the *bla*$_{OXA-232}$ gene in carbapenemase-producing *Klebsiella pneumoniae* in ST15 isolates from China (Yin et al., 2017a) and the Czech Republic (Skalova et al., 2017), it was localised to a ColE-type conjugative plasmid of 6,141 bp (Yin et al., 2017a). Others have found this gene to be readily mobilizable including studies in China and the Czech Republic (Potron et al., 2013; Jeong et al., 2015; Kwon et al., 2016; Rodrigues et al., 2016; Skalova et al., 2017; Yin et al., 2017a). In our study, the *bla*$_{OXA-232}$ gene was also localised to a ColE-like plasmid, similar plasmid to the novel OXA-232 that was only very recently isolated in France from Enterobacteriaceae of patients who had travelled to India (Potron et al., 2013) and from patients in Singapore (Teo et al., 2013).

However, in our study, we were unable to demonstrate the transfer of *bla*$_{OXA-232}$ associated with ColE plasmids by conjugation with multiple recipients. Again, highlighting the issue of the spread of OXA-232 mediated resistance via the success of the ST231 clone rather than promiscuity of ColE plasmids.

In summary, the ST 231 clone of *K. pneumoniae* is increasingly shown to support the carriage of a number of important β-lactamases and diverse plasmid backbones which include KPC-3, GES-5, SHV-12 Portugal, (Rodrigues et al., 2016) NDM-1-like reported in the UK and India (Teo et al., 2013) and CTX-M-15, TEM-1, TEM-1b, SHV-like reported in Switzerland and Poland. (Mancini et al., 2018) (Izdebski et al., 2018) Currently, OXA-48-like carbapenem-hydrolyzing class D β-lactamases is circulating in Southeast Asia.

Therefore *K. pneumoniae* ST 231 may represent an emerging high-risk multi-drug resistant clone currently disseminating throughout Southeast Asia. Hence, it is important for enhanced surveillance for the international spread of this clone due to its capacity for the carriage of diverse and multiple ESBL and carbapenemase resistance genotypes. The introduction of OXA-48 K-SeT lateral flow assay would be an ideal screening tool for surveillance in carbapenem-resistant *K. pneumoniae* not only in Brunei Darussalam but also in other countries with this problem.

6.4 Surveillance and prevalence of plasmid-mediated polymyxin resistance in *E. coli* isolates from Brunei Darussalam

6.4.1. CHROMagar COL-*APSE* for the isolation and detection of colistin resistance Gram-negative pathogens

Selective culture media have been developed for the detection of colistin-resistant Enterobacteriaceae due to the current emergence of *mcr*-like genes. A sensitive and selective culture media is an important tool in antimicrobial resistance surveillance.

In this study, CHROMagar COL-*APSE* media was developed for the isolation and identification of colistin-resistant Gram-negative bacteria. CHROMagar COL-*APSE* was further evaluated and compared against SuperPolymyxin media (Nordmann et al., 2016b). An alternative media developed for screening and isolation of polymyxin-resistant Gram-negative bacteria.

Both media were able to support the growth of COL (colistin) resistant Gram-negative bacteria while suppressing COL resistant Gram-positive pathogens. The inoculum (clinical isolates) required to support the growth of all COL resistant strains was as low as 10^1 CFU. However, the growth of only 86% (50/58) of *E. coli mcr-1* isolates was supported on SuperPolymyxin at 10^1 CFU, suggesting it may have lower sensitivity in the detection of these strains. Interestingly,

SuperPolymyxin was able to suppress the growth of all COL-susceptible *Salmonella* spp. (colistin MIC ranges from 0.5 – 2 mg/L). While CHROMagar COL-*APSE* was not able to suppress the growth of 1 *Salmonella* spp. isolate (colistin MIC of 2 mg/L). Agerso et al. (Agerso et al., 2012) reported reduced susceptibility to polymyxins in *Salmonella* Enteritidis and other serogroup 9 strains within the epidemiological cut-off (> 2 mg/L) according to EUCAST, henceforth the definition of resistance in this species has been challenged. Although the mechanisms of polymyxin resistance in *Salmonella* spp remain unknown; a more sensitive medium may help in the recovery of COL resistant strains and aid in the elucidation of novel resistance mechanisms.

We developed and evaluated the CHROMagar COL-*APSE* media for the isolation and identification of COL resistant Gram-negative bacterial pathogens. It was observed that CHROMagar COL-*APSE* was similar to SuperPolymyxin media for selective growth of COL resistant organisms. However, CHROMagar COL-*APSE* was found to be slightly more sensitive in the detection of *Enterobacteriaceae* producing MCR-1 and provides the benefit of presumptive chromogenic identification. Furthermore, the LLD of 10^1 CFU for both medium, suggests either could be used to confirm polymyxin resistance in organisms recovered from other growth media or if employed as a primary isolation medium.

In this study we observed differences in the growth of *P. aeruginosa* ATCC 27853 on both media, where CHROMagar COL-*APSE* showed a lower limit of detection (LLD) of 10^2 CFU when compared to SuperPolymyxin which required 10^5 CFU. This

is most likely due to the heteroresistant properties of this strain and a high frequency of COL resistance by spontaneous mutation (Li et al., 2006b). In *A. baumannii* strains, there was also the differential ability of CHROMagar COL-*APSE* and SuperPolymyxin to identify heteroresistant strains, and this may be due to a synergy between the selective antimicrobials in the media (Phee et al., 2013). The clinical relevance of heteroresistant to polymyxins is still unclear, although the ability to identify such strains maybe useful in optimizing or selecting COL based therapies on an individual basis.

Of note, the addition of PNPG in our media was beneficial in suppressing the swarming of colistin-resistant *Proteus spp* without affecting the performance of the media. Hence, this media may provide a more robust means of selecting out COL resistant isolates within mixed specimens.

More recently, another selective culture medium, LBJMR (Lucie Bardet-Jean-Marc Rolain), has been described, for the detection of colistin-resistant Gram-negative bacteria, including Enterobacteriaceae strains harboring the *mcr-1* gene and also isolation of vancomycin-resistant Gram-positive bacteria (Bardet et al., 2017). Bardet et al. (Bardet et al., 2017) have included detection of vancomycin-resistant enterococci isolates in their media due to the increasing prevalence of this pathogen in Europe (Mendes et al., 2016), especially *Enterococcus faecium*, and causing nosocomial infection in the USA (Reyes et al., 2016) with high mortality rate due to their dissemination (Chiang et al., 2017).

LBJMR medium was then evaluated against SuperPolymyxin media, similar sensitivity with LBJMR was observed for Enterobacteriaceae for all colistin resistant strains with a detection limit of 10^1 CFU, including the 30 *mcr*-1 strains tested, and the inhibition of all susceptible strains (Bardet et al., 2017). Additionally, 68 stools samples (56 humans and 10 chickens from different countries; 2 VRE clinical isolate from La Timone Hospital) that were previously screened for *mcr-1* by RT-PCR (56 positive and 10 negative) were evaluated (Bardet et al., 2017). The results for growth of colistin-resistant isolate with *mcr-1* were similar for both media (Bardet et al., 2017). As with the detection of colistin-resistant non-fermentative Gram-negative strain, higher sensitivity was observed on LBJMR than SuperPolymyxin medium (Bardet et al., 2017). Furthermore, all VRE strains were detected on LBJMR and not on SuperPolymyxin medium because of the presence of daptomycin (Bardet et al., 2017). However, LBJMR and CHROMagar COL-*APSE* medium performances have yet to be assessed.

In this study, the differentiation of colistin-resistant Enterobacteriaceae based on colour compared to SuperPolymyxyin and LBJMR medium, CHROMagar COL-*APSE* showed better differentiation. Additionally, *Proteus* sp. swarming was inhibited by all three media. CHROMagar COL-*APSE* uses PNPG for inhibition while LBJMR inhibited *Proteus* sp. swarming due to the composition of the media which is deprived of electrolytes. In contrast to SuperPolymyxin medium, the inhibition

compound for *Proteus* sp. swarming was not clearly stated. The lower limit of detection was similar for all media 10^1 CFU. Heteroresistant strains were able to be identified with CHROMagar COL-*APSE*, however, warrants further testing for all medium.

In summary, the CHROMagar COL-*APSE,* SuperPolymyxin and LBJMR media will be ideal for under-resourced laboratories due to their lower cost as initial screening tools (Osei Sekyere, 2018). Although molecular methods that can identify *mcr-like* genes in samples are available, they are more expensive, require advanced skill and in most cases, cannot detect resistant strains with novel resistance mechanisms or chromosomal mutations-mediated resistance (Osei Sekyere, 2018). Furthermore, the presence of *mcr-like* genes does not always translate phenotypically into colistin resistance (Nordmann et al., 2016a; Nordmann et al., 2016b; Bardet et al., 2017). More importantly, these culture media will act as tools for the surveillance and quick recovery of isolating COL resistant bacteria from complex human, veterinary and environmental samples.

6.4.2 Surveillance of MCR-1 in poultry farms and human clinical faecal samples

A very high prevalence of *E. coli mcr-1* isolate was identified from poultry farms 58% recovered from 300 ceacal samples (174/300) and 41.7% in human clinical faecal samples (106/254).

Around Southeast Asia, Malaysia has reported *E. coli mcr-1* isolates in 16% (5/31) animal, 1% (1/95) in humans and 40% (2/5) in the environment (Yu et al., 2016). These results were obtained retrospectively from *in silico* screening of more than 900 bacterial isolates from human, animal and environmental sources collected from different states in Malaysia, some of which have been archived since 2009 (Yu et al., 2016). The MICs of colistin for the *E. coli mcr-1* positive isolates range from 8- 12 mg/L determined by agar dilution method (Yu et al., 2016). Clonal diversity was observed in all isolates analysed by pulsed-field gel electrophoresis (PFGE); ST744 in the human clinical sample and ST3489, ST3014, ST410, ST117 from animal and environmental sample (Yu et al., 2016). Additionally, S1 nuclease–PFGE analysis further revealed the presence of multiple plasmids in all isolates (Yu et al., 2016). This correlates with the detection of different types of plasmid replicons in the draft genomes (Yu et al., 2016). Co-resistance were observed with aminoglycosides, fluoroquinolones, florfenicol, sulphonamides, tetracyclines and trimethoprim (Yu et al., 2016). More importantly, the presence of the florfenicol resistance gene *floR* was reported in all of the isolates, including the human isolate (Yu et al., 2016). Hence, the importance to regulate antibiotic

use in animal husbandry to prevent an animal-to-human transfer of resistance (Yu et al., 2016).

In Singapore, two *E. coli mcr-1* isolates (EC249 and EC362) were isolated from human clinical samples (surveillance rectal swab and intraoperative sacral sore tissue) from patients admitted into different wards in a public tertiary care hospital with a range of comorbidities (Teo et al., 2016). Patients had no travel history, previous hospital admission with only exposure to broad spectrum antibiotics and none had prior receipt of polymyxin B (Teo et al., 2016).

One patient was associated with *E. coli* (EC362) infection (Teo et al., 2016). Interestingly, these patients were infected or colonized with other Enterobacteriaceae before/after the isolation of the index *mcr-1* positive isolates (Teo et al., 2016). Additionally, these *E. coli mcr-1* isolates also co-harboured $bla_{\text{KPC-2}}$ (Teo et al., 2016). Analysis of *mcr-1* neighbouring region revealed the *mcr-1-pap2* element was present in all isolates (Teo et al., 2016) similar to our studies. However, the IS*ApI*1 mobile element was observed only in EC362 (Teo et al., 2016) which has been suggested by several studies that the IS*ApI*1 may not always be associated with *mcr-1* (Liu et al., 2016; Ye et al., 2016). The *nikB* gene was only present in EC249 (Teo et al., 2016).

In Thailand, 317 non-duplicate colistin-resistant Enterobacteriaceae clinical isolates (37 *E. coli* and 280 *K. pneumoniae*) were screened by PCR for the *mcr-1* gene (Eiamphungporn et al., 2018). 29.7% (11/37) were positive for *mcr-1* in *E. coli* isolates with colistin MIC of 4-32 mg/L whilst only 1.4% (4/280) *mcr-1* positive in *K. pneumoniae* (Eiamphungporn et al., 2018).

In Laos, Olaitan et al. have reported the presence of *E. coli mcr-1* gene in 6 human clinical samples (colistin MICs 6 to 12 mg/L) and 3 in pigs (colistin MICs 4 to 6 mg/L) (Olaitan et al., 2016a). In another study, Malhotra-Kumar et al. screened 24 Extended-Spectrum β-lactamase (ESBL, $bla_{\text{CTX-M}}$) harbouring *E coli* recovered from 22 rectal swabs taken from chickens on two farms in the Van Lam district of the Hung Yen province, and from a pig farm and a pig slaughterhouse located in the Hoai Duc region of the Hanoi province, Vietnam (Malhotra-Kumar et al., 2016). 37.5% (9/24) of ESBL *E. coli* ESBL strains were positive for *mcr-1*, six were isolated from the rectal swabs of pigs on the farm, one from a rectal swab from a pig about to be slaughtered, and two strains from swabs collected from the lairage area of the slaughterhouse (Malhotra-Kumar et al., 2016). However, the media for screening of the rectal swabs was not stated. The MICs for colistin in all 9 strains by macrobroth dilution were 4 or 8 mg/L (Malhotra-Kumar et al., 2016). MCR-1 sequence showed 100% similarity to the gene reported in China (Liu et al., 2016). In our study, the prevalence of ESBLs reported phenotypically (cefpodoxime 10 μg, as marker) versus WGS analysis data in chickens are 52% and 23% while in humans 11% and 8% a higher rate of ESBLs in colistin-resistant *E. coli* strains

isolated from chicken than in humans. However, two-sample independent t-test (performed on Microsoft Excel for Mac, Version 16.20) of ESBL genes carried in colistin-resistant *E. coli* strains isolated from chickens and humans (WGS analysis results) showed the difference was not associated with a statistically significant effect, t (50) = 1.54, p = 0.06.

Outside of Southeast Asia, China where *mcr-1* was first reported by Liu et al., clinical isolates (902 *E coli* and 420 *K pneumoniae*) recovered from two tertiary hospitals in Guangdong and Zhejiang provinces were screened retrospectively, while samples were collected from pigs at slaughter and recovered 804 isolates and 523 isolates from raw meat (Liu et al., 2016). All isolates were screened for the presence of *mcr-1* gene, to understand the spread of *mcr-1* in food animals and food (Liu et al., 2016). The prevalence of *mcr-1* in animals was shown to be much higher than in human clinical isolates and mostly reported in *E. coli* isolates (Liu et al., 2016). The number of *E. coli mcr-1* positive isolates were as follows; pigs at slaughter 20.6% (166/804), retail meat 14.9% (78/523) and humans inpatient 1.4% (13/902) while 0.7% (3/420) from inpatient in *K. pneumonia* isolates (Liu et al., 2016). Since then, further reports of *mcr-1* from China has been observed from human clinical isolates (Li et al., 2018a; Li et al., 2018b), animals (Liu et al., 2019) and the environment (Shen et al., 2018).

In the United Kingdom, Doumith et al. identified 15 isolates with the *mcr-1* gene (13 isolates from 12 humans and 2 isolates from poultry meat) from genome sequences screening of >24000 *Salmonella*, *E. coli*, *Klebsiella* spp., *Enterobacter* spp., *Campylobacter* spp. and *Shigella* spp. from human and food isolates from the Public Health England (PHE) sequence archive (Doumith et al., 2016). These 15 isolates include 10 unrelated *Salmonella* from human gastrointestinal infections submitted between 2012 and 2015 (8 *Salmonella* Typhimurium isolates, 1 isolate of *Salmonella* Paratyphi B var Java and 1 isolate of *Salmonella* Virchow); 2 isolates of *Salmonella* Paratyphi B var Java phage type Colindale from poultry meat imported from the EU and 3 isolates of *E. coli* from 2 patients (Doumith et al., 2016). The colistin MICs of 14 *mcr-1* positive isolates range from 4-8 mg/L (Doumith et al., 2016). Unfortunately, one negative *E. coli mcr-1* isolate by PCR after recovery from storage was now colistin susceptible (MIC 1 mg/L), hence suggestive of plasmid loss (Doumith et al., 2016).

Other resistance determinants were reported in three of the *E. coli* isolates which include CTX-M-27 or CTX-M-14 and one of the *Salmonella* spp. isolates produce $bla_{\text{CMY-2}}$ (Doumith et al., 2016). Chromosomal colistin resistance caused by mutations in the *pmrAB, phoPQ* and *mgrB* genes were not observed in all the 15 *mcr-1* positive isolates (Doumith et al., 2016).

In France, the prevalence of *E. coli mcr-1* in livestock (turkey, broiler and pigs) from 2007-2014 were 1.6% (23/1045) (Perrin-Guyomard et al., 2016). As with Germany, the prevalence of *mcr-1* in German broilers from 2010 to 2014 were 6.7% (total

number of isolates investigated n=2,555 and total number of *mcr-1* positive isolates, n=173) (Irrgang et al., 2016). Additionally, in the Netherlands, *mcr-1* was detected at low prevalence in *E. coli* isolates from livestock (<1%) (Veldman et al., 2016).

Looking at all these reports from various countries, our study has reported the highest rate of *E. coli mcr-1-like* genes isolated from animals and human clinical samples. Furthermore, out of 162 colistin-resistant *E. coli* isolates (60 from chickens and 102 from humans) that were RAPD typing, 52 distinguishable profile were observed showing considerable diversity. Interestingly, there were clones (ST48, ST115, ST117, ST359, ST1011 and ST1642) found in chicken that was also present in humans. Hence, there might be a possible movement of resistance between livestock and human populations, although such studies have not been established (Woolhouse et al., 2015). However, one study showed by WGS analysis, methicillin-resistant *Staphylococcus aureus* (MRSA) lineage with clonal complex, CC97 has entered the human population from a livestock source on more than one occasion over the past 100 years (Spoor et al., 2013). In another study, there is evidence of *S. aureus* CC398 from livestock-to-human jumps have occurred more frequently human-to-livestock jumps over the evolutionary history of that lineage (Ward et al., 2014). In our study, there is a possible movement of clones through the food-borne route, although this warrants further investigation.

The ST48 clone (part of a clonal complex, CC10, that differs from ST10 by only a single nucleotide) in *E. coli mcr*-positive has been reported from hospital sewage

and human clinical samples in China and Switzerland, respectively (Zhao et al., 2017; Zurfluh et al., 2017). *E. coli* MCR-1 belonging to ST115 has been reported in animals from Germany, Denmark, Slovakia and United States and only one human case from Denmark (Drali et al., 2018). With regards to the environment, ST115 was reported in *E. coli mcr-1* reported in sea water of Algiers coast and ST359 from river water in Switzerland (Zurfuh et al., 2016; Drali et al., 2018). The ST117 and ST1011 clones were reported in clinical *E. coli mcr-1* isolates from New York and Egypt, respectively (Elnahriry et al., 2016; Macesic et al., 2017). Interestingly, ST117 was observed in diseased chickens from China (Ding et al., 2018). From another study, ST1011 was reported in *E. coli* strains co-harbouring *mcr-1* and bla_{NDM} from chickens in China (Liu et al., 2017). Again, in China, ST1642 was seen in ESBL-producing *E. coli* (CTX-M-65) in well water which a primary source of irrigation water and drinking water for humans and farm animals (Sun et al., 2017b). Therefore, ST48, ST115, ST117, ST359, ST1011 and ST1642 are important clones as it exists in all three compartments (humans, animals and environment), that warrants further investigation.

In our study, we also identified different plasmid backbones for *mcr-1-like* (IncI2, IncHI2 and IncX4), in all colistin-resistant *E. coli* isolates although the localization of *mcr-1-like* genes was not performed for all but only one in *E. coli* (EC C22) isolated from chickens that were located on IncI2 plasmid, similar to the plasmid replicon type reported in China (Liu et al., 2016).

Additionally, the predicted serotypes from WGS in our studies did not show the classical Enterohemorrhagic *E. coli* with serotype O157:H7 which is major foodborne pathogen causing severe disease in humans worldwide (Lim et al., 2010). Serotype O157:H7 is usually associated with Shiga toxins genes (stx_1 or stx_2) (Lim et al., 2010). However, in our study, there are two *E. coli mcr-1-like* isolates from humans that express somatic (O) antigen 157 but different flagella (H) antigen, H16 (*E. coli* 61, ST1011) and H9 (*E. coli* 124, ST1011). Interestingly, these two *E. coli* isolates did not acquire the Shiga toxin genes. Virulence factor genes were diverse for all the colistin-resistant *E. coli* isolates. *In silico* ClermoTyping have shown some phylotypes belonged to Extra-intestinal pathogenic *E. coli* (ExPEC), which clusters mostly in groups B2 and D, showing a link between phylogeny and virulence (Escobar-Paramo et al., 2004). In chickens, 4% B2 (1/26) and 23% D (6/26) while in humans, 4% B2 (1/26) and 31% D (8/26) phylotypes which shows most of our isolates were commensals. Despite these findings, there were discrepancies in the results for colistin-resistant *E. coli* isolated from chickens obtained by PCR. Although the PCR used was only for detecting phylogenetic groups A, B1, B2 and D (Clermont et al., 2000). Phylotype groups B2 and D were observed in our study, the pathogenicity also depends on other factors such as virulence factors, resistance determinants and plasmid types and this could not be disregarded. Additionally, these chickens were healthy when sampling was carried out. In contrast, the sampling of human faecal samples was done without the patient's data and diagnosis.

Serotyping of O antigens was performed on 46 colistin-resistant *E. coli* isolates from chickens using antisera pools specific for human enterotoxigenic *E. coli* (ETEC) (O11, O20, O21, O27, O32, O48, O78, O80) and invasive O serotypes common to poultry, pigs and sheep (O10, O18ab, O35, O83, O87, O95, O116, O120). Out of 19 negative colistin-resistant *E. coli* isolates tested with human ETEC O serotype, a discrepancy was seen in one isolate (*E. coli* A8) where the predicted serotype was O21. Hence false negative result was observed with the conventional serotyping. Similarly, with the invasive O serotype, another false negative result was seen with one of the negative colistin-resistant *E. coli* isolates (*E. coli* A28), where the predicted serotype showed O18. In contrast, six positive colistin-resistant *E. coli* isolates were tested for invasive O serotype, discrepancies were seen with WGS predicted O antigen serotypes (O168, O33, O45, O134, O17/77, and O37) that are not present in the invasive O serotype pool. Joenson and colleagues (Joensen et al., 2015), have shown *in silico* serotyping (on the basis of WGS data by SerotypeFinder) of *E. coli* isolates was more superior to conventional serotyping in terms of reproducibility, discriminatory and definitive power. However, discrepancies were seen and caused by several factors such as cross-reactions of O groups phenotypically, suspected transcription errors or typing mistakes, isolates had to be conventionally retested, poor sequence data and assemblies (Joensen et al., 2015). Therefore, in our study, the negative isolates performed conventionally and predicted to be positive by WGS, requires further conventional serotyping of specific individual O-antisera. The discrepancies with the positive isolates performed by conventional methods against the predicted serotype would rule out cross-reaction of the O groups.

The phylotypes of colistin-resistant *E. coli* isolates in our study were mostly commensals and few belonged to Phylogroup B2. However, discrepancies were observed using triplex PCR phylogroup and *in silico* ClermonTyping methods. The differences in the result could be due to not performing the updated PCR Phylogroup version which include additional Phylogroups C, E and F (Clermont et al., 2013). In contrast, given the high accuracy with *in silico* ClermonTyping, discrepancies have been reported resulting from SNPs in the primers, horizontal gene transfer, strain contamination and insertion elements (IS*1*) (Beghain et al., 2018).

There is one *E. coli mcr-1* isolated from humans (EC 84) in our study predicted by WGS to carry the *vanC1XY* resistance gene cluster, which is an intrinsic gene found on the chromosome of *Enterococcus gallinarum* (Reynolds et al., 1999). Although there have not been any studies that show such genes can be acquired by an *E. coli* isolate, hence this could be contamination (mixed culture) when sending this isolate for WGS.

Additionally, the *fosA-like* gene was found more abundant in colistin-resistant *E. coli* isolated from chickens than in humans. This is shown with independent samples *t*-test (performed on Microsoft Excel for Mac, Version 16.20) associated with a statistically significant effect, t (50) = 4.12, p = 0.00007. Thus, the acquired *fosA-like* genes in *E. coli mcr-1-like* isolates were associated with a statistically

significant larger mean value in chickens (mean, M= 0.81, standard deviation, SD= 0.40) than in humans (M= 0.31, SD= 0.47).

Hence, from all these findings, they may be a possible movement of resistance from animals to human which represents a public health concern. Given the high rate of MCR-1 in poultry, it could serve as a reservoir for the spread of plasmids harboring resistance and virulence genes through the food chain which warrants further investigation.

6.4.3. Novel MCR-1.8 variant recovered in *E. coli* isolate from chicken caecal samples

Polymyxins are among the earliest antimicrobial agents described, yet they are increasingly being relied upon as therapies of last resort in the treatment of Gram-negative infections (Olaitan et al., 2014). Polymyxin resistance is well known to be intrinsic to some bacterial species, however, recently polymyxin resistance has been shown to spread via plasmid-mediated mechanisms and has subsequently spread across the globe (Di Pilato et al., 2016; Liu et al., 2016; Xavier et al., 2016; Borowiak et al., 2017; Carattoli et al., 2017; Hernandez et al., 2017; Ling et al., 2017; Litrup et al., 2017; Roer et al., 2017; Yin et al., 2017b; Fukuda et al., 2018; Wang et al., 2018a). The most widespread plasmid-mediated resistance determinants are PEtN enzymes (MCR-like) that modify native LPS and reduce the binding affinity of polymyxins and other antimicrobial peptides (Olaitan et al., 2014). We identified a novel gene variant *mcr-1.8* in a plasmid carried by an avian *E. coli* strain. This variant was located on a unique IncI2 like plasmid in a MDR *E. coli* sequence type (ST) 101 isolate.

E. coli C22 isolate was obtained as part of a surveillance study (2017), pre-slaughter from healthy birds. Screening of this isolate by PCR revealed a variant with a single non-synonymous nucleotide difference (A8G) identified as *mcr-1.8.* Colistin and polymyxin B MICs were 8 mg/L and 12 mg/L, respectively. When *mcr-1.8* was cloned and expressed in *E. coli* TOP10 under the native promoter, colistin and polymyxin B MICs were increased by 4- to 16- fold, clearly implicating the functionality of this enzyme in *E. coli*.

MCR 1.8 was encoded by pEC-MCR1.8, a 63,056 bp with an IncI2 replicon. Apart from IncI2, *mcr-1* has been observed in IncHI2 and IncX4 (Matamoros et al., 2017). Plasmid pEC-MCR1.8 also encodes a typical conjugative (*tra*) and pilus (*pil*) apparatus for its mobilisation and transfer, as well as both *hicA/B* and *stbD/E* toxin /antitoxin modules involved in maintenance and plasmid stability. Both pEC-MCR1.8 and polymyxin resistance was readily transferable to susceptible strains of *E. coli* J53 with a high frequency for *in vitro* transfer (10^{-4} t/d). Similarly, reports of the *mcr-1.2* gene carried by IncX4 plasmids are highly transferable by conjugation to *E. coli* J53 with a frequency of 5 x10^{5} (t/r) (Di Pilato et al., 2016). This suggests pEC-MCR1.8 could readily be transferred to susceptible strains in nature and has the potential to spread into key human pathogens.

Levels of polymyxin resistance mediated by PEtN are moderate (MIC 2 – 4 mg/L) when compared with those seen in isolates with chromosomal (*phoP/*Q, *pmrA/B*) mechanisms of resistance (Liu et al., 2016; Olaitan and Li, 2016; Jeannot et al., 2017). A recent study by Yang et al. highlighted amino acid substitutions in PmrA (R81S) (Quesada et al., 2015) and PmrB (V161M) (Sun et al., 2009) were responsible for reducing susceptibility to colistin in *E. coli* (Yang et al., 2017). In C22, we found mutations in *phoP/Q* and *pmrA/B* that are predicted to encode the amino acid changes I44L (*phoP)*, I165F (*phoQ),* S29G (*pmrA)*, D282G and Y358N *(pmrB)*. These substitutions have not previously been linked with reduced susceptibility to polymyxins in *E. coli,* but D282G and Y358N are predicted to occur in the ATP binding domain of *pmrB* in *Salmonella* spp (Sun et al., 2009) and could contribute to polymyxin resistance.

Immediately downstream of *mcr-1.8* was the *pap2* gene predicted to encode a membrane-associated phosphatase enzyme, an enzyme that catalyses the removal of terminal phosphate groups from lipid carriers essential in the transport of hydrophilic small molecules across the outer membrane (Fan et al., 2014). The role of PAP2-like phosphatases in polymyxin resistance is unclear, although a study by Zurfluh and colleagues suggests that plasmid encoded PAP2 does not influence susceptibility to polymyxins (Zurfluh et al., 2016).

In silico analysis of the resistome of C22 revealed co-carriage of many other plasmid-mediated resistance genes. Additionally, fosfomycin- (*fosA4*) and sulfonamide-resistance (*sul3*) that were localised to a HI mulitreplicon (HIA, HIB and FIA) plasmid could not be transferred to *E. coli* J53 by conjugation (data not shown) and we were unable to transfer co-resistance to any other antimicrobial following selection on sodium azide/colistin supplemented media. Highlighting that the *mcr-1.8* gene was localized solely to pEC-MCR 1.8.

E. coli C22 was assigned to ST101. This sequence type, associated with polymyxin resistance has been reported across Korea (Yoo et al., 2013), China (Yang et al., 2017) but also in Brazil (Fernandes et al., 2016). In one report originating from Brazil (Fernandes et al., 2016), *E. coli* ST101 carrying *mcr-1* on a highly transmissible IncX4 plasmid was isolated from a patient with a 2-month history of a right calcaneal ulcer. Polymyxin resistant *E. coli* ST101 isolates with *mcr-1* were also recovered from retail meats on sale in a traditional market in Taiwan (Kuo et al., 2016). Of more concern, are reports that highlight the potential for *E. coli*

ST101 to act as a strain able to carry additional resistances including to carbapenems (NDM-1) (Yoo et al., 2013). This suggests that ST101 could represent a hi-risk clone able to promote global dissemination of polymyxin and multi-drug resistance in *E. coli.*

It is unclear whether modification of PEtN will attenuate or enhance virulence as well as generating resistance. John et al. found phosphoethanolamine and sialic acid substitutions to be more common amongst invasive strains of *Neisseria meningitidis* suggesting that PEtN lipid A modification may promote virulence (John et al., 2016). Colistin resistance due to an *mgrB* mutation was also shown to enhance the virulence of *Klebsiella pneumoniae* by decreasing the affinity for colistin and attenuating the host immune response (Kidd et al., 2017). In contrast, a study done by Yang et al. in which *mcr-1* producing plasmids were transferred into susceptible strains of *E. coli* of different STs, virulence in animal models was markedly depleted (Yang et al., 2017). MCR-1 was shown to provide a significant fitness cost on *E. coli* both by impairing cell growth and alteration of the cellular architecture despite conferring resistance to polymyxins (Yang et al., 2017).

Plasmid-encoded PEtn enzymes, able to mediate clinically relevant levels of polymyxin resistance in Enterobacteriaceae are an alarming development in the evolution of the problem of antimicrobial resistance (AMR). Although only recently identified as a novel mechanism of antimicrobial resistance (Liu et al., 2016), 15 functional variants of the MCR-1 enzyme have now been described, including the *mcr-1.8* allele encoded by the pEC-MCR1.8 plasmid that was

characterised here. This highlights the need for continual and enhanced surveillance for both the plasmids, host strains and bacterial species able to support the success and dissemination of this resistance determinant. Given existing knowledge of the global epidemiology of MCR-producing strains this may be particularly important for countries in Southeast Asia.

6.5 Comparative virulence of avian and human origin in *Galleria mellonella* model

The *G. mellonella* infection model has been used to study virulence and treatment combinations over a range of a clinically relevant bacterial and fungal pathogen (Junqueira, 2012; Hornsey et al., 2013a; Wei et al., 2017; Betts et al., 2017).

Heterogeneity in all forty colistin-resistant *E. coli* strains (resistance gene determinant, sequence type (ST), serotype, phylotype, virulence factor and plasmid replicon types) isolated from chickens and humans have shown differences in *G. mellonella* killing. Of note, acquiring antibiotic resistance genes by mutation or horizontal gene transfer may impact on the fitness cost (Andersson and Levin, 1999; Andersson and Hughes, 2010; Vogwill and MacLean, 2015). Additionally, Yang and colleagues have shown *mcr-1* can impose a fitness cost on its *E. coli* hosts whereby increased expression of *mcr-1* not only impaired cellular growth but diminished bacterial fitness and results in the critical changes in cellular architecture lethal to the bacterium (Yang et al., 2017).

In our findings, differences in *G. mellonella* killing was observed with colistin-resistant *E. coli* isolated from chickens and humans. Important to note exotoxins, endotoxins, adherence factors, secretion systems, iron acquisition and fimbria are commonly considered to be important virulence features in Gram-negative bacteria (Cosentino et al., 2013). In this study, *cvaB* (colicin V secretion/processing ATP-binding protein) was associated with the most virulent strain and the least

virulent were *gadA* (glutamate decarboxylase alpha) according to the calculated Hazard Ratio. CvaB, is part of an ABC transporter system, which transport colicin V (ColV) an antibacterial peptide toxin active against many *E. coli* strains and other members of the Enterobacteriaceae family (Hwang et al., 1997). While *gadA* is part of glutamate decarboxylase structural genes, which function as an important mechanism of acid resistance (Smith et al., 1992; Feehily and Karatzas, 2013).

Other VF that demonstrates virulence and high mortality includes *vat*, *hylE*, *AfaB*, *senB*, *tsh*, *eptD*, *cba*, *pic*, *iatB*, *iss* and *cma*. However, carriage of *lpfA*, *gadB*, *gad*, *invasin*, *air*, *celB* and *gad* (associated with low virulence and low mortality) also shown to be protective according to calculated Hazard Ratio.

In *G. mellonella* infection study done by Alghoribi et al. demonstrated clones of ST69 and ST127 of uropathogenic *E. coli* (UPEC) showed greater killing than the more recognised clones including ST73, ST95 and ST131 (Alghoribi et al., 2014). However, when we investigated our *E. coli* strains (transconjugant versus ECJ53 recipient) the varied survival was not clonal in nature. *G. mellonella* killing with *E. coli* C22/EC J53 transconjugant showed enhanced virulence compared to *E. coli* B28/EC J53, *E. coli* A53/EC J53 and the ECJ53 background. All transconjugants had similar sequence type, ST10 to the background ECJ53 recipient.

Wu and colleagues have looked at the fitness advantage of bearing IncI2 plasmid *in vitro* and reported that acquiring IncI2-type plasmid was more beneficial for

host *E. coli* DH5α than either IncHI2 or IncX4 plasmid (Wu et al., 2018). In contrast, the presence of IncHI2, IncFII and IncY plasmids showed a fitness cost (Wu et al., 2018). In our findings, the *E. coli* B28/EC J53 transconjugant, have acquired additional plasmids, IncFIB(AP001918) and IncFII(pCoo) during the conjugation process which may lead to the fitness cost and less *G. mellonella* killing compared to *E. coli* C22/EC J53. However, the growth kinetics in our study between transconjugants and EC J53 recipients did not show any obvious differences; hence, acquisition of a *mcr-1-like* bearing plasmid may not affect a fitness cost.

The *E. coli* A53/EC J53 transconjugant did show less *G. mellonella* killing compared to *E. coli* C22/EC J53 despite the number of virulence genes acquired, and plasmid replicon type was similar for both. Hence, the only difference observed was in the *mcr* gene determinant, *mcr-1.1* in *E. coli* A53/EC J53 and *mcr-1.8* in *E. coli* C22/EC J53. Furthermore, three other *E. coli mcr-1.8* wildtypes (*E. coli* H124, *E. coli* H112 and *E. coli* H109) were identified from humans that ranked higher in the *Galleria* virulence lists seen in Table 20. Therefore, one hypothesis for the enhanced virulence could be due to the acquisition of the *mcr-1.8* gene. This could be worthy of further investigation.

Interestingly, on analysis of the predicted serotypes, we noted frequent absence of the O antigen which may affect the architecture of the outer cell membrane. Strains with the higher colistin MICs were also noted to be lower down the

virulence rank according to the calculated Hazard Ratios (Table 28). Another hypothesis for the lower rank in virulence with high the colistin MICs is associated with the loss of O antigen resulting in reduced virulence. However, further investigation is still warranted.

Colistin-resistant *E. coli* strains	Origin	Ranking (Hazard Ratio)	Serotyping	Colistin MIC (mg/L)
B52	Avian	1	Novel	4
67	Human	2	:H31	4
106	Human	8	O35:	>32
84	Human	16	:H29	2
B7	Avian	18	:H25	2
B28	Avian	20	:H7	>32
C19	Avian	25	:H32	2
64	Human	27	:H31	4
86	Human	32	:H38	4
A12	Avian	39	:H10	>16
63	Human	-	:H16	>32
A25	Avian	-	:H10	2
B42	Avian	-	:H11	2
B12	Avian	-	:H25	2
121	Human	-	O8:	2

Table 28. Colistin-resistant *E. coli* strains in relations to their serotypes, colistin MICs and virulence ranking.

In conclusion, we have demonstrated varied *G. mellonella* killing by colistin-resistant *E. coli* was not attributable to any specific clone. Acquisition of polymyxin resistance gene did not uniformly affect fitness or virulence with the possible exception of the transfer of plasmids encoding MCR-1.8. Strains studied were obtained from healthy poultry and from routine microbiology sampling of human clinical faecal samples. Although, the diagnosis of the patient was not obtained.

Further studies should focus on diseased animals or humans with severe *E. coli* infections.

6.6 Conclusion

The work in the thesis demonstrates the high rate of resistance to carbapenems and polymyxins in critical Gram-negative bacteria in Brunei Darussalam. Carbapenem-resistant *A. baummanni*, OXA-232-producing *K. pneumoniae* belonging ST231 clone causes a hospital outbreak in Brunei's hospital. Plasmid-mediated polymyxin resistance in *E. coli* was found to be endemic in poultry (58%) and highly prevalent (41.7%) in the human samples studied.

Unlike carbapenem resistance, polymyxin resistance was not associated with any predominant clone and did not readily correlate with virulence properties of the strain. The enhanced virulence seen in one of the transconjugant isolates may be due to the acquisition of the *mcr-1.8* gene. Further, extensive research in this area are needed to elucidate the underlying mechanism. However, given the high background rates, enhanced surveillance for MCR-1 within more virulent Enterobacterial backgrounds is warranted, particularly in Southeast Asian countries.

6.7 Future developments

- To introduce AMR surveillance in Brunei Darussalam using existing diagnostic tools developed in the study such as CHROMagar COL-*APSE* culture media, lateral flow assay and molecular techniques (PCR and WGS).
- To develop Brunei's National Action Plan (NAP) on AMR surveillance focusing on pathogens listed in the WHO Global Antimicrobial Surveillance System (GLASS) (*A. baumannii*, *E. coli*, *K. pneumoniae and Salmonella* spp among the Gram-negatives) and WHO critical pathogen lists. The NAP must include stakeholders from the Ministry of Health, Ministry of Primary Resources and Tourism (Department of Agriculture and Agrifood) and Ministry of Development (Department of Environment, Parks and Recreation) working together to eventually come up with a policy that can be implemented in other districts in Brunei.
- To carry out AMR surveillance in patients with sepsis from blood cultures for the presence of plasmid-mediated polymyxin resistance. And also, to include AMR surveillance of CRE (OXA-232) and plasmid-mediated polymyxin resistance from other infected sites in patients admitted to the Intensive Care Unit (ICU) in Brunei's hospital.

Chapter 7

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Appendices

Appendix A (List of primers)

Primer		Primer sequence (5'-3')	Expected size (bp)	Reference
		Chapter 2		
RAPD typing				
ERIC2	N/A	AAGTAAGTGGACTGGGGTGAGCG	Variable	(Renders et al., 1996)
OXA carbapenemases				
OXA-51-like	F	TAATGCTTTGATCGGCCTTG	353	(Woodford et al., 2006)
	R	TGGATTGCACTTCATCTTGG		
OXA-23-like	F	GATCGGATTGGAGAACCAGA	501	
	R	ATTTCTGACCGCATTTCCAT		
OXA-24-like	F	GGTTAGTTGGCCCCCTTAAA	246	
	R	AGTTGAGCGAAAAGGGGATT		
OXA-58-like	F	AAGTATTGGGGCTTGTGCTG	599	
	R	CCC CTC TGCGCTCTACATAC		
Aminoglycoside methyltransferases				
rmtA	F	CTAGCGTCCATCCTTTCCTC	633	(Fritsche et al., 2008)
	R	TTTGCTTCCATGCCCTTGCC		
rmtB	F	TCAACGATGCCCTCACCTC	459	
	R	GCAGGGCAAAGGTAAAATCC		
rmtC	F	GCCAAAGTACTCACAAGTGG	752	
	R	CTCAGATCTGACCCAACAAG		
rmtD	F	CTGTTTGAAGCCAGCGGAACGC	376	
		GCGCCTCCATCCATTCGGAATAG		
armA	F	TATGGGGGTCTTACTATTCTGCCTAT	514	
	R	TCTTCCATTCCCTTCTCCTTT		
npmA	F	CTCAAAGGAACAAAGACGG	641	
	R	GAAACATGGCCAGAAACTC		
AmpC				
ACI5	F	ACTTACTTCAACTCGCGACG	653	(Corvec et al., 2003)
ACI6	R	TAAACACCACATATGTTCCG		
Integrons				
Int1	F	CAGTGGACATAAGCCTGTTC	160	(Dillon et al., 2005)
	R	CCCGAGGCATAGACTGTA		
Int2	F	GTAGCAAACGAGTGACGAAATG	788	
	R	CACGGATATGCGACAAAAAGGT		
Int3	F	GCCTCCGGCAGCGACTTTCAG	979	
	R	ACGGATCTGCCAAACCTGACT		
Class 1 integron gene cassette				

5’ CS	F	GGCATCCAAGCAGCAAG	Variable	(Lévesque et al., 1995)
3’ CS	R	AAGCAGACTTGACCTGA		
Insertion element, IS_{Aba1}				
ISAba1	F	CATTGGCATTAAACTGAGGAGAAA	451	(Ruiz et al., 2007)
ISAba2	R	TTGGAAATGGGGAAAACGAA		
		Chapter 3		
Virulence factor				
ybtS_for	F	GACGGAAACAGCACGGTAAA	242	(Compain et al., 2014)
ybtS_rev	R	GAGCATAATAAGGCGAAAGA		
mrkD_for	F	AAGCTATCGCTGTACTTCCGGCA	340	
mrkD_rev	R	GGCGTTGGCGCTCAGATAGG		
entB_for	F	GTCAACTGGGCCTTTGAGCCGTC	400	
entB_rev	R	TATGGGCGTAAACGCCGGTGAT		
rmpA_for	F	CATAAGAGTATTGGTTGACAG	461	
rmpA_rev	R	CTTGCATGAGCCATCTTTCA		
kfu_for	F	GGCCTTTGTCCAGAGCTACG	638	
kfu_rev	R	GGGTCTGGCGCAGAGTATGC		
allS_for	F	CATTACGCACCTTTGTCAGC	764	
allS_rev	R	GAATGTGTCGGCGATCAGCTT		
iutA_for	F	GGGAAAGGCTTCTCTGCCAT	920	
iutA_rev	R	TTATTCGCCACCACGCTCTT		
magA_for	F	GGTGCTCTTTACATCATTGC	1283	
magA_rev	R	GCAATGGCCATTTGCGTTAG		
K2_for	F	CAACCATGGTGGTCGATTAG	531	
K2_rev	R	TGGTAGCCATATCCCTTTGG		
Beta- lactamases				
Bla1				
TEM-like	F	CATTTCCGTGTCGCCCTTATTC	800	
	R	CGTTCATCCATAGTTGCCTGAC		
SHV-like	F	AGCCGCTTGAGCAAATTAAAC	713	
	R	ATCCCGCAGATAAATCACCAC		
OXA-1/4/30	F	GGCACCAGATTCAACTTTCAAG	564	
	R	GACCCCAAGTTTCCTGTAAGTG		
Bla2				
CTX-M-1 Gp	F	TTAGGAARTGTGCCGCTGYA	688	(Dallenne et al., 2010)
	R	CGATATCGTTGGTGGTRCCAT		
CTX-M-2 Gp	F	CGTTAACGGCACGATGAC	404	
	R	CGATATCGTTGGTGGTRCCAT		
CTX-M-9 Gp	F	TCAAGCCTGCCGATCTGGT	561	
	R	TGATTCTCGCCGCTGAAG		
Bla3				

ACC	F	CACCTCCAGCGACTTGTTAC	346	
	R	GTTAGCCAGCATCACGATCC		
FOX	F	CTACAGTGCGGGTGGTTT	162	
	R	CTATTTGCGGCCAGGTGA		
MOX/CMY	F	GCAACAACGACAATCCATCCT	895	
	R	GGGATAGGCGTAACTCTCCCAA		
DHA	F	TGATGGCACAGCAGGATATTC	997	
	R	GCTTTGACTCTTTCGGTATTCG		
CIT	F	CGAAGAGGCAATGACCAGAC	538	
	R	ACGGACAGGGTTAGGATAGY		
ACT/MIR	F	CGGTAAAGCCGATGTTGCG	683	
	R	AGCCTAACCCCTGATACA		
Bla4				
GES-like	F	AGTCGGCTAGACCGGAAAG	399	
	R	TTTGTCCGTGCTCAGGAT		
PER-like	F	GCTCCGATAATGAAAGCGT	520	
	R	TTCGGCTTGACTCGGCTGA		
VEB-like	F	CATTTCCCGATGCAAAGCGT	648	
	R	CGAAGTTTCTTTGGACTCTG		
Bla5				
GES-like	F	AGTCGGCTAGACCGGAAAG	399	
	R	TTTGTCCGTGCTCAGGAT		
OXA-48-like	F	GCTTGATCGCCCTCGATT	281	
	R	GATTTGCTCCGTGGCCGAAA		
Bla6				
IMP-like	F	TTGACACTCCATTTACDG	139	
	R	GATYGAGAATTAAGCCACYCT		
VIM-like	F	GAT GGT GTT TGG TCG CAT A	390	
	R	CGA ATG CGC AGC ACC AG		
KPC-like	F	CATTCAAGGGCTTTCTTGCTGC	538	
	R	ACGACGGCATAGTCATTTGC		
Carbapenemases				
BIC-like	F	TATGCAGCTCCTTTAAGGGC	537	
	R	TCATTGGCGGTGCCGTACAC		(Poirel et
NDM-like	F	GGTTTGGCGATCTGGTTTTC	621	al., 2011b)
	R	CGGAATGGCTCATCACGATC		
Quinolone				
qnrA	F	AGAGGATTTCTCACGCCAGG	580	
	R	TGCCAGGCACAGATCTTGAC		
qnrB	F	GGMATHGAAATTCGCCACTG	264	(Cattoir et al., 2007)
	R	TTTGCYGYYCGCCAGTCGAA		
qnrS	F	GCAAGTTCATTGAACAGGGT	428	
	R	TCTAAACCGTCGAGTTCGGCG		
Aminoglycoside Duplex				(Noppe-Leclercq
aph(3')-VI	F	CGGAAACAGCGTTTTAGA	716	

	R	TTCCTTTTGTCAGGTC		et al.,
ant(2'')-Ia	F	T		1999)
	R	CGAGCCTGTAGGACT	404	
Duplex				
aac(6')-Ib	F	TATGAGTGGCTAAATCGAT	395	
	R	CCCGCTTTCTCGTAGCA		
aph(3')-Ia	F	CGAGCATCAAATGAAACTGC	623	
	R	GCGTTGCCAATGATGTTACAG		
Triplex				
aac(6')-Ih	F	TGCCGATATCTGAATC	407	
	R	ACACCACACGTTCAG		
aac(3)-Ia	F	GACATAAGCCTGTTCGGTT	372	
	R	CTCCGAACTCACGACCGA		
aac(3)-IIa	F	ATGCATACGCGGAAGGC	822	
	R	TGCTGGCACGATCGGAG		
Plasmid replicon				
IncX4				
IncX4_F	F	AGCAAACAGGGAAAGGAGAAGACT	569	(Johnson
	R	TACCCCAAATCGTAACCTG		et al., 2012)
ColE-like				
RepCol181-For	F	TACGGATTGCGGATGTTGCC	600	(Potron et
RepCol181-Rev	R	GTGCTGGTGCGTCCATTTGG		al., 2013)
OXA-232 primer walking				
OXA-232invL		CCGATAATCGATGCCACCAA	Variable	
pKP41invL1		GGAGTTAGCATGTCTGATTC	Variable	
pKP41invL2		GATCCACGTTTTGAGCAACA	Variable	
pKP41invL3		CCAACAAGTAGCCAACAGG	Variable	
OXA-232invR		GCCATCACAAAAGAAGTGCTC	Variable	This study
pKP41invR1		AGGGTGAAAGGTTACATGCA	Variable	
pKP41invR2		AACTGTACCCTGTTCGAGAA	Variable	
pKP41invR3		CTTCTTGGCTCTGGAGAATG	Variable	
		Chapter 4		
Plasmid-mediated Polymyxin				
mcr-1-like	F	AGTCCGTTTGTTCTTGTGGC	320	
	R	AGATCCTTGGTCTCGGCTTG		(Cavaco et
mcr-2-like	F	CAAGTGTGTTGGTCGCAGTT	715	al., 2016)
	R	TCTAGCCCGACAAGCATACC		
Phyloytyping				
ChuA.1	F	GACGAACCAACGGTCAGGAT	279	
ChuA.2	R	TGCCGCCAGTACCAAAGACA		(Clermont
YjaA.1	F	TGAAGTGTCAGGAGACGCTG	211	et al.,
YjaA.2	R	ATGGAGAATGCGTTCCTCAAC		2000)
TspE4C2.1	F	GAGTAATGTCGGGGCATTCA	152	

TspE4C2.2	R	CGCGCCAACAAAGTATTACG

Appendix B (Antibiotic Susceptibility Testing table of colistin-resistant *E. coli* isolated from chickens and humans)

E. coli mcr-1-like isolates	Amikacin (30 µg)	Augmentin (30 µg)	Colistin (25 µg)	Cefpodoxime (10 µg)	Cephalothin (30 µg)	Ciprofloxacin (5 µg)	Cefepime (30 µg)	Minocycline (30 µg)
A8	23	6	13	6	6	12	51	25
A12	16	6	11	6	6	9	23	16
A13	23	6	12	11	6	14	34	11
A25	17	6	11	21	6	11	26	13
A28	19	6	10	6	6	12	20	6
A31	17	6	11	6	6	6	13	6
A33	19	16	6	24	6	9	31	6
A41	18	6	11	6	6	12	32	14
A53	20	6	6	6	6	11	30	8
B1	23	6	11	6	6	6	51	24
B5	22	6	13	13	14	26	37	13
B7	23	6	13	6	6	6	37	14
B11	16	11	10	6	6	12	25	13
B12	15	6	11	22	6	18	25	12
B23	16	6	11	12	6	6	28	10
B25	14	6	11	18	6	20	23	6
B28	24	6	11	6	6	6	35	11
B42	23	6	12	6	6	39	55	22
B47	23	9	12	6	6	6	28	15
B52	17	8	11	6	6	15	29	10
C1	24	6	12	36	6	27	35	18
C19	17	6	11	22	6	19	25	8

C22	19	6	12	6	6	11	17	11
C28	18	6	11	13	6	6	29	6
C45	24	19	12	6	6	35	22	11
C55	20	14	11	24	6	20	30	13

Antibiotic susceptibility testing of colistin-resistant *E. coli* isolated from chickens. Green colour- sensitive, Yellow colour- intermediate, Red colour- resistance. Bacteria that grows towards disc are resistance and measured as the size of the disc (6 mm). Columns that are not shaded - zone diameter (mm) breakpoints are not available.

E. coli mcr-1-like isolates	Kanamycin (30 µg)	Ertapenem (10 µg)	Imipenem (10 µg)	Meropenem (10 µg)	Chloremphenicol (30 µg)	Apramycin (15 µg)	Florfenicol (30 µg)	Pefloxacin (5 µg)
A8	6	25	24	45	6	14	6	6
A12	6	19	23	28	6	6	6	6
A13	6	20	34	20	6	19	8	6
A25	6	24	25	27	11	14	18	6
A28	6	23	23	28	6	6	6	6
A31	6	20	22	26	6	11	6	6
A33	6	27	28	33	6	14	6	6
A41	6	23	25	29	6	14	6	6
A53	6	23	27	28	6	6	6	6
B1	6	30	26	42	6	6	6	6
B5	6	6	34	18	6	6	12	6
B7	6	19	33	19	6	6	10	6
B11	6	23	22	27	6	6	6	14
B12	15	24	24	28	6	14	6	15
B23	6	21	25	28	6	6	6	6
B25	18	25	21	22	6	6	6	21
B28	6	18	29	19	6	6	6	6
B42	14	33	25	46	6	6	6	37
B47	6	21	25	29	6	6	6	6
B52	6	23	25	29	6	6	6	10
C1	6	20	34	20	6	6	6	6
C19	6	25	25	28	6	13	6	14

C22	6	22	25	25	6	6	6	6
C28	6	28	25	29	6	6	6	6
C45	20	24	33	35	6	6	6	19
C55	6	29	29	32	6	6	6	13

Antibiotic susceptibility testing of colistin-resistant *E. coli* isolated from chickens. Green colour- sensitive, Yellow colour- intermediate, Red colour- resistance. Bacteria that grows towards disc are resistance and measured as the size of the disc (6 mm). Columns that are not shaded - zone diameter (mm) breakpoints are not available.

E. coli mcr-1-like isolates	Enroflaxacin (5 µg)	Ceftazidime 30 µg/AVI 20 µg	Ceftolozane 30 µg/Tazobactam 10 µg	Cefoxitin (30 µg)	Ceftiofur (30 µg)
A8	6	31	27	9	18
A12	6	25	18	6	14
A13	6	29	26	23	25
A25	6	24	28	21	22
A28	6	22	16	6	14
A31	6	25	22	7	15
A33	10	29	28	23	27
A41	8	28	22	6	20
A53	6	30	23	6	21
B1	6	29	25	6	22
B5	6	32	30	25	27
B7	6	30	26	6	22
B11	16	25	21	11	20
B12	17	26	22	20	22
B23	6	26	23	6	22
B25	22	25	21	21	18
B28	6	29	24	6	22
B42	34	28	25	6	21
B47	6	27	21	10	15
B52	11	27	24	6	22
C1	6	30	26	22	26
C19	16	27	24	19	22

C22	8	27	22	25	6
C28	6	30	25	18	22
C45	22	37	28	32	6
C55	16	28	27	21	24

Antibiotic susceptibility testing of colistin-resistant *E. coli* isolated from chickens. Green colour- sensitive, Yellow colour- intermediate, Red colour- resistance. Bacteria that grows towards disc are resistance and measured as the size of the disc (6 mm). Columns that are not shaded - zone diameter (mm) breakpoints are not available.

E. coli mcr-1-like isolates	Amikacin (30 µg)	Augmentin (30 µg)	Colistin (25 µg)	Cefpodoxime (10 µg)	Cephalothin (30 µg)	Ciprofloxacin (5 µg)	Cefepime (30 µg)	Minocycline (30 µg)
1	21	15	12	25	14	6	28	15
8	19	6	12	25	13	28	28	12
25	20	6	13	25	9	12	28	16
46	18	6	12	25	13	6	26	13
49	19	6	13	15	6	6	26	11
50	20	18	12	27	15	23	28	12
53	20	6	12	26	14	13	28	17
54	19	6	11	24	12	21	18	29
59	20	6	12	25	12	30	27	9
60	19	6	12	25	13	23	26	16
61	22	6	13	24	12	10	27	15
63	6	6	6	26	6	13	30	6
64	20	6	12	24	11	11	27	11
67	20	6	12	26	9	28	32	11
70	20	6	12	24	17	12	28	12
84	20	19	12	27	18	30	33	19
86	20	6	12	25	14	23	27	13
92	18	6	12	25	13	10	16	12
96	20	6	12	26	16	24	28	17
106	19	6	6	11	6	19	23	9

109	24	6	6	25	6	14	31	11
111	23	6	13	25	12	13	28	11
112	22	8	13	26	17	27	30	16
113	20	6	12	25	14	14	28	16
121	20	6	12	23	11	20	27	13
124	18	6	6	25	6	13	24	11

Antibiotic susceptibility testing of colistin-resistant *E. coli* isolated from humans. Green colour- sensitive, Yellow colour- intermediate, Red colour- resistance. Bacteria that grows towards disc are resistance and measured as the size of the disc (6 mm). Columns that are not shaded - zone diameter (mm) breakpoints are not available.

E. coli mcr-1-like isolates	Kanamycin (30 µg)	Ertapenem (10 µg)	Imipenem (10 µg)	Meropenem (10 µg)	Chloremphenicol (30 µg)	Apramycin (15 µg)	Florfenicol (30 µg)	Pefloxacin (5 µg)
1	20	21	30	23	6	15	6	6
8	6	20	27	21	6	6	6	16
25	19	20	31	23	6	6	6	6
46	6	26	27	21	6	14	6	6
49	6	23	25	20	6	15	6	6
50	22	22	31	23	15	15	6	17
53	6	25	28	21	6	14	6	6
54	6	21	28	29	6	13	6	19
59	18	19	30	22	9	6	6	29
60	6	24	27	22	6	14	6	14
61	6	26	30	24	6	6	6	6
63	6	24	26	19	6	6	6	6
64	19	26	26	22	6	6	6	6
67	18	24	29	21	24	15	22	26
70	19	21	30	25	6	14	6	6
84	20	24	31	32	25	15	24	27
86	19	24	26	22	6	13	6	17
92	6	26	29	20	6	13	6	6
96	20	22	29	23	10	16	6	16
106	6	20	16	17	9	6	10	11

109	6	19	26	20	6	6	6	6
111	6	27	30	23	6	6	6	6
112	21	21	30	25	25	15	22	17
113	6	27	29	22	6	14	6	6
121	6	27	28	23	6	15	6	11
124	6	21	22	16	6	6	6	6

Antibiotic susceptibility testing of colistin-resistant *E. coli* isolated from humans. Green colour- sensitive, Yellow colour- intermediate, Red colour- resistance. Bacteria that grows towards disc are resistance and measured as the size of the disc (6 mm). Columns that are not shaded - zone diameter (mm) breakpoints are not available.

E. coli mcr-1-like isolates	Enroflaxacin (5 µg)	Ceftazidime 30 µg/AVI 20 µg	Ceftolozane 30 µg/Tazobactam 10 µg	Cefoxitin (30 µg)	Ceftiofur (30 µg)
1	6	28	25	21	23
8	19	28	24	20	22
25	6	29	27	21	24
46	6	30	25	19	22
49	6	29	24	6	20
50	17	30	27	21	25
53	13	29	24	22	23
54	22	29	26	20	27
59	32	27	26	22	24
60	18	28	25	21	22
61	6	31	25	18	23
63	8	30	23	15	24
64	6	30	26	20	22
67	27	29	25	21	24
70	6	27	25	20	23
84	29	30	27	22	25
86	22	28	25	21	23
92	6	29	25	21	24
96	19	28	25	22	23
106	11	26	22	6	15

109	9	32	28	10	26
111	6	29	26	21	25
112	20	27	25	21	23
113	9	28	25	22	24
121	15	29	25	20	22
124	6	24	24	18	22

Antibiotic susceptibility testing of colistin-resistant *E. coli* isolated from humans. Green colour- sensitive, Yellow colour- intermediate, Red colour- resistance. Bacteria that grows towards disc are resistance and measured as the size of the disc (6 mm). Columns that are not shaded - zone diameter (mm) breakpoints are not available.

Antimicrobial Discs	Potency (μg)	Zone diameter breakpoint (mm)		Reference
		S≥	R<	
Amikacin	30	18	15	(EUCAST, 2018)
Augmentin (Amoxycillin-Clavulanic Acid)	30	19	19	(EUCAST, 2018)
Colistin	25	N/A	N/A	N/A
Cefpodoxime	10	21	21	(EUCAST, 2018)
Cephalothin	30	18	15	(CLSI, 2015)
Ciprofloxacin	5	26	24	(EUCAST, 2018)
Cefepime	30	27	24	(EUCAST, 2018)
Minocycline	30	16	13	(CLSI, 2018)
Kanamycin	30	18	14	(CLSI, 2018)
Ertapenem	10	25	22	(EUCAST, 2018)
Imipenem	10	22	16	(EUCAST, 2018)
Meropenem	10	22	16	(EUCAST, 2018)
Chloramphenicol	30	17	17	(EUCAST, 2018)
Apramycin	15	N/A	N/A	N/A
Florfenicol	30	N/A	N/A	N/A
Pefloxacin	5	24	24	(CLSI, 2018)
Enrofloxacin	5	23	17	(CLSIVET08, 2018)
Ceftiofur	30	21	18	(CLSIVET08, 2018)
Cefoxitin	30	18	15	(CLSI, 2018)
Ceftazidime 30 Avibactam 20	30/20	21	21	(CLSI, 2018)
Ceftolozane 30 Tazobactam 10	30/10	21	18	(CLSI, 2018)

Zone diameter breakpoints (mm) of antibiotics tested for 52 colistin-resistant *E. coli* isolates. N/A – Not Available.

Appendix C (Publications)

i) Evaluation of an Immunochromatographic Lateral Flow Assay (OXA- 48 *K*-SeT) for Rapid Detection of OXA-48-Like Carbapenemases in *Enterobacteriaceae*

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Evaluation of an Immunochromatographic Lateral Flow Assay (OXA-48 *K*-SeT) for Rapid Detection of OXA-48-Like Carbapenemases in *Enterobacteriaceae*

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We evaluated an immunochromatographic lateral flow assay to detect OXA-48-like carbapenemases (OXA-48 *K*-SeT) in *Enterobacteriaceae* ($n = 82$). One hundred percent sensitivity and specificity were observed using bacteria recovered from both solid medium and spiked blood culture bottles, and the results were obtained in <10 min.

The identification, treatment, and control of multidrug-resistant (MDR) bacterial infections are global health priorities. *Enterobacteriaceae* with plasmids carrying genes encoding class A (*Klebsiella pneumoniae* carbapenemases [KPC]), B (IMP, VIM, and NDM), and D (OXA) carbapenemases (carbapenemase-producing *Enterobacteriaceae* [CPE]) are one of the most important groups of pathogens due to the burden of disease, lack of any new treatments, and potential for dissemination (1). Rapid and effective diagnostics therefore underpin any strategy aimed at tackling the problem of CPE. Considerable effort has been made to develop novel assays using both genotypic and phenotypic approaches. These approaches include the genetic detection of resistance gene profiles (PCR, loop-mediated isothermal amplification [LAMP], microarrays, and genome sequencing) (2), selective culture medium (chromogenic or supplemented) (3), combination disk testing (4), and direct or indirect detection of carbapenem-hydrolyzing enzymes (matrix-assisted laser desorption ionization–time of flight mass spectrometry [MALDI-TOF MS], acidometric Carba NP, and Blue-Carba) (5–7). These methods require varied levels of technical skill, investment into equipment, and quality assurance optimization. Strains producing OXA-48-like carbapenem-hydrolyzing class D enzymes (CHDL) have proven to be particularly difficult to detect in clinical laboratories. This is due in part to relatively low MICs, conflicting interpretive rules associated with automated systems (8), and a lack of suitable inhibitor compounds for use in confirmatory tests. A novel means of detecting OXA-48-like enzymes using an antibody-mediated approach was recently developed (9). The OXA-48 *K*-SeT assay relies on the immunological capture of two epitopes specific to the OXA-48 enzyme using colloidal gold nanoparticles bound to a nitrocellulose membrane within a lateral flow device. Capture and detection antibodies were designed to bind all current CHDL OXA-48-like variants (OXA-48, -181, -204, -232, and -244).

In this study, we assessed the performance of the Coris OXA-48 *K*-SeT assay for detecting OXA-48-like-mediated carbapenem resistance in a large collection of carbapenem-resistant *Enterobacteriaceae* and also determined whether it could provide robust results when working directly with organisms recovered from blood culture bottles.

Eighty-two enterobacterial isolates were used in the evaluation. Seventy-eight were clinical isolates (*K. pneumoniae*, $n = 60$; *Escherichia coli*, $n = 11$; *Enterobacter cloacae*, $n = 6$; *Enterobacter aerogenes*, $n = 1$) with resistance to one or more carbapenems (ertapenem, imipenem, and/or meropenem) along with a susceptible type strain as a representative control for each bacterial species (*K. pneumoniae* NCTC 9633, *E. coli* NCTC 12241, *E. cloacae* 13380, and *E. aerogenes* NCTC 9375). Resistance or reduced susceptibility to carbapenems in clinical isolates was identified by disk diffusion and/or Etest (bioMérieux, Marcy L'Etoile, France) and confirmed by broth microtiter dilution (ertapenem MIC, ≥1 μg/ml), ac-

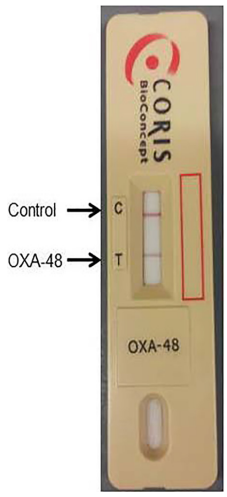

FIG 1 Detection of OXA-48-producing *K. pneumoniae* using OXA-48 *K*-SeT assay cassettes. The device was inoculated with 2×10^6 CFU/ml of OXA-48-producing KP41 and read after 5 min.

Received 29 October 2015 **Returned for modification** 15 November 2015
Accepted 18 November 2015
Accepted manuscript posted online 25 November 2015
Citation Wareham DW, Shah R, Betts JW, Phee LM, Momin MHFA. 2016. Evaluation of an immunochromatographic lateral flow assay (OXA-48 *K*-SeT) for rapid detection of OXA-48-like carbapenemases in *Enterobacteriaceae*. J Clin Microbiol 54:471–473. doi:10.1128/JCM.02900-15.
Editor: K. C. Carroll
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cording to Clinical and Laboratory Standards Institute (CLSI) methodology. Multiplex PCRs were used to detect the presence of genes encoding class A, B, C, and D β-lactamases (10). PCR detection and Sanger sequencing of entire bla_{OXA-48} alleles were undertaken and used as the standard for all isolates designated OXA-48-like-positive strains (3, 11). OXA-48 *K*-SeT assay kits (Coris BioConcept, Gembloux, Belgium) were obtained commercially through BioConnections (Knypersley, United Kingdom) at the catalogue price in August 2015 (£5/$7.6 per cassette).

Isolates were grown and maintained on Mueller-Hinton II cation-adjusted agar plates (Sigma-Aldrich, Gillingham, United Kingdom). The detection of CHDL-producing isolates from solid medium was undertaken, according to the manufacturer's instructions. Briefly, a single colony was resuspended in 10 drops of LY-A buffer (Tris-HCl, NaN_3 [pH 7.5]), and 3 drops of the homogenized solution was then applied to the sample well. The tests were read by eye within 15 min (Fig. 1). The lower limit of detection (in CFU per milliliter) of the OXA-48 *K*-SeT device was determined using *K. pneumoniae* NCTC 9633 and KP41 (bla_{OXA-48}) grown in Trypticase soy broth (TSB) (Sigma). Cells from 1 ml of overnight cultures were harvested by centrifugation, resuspended in phosphate-buffered saline (PBS), plated in serial dilutions (10^{-1} to 10^{-9}) onto MH agar, and viable counts (in CFU per milliliter) were recorded after 18 h of incubation. Ten drops of LY-A buffer were added to the cells pelleted from each dilution and used to inoculate OXA-48 *K*-SeT assay cassettes, as described above.

The ability of the OXA-48 *K*-SeT system to directly detect OXA-48-producing isolates recovered using commercial microbial blood culture medium was performed using the BacT/Alert system (bioMérieux, Durham, NC). Aerobic FA Plus 30-ml bottles were supplemented with 10 ml of heparinized horse blood (Oxoid, Basingstoke, United Kingdom) before inoculation with 10^2 CFU of each of the test organisms. The bottles were incubated at 37°C for up to 18 h before 0.1-ml aliquots were harvested, lysed, and analyzed directly without further dilution. Adequate growth of each isolate under simulated blood culture conditions was confirmed by subculture on MH II agar.

Molecular analysis of the isolates used in the analysis confirmed that 53 isolates produced an OXA-48-like carbapenemase, including 2 *K. pneumoniae* isolates with the OXA-181 and OXA-232 variants. Twenty-five isolates were carbapenem resistant due to the production of either KPC, VIM, or NDM ($n = 13$) β-lactamases or had hyper-*ampC* and/or permeability lesions. The OXA-48 *K*-SeT assay gave a positive result for all 53 OXA-48-producing strains and was negative for all of the other isolates tested, including 5 isolates that produced OXA-1. A correlation of the viable colony counts with the OXA-48 *K*-SeT assay results obtained with *K. pneumoniae* NCTC 9633 and KP41 assessed the lower limit of detection of the assay to be 2.41×10^6 CFU/ml. The results were identical whether the cassettes were inoculated with lysates prepared from MH II plates or from BacT/Alert blood culture bottles. In this evaluation, complete agreement between the molecular and OXA-48 *K*-SeT assay results was demonstrated (Table 1), and the sensitivity and specificity of the assay for detecting OXA-48-producing isolates were both calculated to be 100% (95% confidence interval, 91.9 to 100% and 84.2 to 100%, respectively). Positive and negative results were clearly differentiated within 10 min. Furthermore, the reads were not obscured by the presence of red cells when using lysates from blood culture sets.

TABLE 1 Detection of OXA-48 production in carbapenem-resistant *Enterobacteriaceae* using OXA-48 *K*-SeT cassettes

OXA-48 *K*-SeT result (*n*) by organism	Type (no.) of carbapenem-resistant *Enterobacteriaceae* ($n = 78$) by type		
	OXA-48-like carbapenemase	Other carbapenemase	No carbapenemase[a]
Positive (53)			
K. pneumoniae	46; OXA-181 (1), OXA-232 (1)	0	0
E. coli	5	0	0
E. cloacae	2	0	0
Negative (25)			
K. pneumoniae	0	8; KPC (2), NDM (3), VIM (2), KPC+NDM (1)	6
E. coli	0	2; KPC (1), NDM (1)	4
E. cloacae	0	3; NDM (2), VIM (1)	1
E. aerogenes	0	0	1

[a] Porin loss ± hyper-*ampC* production.

The OXA-48 *K*-SeT assay required minimal hands-on analytical time and no investment in any special equipment outside that available in a routine microbiology laboratory. The assay was highly sensitive and specific and was able to either rule in or rule out the presence of an OXA-48-producing strain within minutes. With a limit of detection of 10^6 CFU/ml, it might also be useful for the direct detection of OXA-48-producing strains in urinary samples or other biological fluids. Although we did not undertake a formal cost/benefit analysis, this assay has clear potential for development as a simple rapid local point-of-care test able to identify and guide the treatment and control of carbapenem-resistant infections due to OXA-48-producing *Enterobacteriaceae*.

ACKNOWLEDGMENTS

No specific funding was used to undertake this study, as it was performed as part of our routine activities.

We declare no conflicts of interest and have no association with Coris BioConcept.

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ii) Emergence and nosocomial spread of carbapenem-resistant OXA-232 -producing *Klebsiella pneumoniae* in Brunei Darussalam

Journal of Global Antimicrobial Resistance 9 (2017) 96–99

Contents lists available at ScienceDirect

Journal of Global Antimicrobial Resistance

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

Short Communication

Emergence and nosocomial spread of carbapenem-resistant OXA-232 -producing *Klebsiella pneumoniae* in Brunei Darussalam☆

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ARTICLE INFO

Article history:
Received 15 November 2016
Received in revised form 6 February 2017
Accepted 10 February 2017
Available online 27 April 2017

Keywords:
Enterobacteriaceae
Southeast Asia
Carbapenem resistance
OXA-232
ST231 *Klebsiella pneumonia*
High-risk clone

ABSTRACT

Objectives: Carbapenem-resistant Enterobacteriaceae (CRE) are identified as a major global health concern. The success of CRE is facilitated by the emergence, acquisition and spread of successful clones carrying plasmid-encoded resistance genes. In this study, an outbreak of carbapenem-resistant *Klebsiella pneumoniae* (CRKP) infections in patients hospitalised in Brunei Darussalam was investigated.
Methods: Over a 3-month period (May–July 2015), five multidrug-resistant *K. pneumoniae* were recovered from individual patients admitted to intensive care units at two hospitals (RIPAS and PMMPMHAB) in Brunei. Antimicrobial susceptibility was determined by broth microtitre dilution using a Micronaut-S β-lactamase VII kit or by Etest. Carbapenemase production was confirmed using the RAPID CARB Blue screen, and classes A–D β-lactamases were detected by multiplex PCR. Molecular typing was performed by random amplified polymorphic DNA (RAPD) PCR and multilocus sequence typing (MLST), with associated virulence and capsular types identified by PCR and sequencing. Plasmids were extracted, sized and characterised by PCR-based replicon typing.
Results: All isolates were resistant to cephalosporins, carbapenems, aminoglycosides, quinolones and sulfonamides but remained susceptible to polymyxins. Isolates were indistinguishable by RAPD-PCR and all belonged to sequence type (ST231). Resistance was due to the production of OXA-232 and CTX-M-15 β-lactamases, with the $bla_{OXA-232}$ carbapenemase gene located on a ColE-like plasmid.
Conclusions: This is the first report of plasmid-encoded OXA-232-producing CRKP in Brunei hospitals. All isolates were members of ST231, which may be representatives of a high-risk CRKP clone disseminating across Southeast Asia.

1. Introduction

Carbapenem-resistant Enterobacteriaceae (CRE) are identified as a major global health concern [1]. The most worrying are those with plasmid-encoded carbapenemases of the KPC, IMP, VIM, NDM and OXA-48-like families. CRE infections are extremely difficult to control in hospitals and in the community, hindered by the lack of effective treatments, spread between bacterial species and the dissemination of resistance through successful epidemic clones.

Carbapenemases are usually classified as belonging to either the serine (class A), metallo (class B) or oxacillinase (class D) family of enzymes. Amongst the carbapenem-hydrolysing class D β-lactamases (CHDLs), the OXA-48 variant is most prevalent and is found in numerous enterobacterial species that are also often multidrug-resistant (MDR) [2]. A number of variants of OXA-48, with different peptide sequence and carbapenem-hydrolysing properties, have been reported in recent years. These include OXA-162 in Turkey, OXA-163 in Argentina, OXA-181 in India and OXA-204 in Tunisia [2]. Recently, another variant (OXA-232) was found

☆ This work was presented in part at the 26th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), 9–12 April 2016, Amsterdam, The Netherland.

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http://dx.doi.org/10.1016/j.jgar.2017.02.008
2213-7165/

in France [2] and in a number of Southeast Asian countries [3,4]. The OXA-232 carbapenemase differs from OXA-48 by five amino acids substitutions [2]. Of note, OXA-232 has relatively weak carbapenem-hydrolysing activity evidenced by the lower minimum inhibitory concentrations (MICs) of imipenem and meropenem required to inhibit clinical isolates. This makes strains producing an OXA-232 carbapenemase difficult to detect in the microbiology laboratory [2].

Here we report on a MDR strain of *Klebsiella pneumoniae* responsible for a cluster of infections in patients hospitalised in Brunei Darussalam.

2. Materials and methods

2.1. Bacterial isolates and antimicrobial susceptibility testing

Over a 3-month period (May–July 2015), five MDR *K. pneumoniae* isolates were recovered from an equal number of individual patients admitted to intensive care units at two hospitals (RIPAS and PMMPMHAB) in Brunei. These isolates were obtained from blood culture ($n = 1$), endotracheal samples ($n = 1$), pus ($n = 2$) and sputum samples ($n = 1$). All CRE from clinical samples are prospectively stored by the laboratory in Brunei and these were the only five *K. pneumoniae* recovered during that period.

Antimicrobial susceptibility testing was performed using a VITEK® 2XL system (bioMérieux, Marcy-l'Étoile, France). The MICs of cefotaxime, cefepime, ceftazidime and meropenem were determined using a Micronaut-S β-lactamase VII panel (Merlin Diagnostika GmbH, Bornheim-Hersel, Germany), and those for colistin, polymyxin B and the combination ceftazidime/avibactam by Etest (bioMérieux). Resistance was defined according to Clinical and Laboratory Standards Institute (CLSI) breakpoints [5]. A modified Hodge test and RAPID CARB Blue test (Rosco Diagnostica A/S, Taastrup, Denmark) were performed for carbapenemase detection. Production of an OXA-48-like class D enzyme was then directly identified using an immunochromatographic lateral flow assay (Coris BioConcept, Gembloux, Belgium) [6].

2.2. Molecular characterisation

The genetic relatedness of each isolate was assessed using random amplified polymorphic DNA (RAPD) PCR as previously described [7]. Molecular typing was performed by multilocus sequence typing (MLST) of seven conserved *K. pneumoniae* housekeeping genes (*gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB* and *tonB*) [8].

2.3. Detection of virulence and antibiotic resistance genes

PCR was used for the detection of *K. pneumoniae* virulence (*kfu*, *fimH*, *uge*, *wabG* and *ureA*) determinants and the K1 hypermucoviscous (*cps*) phenotype [8]. Genes encoding classes A–D β-lactamases were identified with a series of multiplex PCRs [6,9]. The full coding sequence of all $bla_{\text{OXA-48-like}}$ genes was amplified using external primers [10] and was sequenced using the Sanger method. BLASTN analysis was performed on the assembled sequence.

2.4. Plasmid analysis

Plasmid replicon typing was performed using a commercial PCR-based replicon typing kit (Diatheva S.R.L., Cartoceto, Italy) with additional single PCRs for IncX4 and ColE-like plasmid replicons [2,11]. The plasmid location of each β-lactamase gene and the plasmid sizes were determined by pulsed-field gel electrophoresis (PFGE) of *S*1-digested DNA and Southern hybridisation with specific intragenic DNA probes.

2.5. Conjugation assays

Transfer of plasmid-mediated resistance genes by conjugation was investigated using rifampicin-resistant CSH26 and sodium azide-resistant J53 strains of *Escherichia coli* as recipients. Conjugation experiments were set up by mixing 0.5 mL of exponential-phase cultures in 4 mL of Luria–Bertani (LB) (Sigma-Aldrich, Gillingham, Dorset, UK) broth and then incubating overnight at 37 °C without shaking. Transconjugants were selected by growth on LB agar supplemented with rifampicin (128 mg/L) and either ampicillin (32 mg/L), cefotaxime (16 mg/L) or ertapenem (1 mg/L) for conjugation to *E. coli* CSH26 or with sodium azide (100 mg/L) and cefotaxime (0.0625–2 mg/L) using *E. coli* J53.

2.6. Sequencing of the $bla_{OXA-232}$-encoding plasmid

The sequence of the entire $bla_{\text{OXA-232}}$-encoding plasmid (pKP41-OXA-232) was determined using a primer walking approach. Inverse PCR, using long-range *Taq* polymerase (Invitrogen™ Platinum™ SuperFi™ DNA Polymerase; Thermo Fisher Scientific, Paisley, UK), in combination with the primers OXA-232invL (5′-CCGATAATCGATGCCACCAA-3′) and OXA-232invR (5′-GCCATCACAAAAGAAGTGCTC-3′) was used to amplify the entire sequence. Additional primers (Supplementary Table S1 in the online version at DOI: 10.1016/j.jgar.2017.02.008) were used to sequence the extremities flanking $bla_{\text{OXA-232}}$.

3. Results

Susceptibility testing confirmed resistance to cephalosporins, carbapenems, aminoglycosides, quinolones and sulfonamides in all isolates. The MICs of cefotaxime, cefepime and ceftazidime (>128 mg/L) and meropenem (>32 mg/L) confirmed resistance to all β-lactams. All isolates remained susceptible to colistin (MIC < 1 mg/L), polymyxin B (MIC < 1 mg/L) and the combination ceftazidime/avibactam (MIC = 1 mg/L). All isolates were positive for carbapenemase production, with an OXA-48-like class D enzyme detected in the lateral flow assay. Molecular typing revealed that the isolates were clonally related and were assigned to sequence type (ST) 231 (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html) following analysis of the MLST data.

Virulence determinants associated with iron uptake (*kfu*), fimbrial-mediated adhesion (*fimH*), lipopolysaccharide biosynthesis (*uge* and *wabG*) and urease production (*ureA*) were found in all isolates, but not the K1 hypermucoviscous (*cps*) phenotype [8] previously associated with heightened virulence.

Each isolate was positive for bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-M}}$ and $bla_{\text{OXA-48-like}}$ alleles. No other carbapenemase or extended-spectrum β-lactamase (ESBL) genes sought (bla_{NDM}, bla_{IMP}, bla_{VIM}, bla_{KPC}, bla_{GES} and bla_{VEB}) were found. BLASTN analysis of the assembled OXA-48-like sequence showed 100% nucleotide identity with the $bla_{\text{OXA-232}}$ allele (GenBank accession no. JX423831.1) that encodes a CHDL enzyme [2]. The class A β-lactamases in each isolate were similarly identified as $bla_{\text{TEM-1b}}$, $bla_{\text{SHV-11}}$ and $bla_{\text{CTX-M-15}}$.

Plasmid replicon typing revealed that the strains contained plasmids with A/C, FIA, FIIK and ColE-like replicons. The $bla_{\text{OXA-232}}$ gene was carried on a small ColE-like plasmid of ca. 6 kb (pKP41-OXA-232). The $bla_{\text{CTX-M-15}}$ ESBL gene was localised to an IncF plasmid of ca. 161.7 kb containing fused FIA-FIIk replicons, and the $bla_{\text{TEM-1b}}$ to a non-typeable plasmid of ca. 8.4 kb. Neither the $bla_{\text{OXA-232}}$- nor $bla_{\text{CTX-M15}}$-containing plasmids could be mobilised to either of the *E. coli* recipient strains under the antibiotic selection methods used.

Sequencing of the extremities flanking $bla_{\text{OXA-232}}$ confirmed that it was located on a 6141-bp plasmid identical to one previously characterised by Potron et al. [2]. The plasmid also carried several

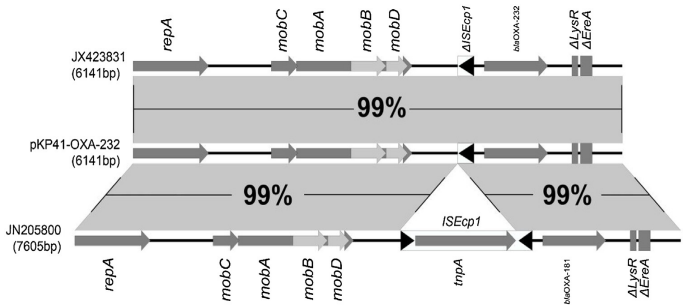

Fig. 1. Genetic structure of pKP41-OXA-232 in comparison with other ColE-like plasmids encoding $bla_{OXA-232}$ (GenBank accession no. JX423831) and $bla_{OXA-181}$ (GenBank accession no. JN205800). The reading frames are shown as grey shaded arrows, with the arrowhead indicating the direction of transcription. The truncated reading frames are shown as grey shaded rectangles. The mobile genetic elements are shown as white shaded rectangles flanked by two (complete) or one (truncated) black triangles. The areas shaded in grey indicate nucleotide identity in plasmid sequences, with the percentage indicated.

mobilisation genes (*mobA–D*), a replication gene (*repA*), truncated parts of genes encoding a putative LysR-type transcriptional regulator (Δ*lysR*) and an erythromycin esterase (Δ*ereA*), and part of an ΔIS*Ecp*1 upstream of the $bla_{OXA-232}$ gene (Fig. 1).

4. Discussion

Local, regional and global spread of antimicrobial resistance can often be linked to specific genes in association with specific bacterial clones. Brunei Darussalam lies in Southeast Asia, in close proximity to Malaysia, Singapore, Indonesia and The Philippines. Antimicrobial resistance surveillance studies conducted across the Asia-Pacific region have frequently reported higher rates of ESBL-producing and carbapenem-resistant bacteria than those seen in Europe and North America [12]. Recent data from the Study for Monitoring Antimicrobial Resistance Trends (SMART) programme identified CTX-M-14/15 and NDM as the most prevalent enzymes in this region [13]. Here we identified the spread of MDR *K. pneumoniae* in Brunei involving a strain of ST231 producing the OXA-232 carbapenemase.

OXA-232-producing *K. pneumoniae* isolates typically display carbapenem MICs of ≥32 mg/L [2,3,14]. Phenotypic resistance is further mediated by the presence of a truncated ΔIS*Ecp1* insertion element upstream of the $bla_{OXA-232}$ gene, which provides a strong −35 and −10 promoter sequence on a high-copy-number plasmid [15] as in the isolates in the current study (Fig. 1).

CRE producing OXA-48 are most prevalent in Europe, the Mediterranean and North Africa [1]. There are multiple reports of nosocomial outbreaks with OXA-48-producing *K. pneumoniae* of various sequence types in European hospitals, a problem that is extremely difficult to control [16]. In terms of pathogenicity, carriage of a number of virulence factors has been associated with more invasive and severe infections. Holt et al. identified a MDR ST231 *K. pneumoniae* clone carrying yersiniabactin (*ybt*) and aerobactin (*iutA*), which was associated with lethal cases of community-acquired pneumonia [17] but was not present in any of the isolates in the present study.

In Southeast Asia, OXA-48 has been reported only in Vietnam. Cases of OXA-232 have been found in Singapore (ST231) [4], Malaysia (ST14) [3] and South Korea [18,19]. A number of these strains [3,18] also carried the New Delhi metallo-β-lactamase (NDM-1), often from patients with a history of travel and medical treatment in India. This was not the case with the OXA-232-producing *K. pneumoniae* recovered in Brunei, as all patients were hospitalised locally and no history of recent travel could be obtained.

Outside of Southeast Asia, *K. pneumoniae* isolates producing both OXA-232 and NDM-1 have been recovered in the USA [20] and France [2,14]. Most belonged to ST14, a sequence type common amongst NDM-1-producing *K. pneumoniae* recovered in Europe [4]. In Germany, an OXA-232- and NDM-1-producing strain of *E. coli* has also been reported [21], with the same patient subsequently developing an infection with OXA-232-producing *K. pneumoniae* months later, suggesting in vivo plasmid transfer between species [21]. Enterobacteriaceae producing OXA-232 in combination with CTX-M-15 ESBLs were also found during surveillance studies in tertiary care hospitals in Mexico City [22]. Molecular analysis did not identify evidence of clonal spread in this setting, again suggesting interspecies and intraspecies transmission of plasmid-mediated resistance [22].

The ST231 clone of *K. pneumoniae* is increasingly shown to support the carriage of a number of important β-lactamases and diverse plasmid backbones. These include KPC-3-, GES-5- and SHV-12- producing *K. pneumoniae* community-acquired infections in Portugal [21], NDM-1 in the UK and India [4] and now OXA-48-like CHDLs in Southeast Asia. We identified a nosocomial outbreak involving a MDR ST231 OXA-232-producing strain of *K. pneumoniae* in Brunei. Although we were unable to demonstrate transfer of pKP41-OXA-232 plasmid by conjugation, others have found the $bla_{OXA-232}$ gene to be readily mobilisable [19].

In conclusion, *K. pneumoniae* ST231 may represent an emerging high-risk MDR clone currently disseminating throughout Southeast Asia. Owing to its capacity for carriage of diverse and multiple ESBL and carbapenemase resistance genotypes, enhanced surveillance for international spread of this clone may be warranted.

Funding

This study forms part of the PhD project of M.H.F. Abdul Momin, funded by a Brunei Darussalam Government in-service training scheme.

Competing interests

None declared.

Ethical approval

Not required.

Acknowledgements

The authors thank the microbiology laboratories of Raja Isteri Pengiran Anak Saleha Hospital (RIPAS) and Pengiran Muda Mahkota Pengiran Muda Haji Al-Muhtadee Billah Hospital (PMMPMHAB), Brunei Darussalam, for providing the clinical isolates.

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iii) CHROMagar COL-*APSE*: a selective bacterial culture medium for the isolation and differentiation of colistin-resistant Gram- negative pathogens

JOURNAL OF MEDICAL MICROBIOLOGY

RESEARCH ARTICLE
Abdul Momin *et al.*, *Journal of Medical Microbiology*
DOI 10.1099/jmm.0.000602

MICROBIOLOGY SOCIETY

CHROMagar COL-*APSE*: a selective bacterial culture medium for the isolation and differentiation of colistin-resistant Gram-negative pathogens

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Abstract

Purpose. A selective chromogenic culture medium for the laboratory isolation and differentiation of colistin resistant *Acinetobacter, Pseudomonas, Stenotrophomonas* and *Enterobacteriaceae* spp. (CHROMagar COL-*APSE)* was developed, evaluated and compared to an existing selective bacterial culture medium (SuperPolymyxin).

Methodology. The medium was challenged with 84 isolates, including polymyxin B (POL B)-susceptible and -resistant type strains and colistin (COL)-resistant organisms recovered from human and animal samples. Susceptibility to COL and POL B was determined by agar dilution and broth microtitre dilution. The lower limit for the detection of COL-resistant organisms was also calculated for both CHROMagar COL-*APSE* and SuperPolymyxin media. The ability to isolate and correctly differentiate COL-resistant organisms within mixed cultures was also assessed and compared using both media.

Results. Using CHROMagar COL-*APSE*, Gram-negative pathogens (n=71) with intrinsic (n=8) or acquired COL (n=63) resistance were recovered with 100 % specificity down to the lower limit of detection of 10^1 colony-forming units (c.f.u.). The growth on SuperPolymyxin was similar, but notably weaker for COL-resistant non-fermentative bacteria (*Acinetobacter, Pseudomonas* and *Stenotrophomonas*). CHROMagar COL-*APSE* was also more sensitive in supporting the growth of *Enterobacteriaceae* with COL resistance associated with the carriage of *mcr-1*.

Conclusion. CHROMagar COL-*APSE* is a sensitive and specific medium for the growth of COL-resistant bacterial pathogens. Due to the low limit of detection (10^1 c.f.u.), it may be useful as a primary isolation medium in the surveillance and recovery of COL-resistant bacteria from complex human, veterinary and environmental samples, especially those with plasmid-mediated MCR-1 or novel mechanisms of polymyxin resistance.

INTRODUCTION

Polymyxin B and E (colistin) are increasingly used in the treatment of multi-drug-resistant bacterial infections. Both are cationic polypeptides that bind the lipopolysaccharides (LPS) of Gram-negative bacteria and disrupt the outer membrane. Polymyxin resistance (PR), although intrinsic amongst Gram-positive and some Gram-negative species (*Proteus, Morganella* and *Serratia* spp.), is an emerging problem in a number of other pathogens (*Acinetobacter baumannii, Pseudomonas aeruginosa, Escherichiacoli, Salmonella enterica* and *Klebsiella pneumoniae)*. Resistance can occur due to mutations/insertions in genes involved in LPS biosynthesis (*lpx*, *pmrA/B, mgrB* and *phoP/Q)* and/or can be acquired by the horizontal transmission of genes encoding phosphoethanolamine transferase (pEtN) enzymes. Plasmid-mediated colistin (COL) resistance due to the *mcr-1* pEtN gene was recently identified in China [1]. Since it was first described, bacteria producing MCR-1 have been found in a wide range of food-producing animal, human and environmental sources. Moreover, MCR-1-producing isolates are often pan- (PDR) or extensively

Received 22 March 2017; Accepted 6 September 2017
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Keywords: CHROMagar COL-*APSE*; colistin; resistance; MCR-1.
Abbreviations: COL, colistin; MIC, minimum inhibitory concentration; pEtN, phosphoethanolomine transferases; PNPG, p-nitrophenyl glycerol; POL B, polymyxin B.

(XDR) drug resistant, which significantly limits the therapeutic options for these organisms.

A sensitive and specific means for the isolation of PR organisms is critical for any strategy aimed at monitoring the spread of these isolates and identifying new resistance determinants. Furthermore, polymyxin resistance may often be low level or unstable (heteroresistance), and there are well-established difficulties (e.g. cation concentrations in broth, adsorption of colistin to plastics, poor diffusion in agar) in the accurate determination of COL MICs [2]. Molecular detection is limited to the small number of mutations (*mcr-1–1.8*) and MCR-like alleles (*mcr-2, mcr-3* and *mcr-4*) discovered to date [1, 3].

Selective bacterial culture media offer a means to rapidly detect and identify organisms with resistance to polymyxins. One such medium, SuperPolymyxin, uses an eosin methylene blue (EMB) agar base, with selection for PR Gram-negative bacteria achieved by the addition of a low concentration of COL (3.5 μg ml^{-1}), along with daptomycin (10 μg ml^{-1}) and amphotericin B (5 μg ml^{-1}) to suppress Gram-positive and fungal growth. This medium has been evaluated and proven to be sensitive and specific in the selective growth of PR-resistant *Enterobacteriaceae*, either from pure cultures or using spiked stool samples [4]. Here we describe an alternative medium, CHROMagar COL-*APSE,* designed to be selective in the isolation and differentiation of all strains of *Acinetobacter* (*A*), *Pseudomonas* (*P),* *Stenotrophomonas* (*S*) and *Enterobacteriaceae* (*E*) with either intrinsic, acquired or uncharacterized mechanisms of resistance. A potential advantage compared with SuperPolymyxin would be the ability to recover and differentiate PR non-fermentative Gram-negative organisms as well as *Enterobacteriaceae*.

METHODS

Media preparation

CHROMagar COL-*APSE* plates were prepared in-house using dehydrated CHROMagar base media (CHROMagar, Paris, France) supplemented as in Table 1 with the CHROMagar COL-*APSE* supplement (X192). This contains antimicrobials (colistin sulfate/oxazolidonones) at concentrations designed to enhance the growth of PR Gram-negative species and suppress the growth of Gram-positive bacteria, whilst avoiding significant synergy between the active compounds. This +medium was not autoclaved, but instead sterilized by boiling at 100 °C in order to preserve the chromogenic compounds included in the mixture, prior to the addition of all of the supplements. All of the additional antibiotics used (colistin sulfate, polymyxin B, daptomycin and amphotericin B) were sourced from Sigma-Aldrich (Gillingham, UK) or Cambridge Bioscience (Cambridge, UK) and prepared as stock solutions (10 000–100 μg ml^{-1}) in distilled water. Those containing COL or polymyxin B (POL B) were only handled in glass tubes and used within 24 h. SuperPolymyxin plates were prepared exactly as described by Nordmann *et al.* [4] using EMB manufactured by Thermo Fisher Oxoid (Basingstoke, UK). CHROMagar COL-*APSE* media for use with samples containing *Proteus* spp. were prepared with the addition of 50 mg l^{-1} p-nitrophenyl glycerol (PNPG).

Table 1. Preparation of CHROMagar COL-*APSE* plates

Compound	Amount/L*
CHROMagar base medium	42.5 g
Distilled water	1000 ml
Sterilize by heating to 100 °C	
CHROMagar growth supplement S1	2 ml
CHROMagar COL-*APSE* supplement (X192)	4 ml

*For the production of 50×20 ml CHROMagar COL-*APSE* plates.

Bacterial isolates and determination of polymyxin minimum inhibitory concentrations (MICs)

Eighty-four isolates were used in the evaluation of CHROMagar COL-*APSE* media (Table 2). These included 8 isolates with intrinsic COL resistance (1 clinical human and 7 type strains), 13 COL susceptible isolates (6 human clinical, 3 veterinary clinical and 4 type strains) and 63 isolates with acquired COL resistance (5 human clinical and 58 veterinary clinical) contained within our collections.

The MICs of COL and POL B (0.006–256 μg ml^{-1}) were determined for each isolate by agar dilution on Mueller–Hinton II (MH 2) agar [5]. The plates were seeded with 10^4 c.f.u. using a multi-point inoculator and examined for growth after incubation at 37 °C for 24 h. Susceptibility (≤2–4 μg ml^{-1}) and resistance (>2–8 μg ml^{-1}) to either COL or POL B were interpreted according to current CLSI and/or EUCAST species-specific breakpoints, where available [6, 7]. Susceptibility to COL was also determined by broth microtitre dilution (BMD) to enable the MICs to be compared to those obtained on solid media.

All clinical isolates were additionally screened for the presence of *mcr*-related genes by PCR using the CLR5-F (5′-CGGTCAGTCCGTTTGTTC-3′), CLR5-R (5′-CTTGG TCGGTCTGTAGGG-3′), MCR_2-F (5′-TGGTACAGCCCC TTTATT-3′) and MCR_2-R (5′-GCTTGAGATTGGGTTA TGA-3′) primers for the detection of *mcr-1–1.8* and *mcr-2*, respectively [1, 3].

Comparative growth on selective media

The ability of CHROMagar COL-*APSE* and SuperPolymyxin to support or suppress growth was first assessed using a fixed inoculum of 10^3 c.f.u. of each strain. Overnight cultures were used to prepare a 0.5 McFarland standard (1.5×10^8 c.f.u.) further diluted in 0.85 % saline (OXOID, United Kingdom) and used to inoculate supplemented and unsupplemented CHROMagar and SuperPolymyxin (EMB) base plates.

Table 2. Characteristics of bacterial isolates

Isolate	MIC (mg l^{-1})/Agar dilution		Polymyxin resistance mechanism	Lowest limit of detection (c.f.u.)	
	Colistin	Polymyxin B		CHROMagar COL-*APSE*	SuperPolymyxin
Intrinsic resistance to polymyxins					
P. mirabilis NCTC 13376	>256	>256	Intrinsic	10^1	10^1
S. marcescens NCTC 10211	>256	>256	Intrinsic	10^1	10^1
M. morgannii MM2	>256	>256	Intrinsic	10^1	10^1
E. faecalis ATCC 2912	>256	256	Intrinsic	$>10^9$	$>10^9$
E. gallinarum ATCC 49573	>256	>256	Intrinsic	$>10^8$	$>10^8$
C. albicans ATCC 10231	256	256	Intrinsic	$>10^7$	$>10^7$
E. cloacae NCTC 10005	128	256	Intrinsic	10^2	10^1†
S. maltophilia NCTC 10258	8	64	Intrinsic	10^1*	10^2
Susceptible to polymyxins					
P. aeruginosa ATCC 27853	2	2	NA	10^2*	10^5
Salmonella enterica group D (non-Typhi) Sal3	2	2	N/A	10^4*	10^6
E. coli E17	1	2	MCR-1	10^7	10^7
Salmonella enterica subsp. *diarizonae* Sal1	1	2	N/A	10^5*	10^6
A. baumannii ATCC 19606	1	2	NA	10^6	10^6
E. coli E44	1	1	MCR-1	$>10^9$	$>10^9$
Salmonella enterica subsp. *diarizonae* Sal4	1	1	N/A	10^5*	10^6
K. pneumoniae KP32	0.5	1	NA	10^6	10^6
Salmonella enterica subsp. *diarizonae* Sal2	0.5	1	N/A	10^5*	10^6
Salmonella enterica group D (non-Typhi) Sal5	0.5	1	N/A	10^3*	10^5
E. coli ATCC 25922	0.5	0.5	NA	10^6	10^6
E. coli 408756	0.5	0.5	MCR-1	10^6	10^6
K. pneumoniae ATCC 9633	0.5	0.5	NA	$>10^9$*	10^5
Acquired resistance to polymxins					
A. baumannii AB219	>256	>256	Unknown	10^1	10^1
A. baumannii AB205	>256	32	Unknown	10^1	10^1
A. baumannii AB287	8	4	Unknown	10^1*	10^2
K. pneumoniae KP6	128	256	Unknown	10^1	10^1
K. pneumoniae KP19	64	64	Unknown	10^1	10^1
E. coli 35 095	>256	>256	MCR-1	10^1	10^1
E. coli 35 175	64	32	MCR-1	10^1	10^1
E. coli 29 881	32	32	MCR-1	10^1	10^1
E. coli 33 907	32	32	MCR-1	10^1	10^1
E. coli 34 936	32	32	MCR-1	10^1	10^1
E. coli 35 593	32	32	MCR-1	10^1	10^1
E. coli E10	32	16	MCR-1	10^1*	10^4
E. coli E9	32	16	MCR-1	10^1	10^1
E. coli E33	32	16	MCR-1	10^1	10^1
E. coli E3	32	16	MCR-1	10^1	10^1
E. coli E1	32	16	MCR-1	10^1	10^1
E. coli E35	32	16	MCR-1	10^1	10^1
E. coli E29	32	16	MCR-1	10^1	10^1
E. coli E32	32	16	MCR-1	10^1	10^1
E. coli E13	32	16	MCR-1	10^1	10^1
E. coli E24	32	16	MCR-1	10^1	10^1
E. coli E20	32	16	MCR-1	10^1	10^1
E. coli E25	32	16	MCR-1	10^1	10^1
E. coli E27	16	32	MCR-1	10^1	10^1
E. coli E8	16	16	MCR-1	10^1	10^1
E. coli E11	16	16	MCR-1	10^1*	10^2

Table 2. cont.

Isolate	MIC (mg l^{-1})/Agar dilution		Polymyxin resistance mechanism	Lowest limit of detection (c.f.u.)	
	Colistin	Polymyxin B		CHROMagar COL-*APSE*	SuperPolymyxin
E. coli E39	16	16	MCR-1	10^1	10^1
E. coli E40	16	16	MCR-1	10^1	10^1
E. coli E34	16	16	MCR-1	10^1	10^1
E. coli E2	16	16	MCR-1	10^1	10^1
E. coli 412049521	16	16	MCR-1	10^1	10^1
E. coli E37	16	16	MCR-1	10^1	10^1
E. coli E41	16	16	MCR-1	10^1	10^1
E. coli E36	16	16	MCR-1	10^1	10^1
E. coli E31	16	16	MCR-1	10^1	10^1
E. coli E30	16	16	MCR-1	10^{1*}	10^3
E. coli E14	16	16	MCR-1	10^1	10^1
E. coli E12	16	16	MCR-1	10^1	10^1
E. coli 14042624	16	16	MCR-1	10^1	10^1
E. coli 412016126	16	16	MCR-1	10^1	10^1
E. coli E22	16	16	MCR-1	10^1	10^1
E. coli E19	16	16	MCR-1	10^1	10^1
E. coli E7	16	16	MCR-1	10^1	10^1
E. coli E47	16	16	MCR-1	10^1	10^1
E. coli E49	16	16	MCR-1	10^{1*}	10^3
E. coli E5	16	16	MCR-1	10^{1*}	10^3
E. coli E16	16	16	MCR-1	10^1	10^1
E. coli E18	16	16	MCR-1	10^1	10^1
E. coli E43	16	16	MCR-1	10^{1*}	10^3
E. coli E46	16	16	MCR-1	10^1	10^1
E. coli E28	16	16	MCR-1	10^1	10^1
E. coli E26	16	16	MCR-1	10^1	10^1
E. coli 27 852	16	16	MCR-1	10^1	10^1
E. coli 35 062	16	16	MCR-1	10^{1*}	10^3
E. coli 37 914	16	16	MCR-1	10^1	10^1
E. coli 41 323	16	16	MCR-1	10^1	10^1
E. coli 41 339	16	16	MCR-1	10^1	10^1
E. coli 41 848	16	16	MCR-1	10^1	10^1
E. coli 412044854	16	8	MCR-1	10^1	10^1
E. coli 413040864	16	8	MCR-1	10^1	10^1
E. coli 34 692	16	8	MCR-1	10^{1*}	10^2
E. coli E45	8	16	MCR-1	10^1	10^1
E. coli 32 218	8	8	MCR-1	10^1	10^1

*Enhanced sensitivity of CHROMagar COL-*APSE*.

†Enhanced sensitivity of SuperPolymyxin.

Lower limit of detection (LLD)

The sensitivity was compared by determining the lowest number (c.f.u.) of COL-resistant bacteria in the inocula required for growth on each media. The lowest limit of detection (LLD) was assessed using serial dilutions (10^{-1}–10^{-9}) of overnight cultures grown at 37 °C for 24 h in 3 ml of Luria–Bertani (LB) broth. Tenfold serial dilutions were made in 0.85 % saline (volume/volume) and 20 µl of each dilution was plated onto unsupplemented MH 2 (control), CHROMagar COL-*APSE* and SuperPolymyxin agar plates using the Miles and Misra procedure [8]. Following incubation for 24 h at 37 °C, the colonies were counted and the number of bacteria recovered were expressed as colony-forming units per ml (c.f.u. ml^{-1}). The colony counts after incubation on CHROMagar COL-*APSE* and SuperPolymyxin agar were subtracted from the number recovered on MH 2 agar to quantify the total number of COL-resistant organisms (c.f.u.) within the plated population required for viable growth on each selective media (LLD).

Isolation and differentiation of organisms in mixed culture

Two pools containing mixtures of COL-resistant (R) and COL-susceptible (S) organisms were used to assess the performance of the media with complex polymicrobial samples. Pool one consisted of *Proteus mirabilis* NCTC 13376, *A. baumannii* AB205 (COL R), *E. coli* E7 (COL R), *E. cloacae* NCTC 10005 (COL R), *E. faecalis* ATCC 2912 (COL R), *K. pneumoniae* KP19 (COL R), *M. morgannii* MM2 (COL R), *S. marcescens* NCTC 10211 (COL R) and *P. aeruginosa* ATCC 27853 (COL heteroresistant). Pool two consisted of *A. baumannii* ATCC 19606 (COL S), *E. coli* ATCC 25922 (COL S), *E. faecalis* ATCC 2912 (COL R), *P. aeruginosa* ATCC 27853 (COL heteroresistant) and *K. pneumoniae* ATCC 9633 (COL S). The pools were made by mixing single colonies of each isolate in 3 ml 0.85 % saline adjusted to a 0.5 McFarland standard (1.5×10^8 c.f.u. ml^{-1}), and were confirmed by viable colony counts. Further dilutions were made to achieve a concentration of 10^5 c.f.u. ml^{-1} and CHROM-agar COL-*APSE* plates were then inoculated with 10 µl (10^3 c.f.u.), either spread across the entire plate using a L-shaped spreader, or streaked across the plate using a 10 µl loop (Fig. 2).

The stability of CHROMagar COL-*APSE* following batch production and storage at 4 °C was assessed using media prepared centrally by E and O laboratories (Glasgow, UK) and tested using *P. mirabilis* (NCTC 13376), *E. coli* (ATCC 25922), *K. pneumoniae* (ATCC 9633) and *E. faecalis* (ATCC 2912) as internal and external quality control and the control pools (10^3 c.f.u.) defined above. The media for use with pools containing *P. mirabilis* were supplemented with PNPG.

RESULTS

The *in vitro* activity (MIC mg l^{-1}) of both COL and POL B when determined by agar dilution was comparable (+/−1 dilution) for all of the *Enterobacteriaceae* isolates tested (Table 2). However, the MICs of COL and POL B differed by at least three dilutions for *S. maltophilia* NCTC 10258 and *A. baumannii* AB205. The *mcr-1* gene was present in 61 of the *E. coli,* 58 (95 %) of which were also phenotypically resistant to COL (MIC >2 µg ml^{-1}). Three isolates containing *mcr-1* had colistin MICs below the pharmacodynamic breakpoint when the MIC was determined by agar dilution. Three COL-resistant *A. baumannii* and two *K. pneumoniae* (MIC 8 ->256 µg ml^{-1}) clinical isolates were also identified and included in the evaluation of both media. The mechanism of resistance in these three strains is yet to be determined, despite sequencing for mutations in *mgrB, pmrA/B* and the *phoP/Q* regulatory genes known previously to be associated with mutational polymyxin resistance [9]. When susceptibility to COL was determined by BMD, the MIC of COL was comparable to that determined by agar dilution (+/−1 dilution vs all strains).

Growth on CHROMagar COL-*APSE* on plates inoculated with 10^3 c.f.u. was observed for all COL R Gram-negative isolates. All of the COL-susceptible *Enterobacteriaceae*, COL-resistant Gram-positive (*Enterococci* spp) and fungal (*Candida* spp) isolates failed to grow at these inocula (Fig. 1). Growth of the *P. aeruginosa* ATCC 27853 type strain (COL MIC 2 µg ml^{-1}), however, was supported on CHROMagar COL-*APSE* at 10^3 c.f.u. Although this isolate is deemed susceptible to COL according to current CLSI breakpoints, and is recommended as a control strain for antimicrobial susceptibility testing, it should be noted that it has frequently been shown to exhibit heteroresistance to COL *in vitro* when population analysis profiling (PAP) is used as the gold standard [10]. The −70 °C stock of ATCC 27853 held in our laboratory and used in this study consistently demonstrates heteroresistance to COL when assessed using the PAP method.

The growth on SuperPolymyxin media with an inoculum of 10^3 c.f.u. was similar, but visibly weaker for each of the COL-resistant non-fermenters. The growth of one strain of COL-resistant *A. baumannii* (MIC 8 µg ml^{-1}) and *S. maltophilia* (MIC 32 µg ml^{-1}) was not supported at all (Fig. 1). It is possible that the antimicrobial synergy known to exist between COL and daptomycin against *A. baumannii* [11] could account for the poor growth or inhibition of these species that we observed on SuperPolymyxin agar. In contrast to growth on SuperPolymyxin, the chromogenic properties of the CHROMagar COL-*APSE* media enabled the clear differentiation of COL-resistant *Enterobacteriaceae* from non-fermenters. Phenotypic identification as either dark pink to reddish (*E. coli*), metallic blue (*Klebsiella, Enterobacter* and *Serratia* spp) or colourless natural pigmentation (*Morganella)* was also possible, whilst on SuperPolymyxin only COL-resistant *E. coli* could be identified by their metallic green appearance. In the limit of detection studies, COL-resistant strains could be recovered on both media at 10^1 c.f.u., whilst the growth of strains deemed susceptible to COL was only possible when using an inoculum of $>10^4$ (Table 1).

The stability of the media confirmed that there was no reduction in performance (10^3 c.f.u. ml^{-1}) for up to 4 weeks. Swarming of *P. mirabilis* NCTC 13376 was observed when it was used to inoculate both CHROMagar COL-*APSE* and SuperPolymyxin plates. The addition of 50 mg l^{-1} p-nitrophenyl glycerol (PNPG) to the CHROMagar COL-*APSE* plates reduced this significantly (Fig. 2).

DISCUSSION

We found that both CHROMagar COL-*APSE* and the SuperPolymxin media were able to support the growth of COL-resistant Gram-negative bacteria whilst suppressing COL-resistant Gram-positive pathogens. Using clinical isolates, CHROMagar COL-*APSE* supported the growth of all of the COL-resistant strains down to an inoculum of as low as 10^1 c.f.u. Growth with an inoculum of 10^1 c.f.u. was also supported on SuperPolymyxin, but only for 50/58 (86 %) of the *E. coli* isolates with COL resistance accompanied by *mcr-1*, suggesting that it may have slightly lower sensitivity

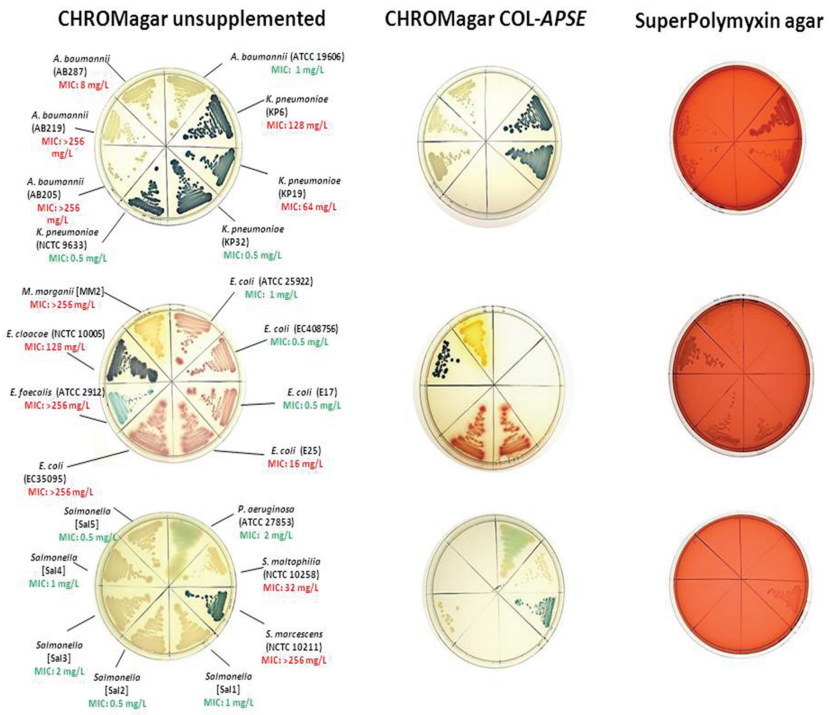

Fig. 1. Comparative growth of COL-susceptible and -resistant isolates on CHROMagar base media, CHROMagar COL-*APSE* and SuperPolymyxin. Strains were streaked on the same segments of corresponding plates.

in the detection of these strains (Table 2). SuperPolymyxin, however, was found to be better ($\geq$1 dilution) at suppressing the growth of COL-susceptible *Salmonella* spp. Reduced susceptibility to polymyxins in *Salmonella enteritidis* and other serogroup 9 strains has been reported, and the epidemiological cut-off (>2 mgl^{-1}) for resistance has been challenged [12]. As the mechanisms for the reduced susceptibility reported in *Salmonella* spp. remain unknown, a more sensitive medium may help in the recovery of COL-resistant strains and aid in the elucidation of alternative resistance mechanisms.

The only other difference we observed between the two media (Table 2) was differential growth of *P. aeruginosa* ATCC 27853 on CHROMagar COL-*APSE* (LLD=10^2 c.f.u.) when compared to SuperPolymyxin (LLD 10^5=c.f.u.). Again, this may represent the heteroresistant properties of this strain and a high frequency of COL resistance by spontaneous mutation. As with *A. baumannii*, synergy between the selective antimicrobials in the media may be responsible for the differential ability of CHROMagar COL-*APSE* and SuperPolymyxin to identify heteroresistant strains. The clinical relevance of heteroresistance to polymyxins is unclear, although the ability to identify such strains easily may be useful in optimizing or selecting COL-based therapies on an individual basis.

Notably, the addition of PNPG was beneficial in suppressing swarming of colistin-resistant *Proteus* spp. without affecting the performance of the media. This provides a more robust method to select out COL-resistant isolates within mixed specimens.

In summary, we developed and evaluated a new chromogenic culture medium for the isolation and identification of COL-resistant Gram-negative bacterial pathogens. CHROMagar COL-*APSE* was similar to SuperPolymyxin as a medium for the selective growth of COL-resistant organisms, although we found CHROMagar COL-*APSE* to be slightly more sensitive in the detection of *Enterobacteriaceae* producing MCR-1, and it also provides the benefit of presumptive chromogenic identification. The low limit of detection (10^1 c.f.u.) of both media suggest either could be used to confirm polymyxin resistance in organisms recovered from other growth media, or if they were employed as a primary isolation medium. Evaluation of the media as tools for the surveillance and recovery of COL-resistant

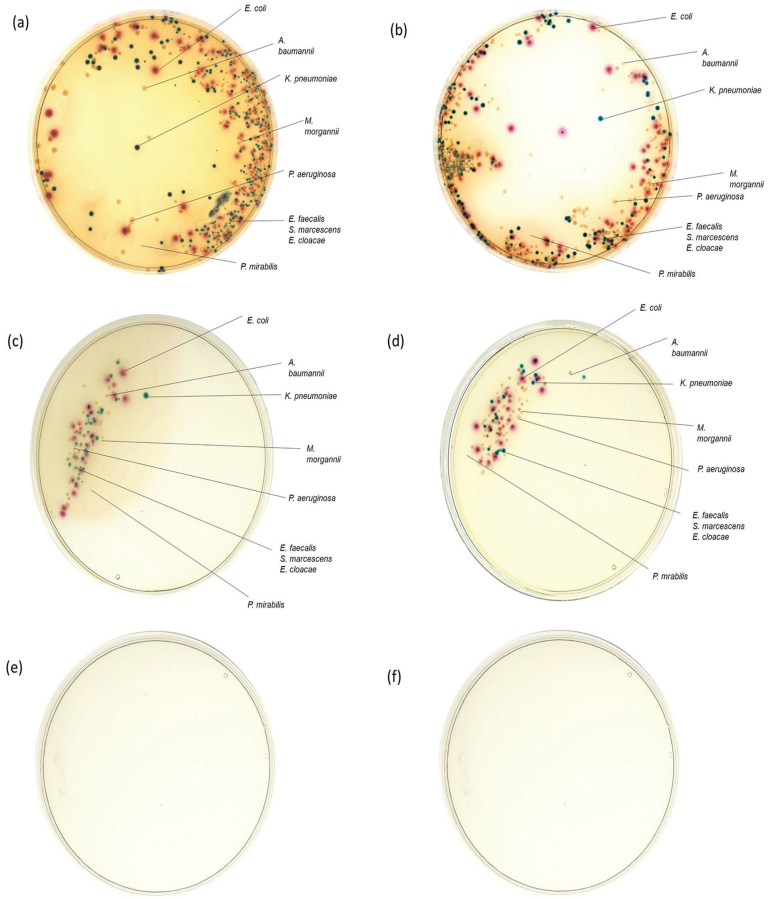

Fig. 2. Growth of simulated pools of COL-susceptible and COL-resistant strains on CHROMagar base media and CHROMagar COL-*APSE*. (a) CHROMagar COL-*APSE* simulated pool containing *A. baumannii* AB205 (COL R), *E. coli* E7 (COL R). *E. cloacae* NCTC 10005 (COL R), *E. faecalis* ATCC 2912 (COL R), *K. pneumoniae* KP19 (COL R), *M. morgannii* MM2 (COL R), *P. aeruginosa* ATCC 27853 (COL heteroresistant), *S. marcescens* NCTC 10211 (COL R) and *P. mirabilis* NCTC 13376 (COL R). The plate was inoculated with an L-shaped spreader. (b) CHROMagar COL-*APSE* + 50 mg l^{-1} p-nitrophenyl glycerol (PNPG) simulated pool containing *A.baumannii* AB205 (COL R), *E. coli* E7 (COL R). *E. cloacae* NCTC 10005 (COL R), *E. faecalis* ATCC 2912 (COL R), *K. pneumoniae* KP19 (COL R), *M. morgannii* MM2 (COL R), *P. aeruginosa* ATCC 27853 (COL heteroresistant), *S. marcescens* NCTC 10211 (COL R) and *P. mirabilis* NCTC 13376 (COL R). The plate was inoculated with an L-shaped spreader. (c) CHROMagar COL-*APSE* simulated pool containing *A. baumannii* AB205 (COL R), *E. coli* E7 (COL R). *E. cloacae* NCTC 10005 (COL R), *E. faecalis* ATCC 2912 (COL R), *K. pneumoniae* KP19 (COL R), *M. morgannii* MM2 (COL R), *P. aeruginosa* ATCC 27853 (COL heteroresistant), *S. marcescens* NCTC 10211 (COL R) and *P. mirabilis* NCTC 13376 (COL R). The plate was inoculated using the streaking method. (d) CHROMagar COL-*APSE* + 50 mg l^{-1} p-nitrophenyl glycerol (PNPG) simulated pool containing *A. baumannii* AB205 (COL R), *E. coli* E7 (COL R). *E. cloacae* NCTC 10005 (COL R), *E. faecalis* ATCC 2912 (COL R), *K. pneumoniae* KP19 (COL R), *M. morgannii* MM2 (COL R), *P. aeruginosa* ATCC 27853 (COL heteroresistant), *S. marcescens* NCTC 10211 (COL R) and *P. mirabilis* NCTC 13376 (COL R). The plate was inoculated using the streaking method. (e) CHROMagar COL-*APSE* simulated pool containing *A. baumannii* ATCC 19606 (COL S), *E. coli* ATCC 25922 (COL S). *E. faecalis* ATCC 2912 (COL R), *P. aeruginosa* ATCC 27853 (COL heteroresistant) and *K. pneumoniae* ATCC 9633 (COL S). The plate was inoculated with an L-shaped spreader. (f) CHROMagar COL-*APSE* + 50 mg l^{-1} p-nitrophenyl glycerol (PNPG) simulated pool containing *A. baumannii* ATCC 19606 (COL S), *E. coli* ATCC 25922 (COL S). *E. faecalis* ATCC 2912 (COL R), *P. aeruginosa* ATCC 27853 (COL heteroresistant) and *K. pneumoniae* ATCC 9633 (COL S). The plate was inoculated with an L-shaped spreader.

bacteria from complex human, veterinary and environmental samples is therefore underway, focusing on the identification of those with MCR-mediated polymyxin resistance.

Funding information
We thank Alberto Lerner for the supply of CHROMagar base media and supplements used in this study, and E and O laboratories for commercial production of the media.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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iv) Rapid Detection of Carbapenemases in *Enterobacteriaceae*: Evaluation of the Resist-3 O.K.N. (OXA-48, KPC, NDM) Lateral Flow Multiplexed Assay

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LETTER TO THE EDITOR

Rapid Detection of Carbapenemases in *Enterobacteriaceae*: Evaluation of the Resist-3 O.K.N. (OXA-48, KPC, NDM) Lateral Flow Multiplexed Assay

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KEYWORDS KPC, NDM, OXA-48, rapid diagnostics, carbapenems

The identification, treatment, and control of carbapenem-resistant *Enterobacteriaceae* (CRE) infections are a major challenge for health care institutions and diagnostic laboratories worldwide. Those producing plasmid-mediated OXA-48, KPC, NDM, or VIM-like carbapenemases (CPE) are most concerning, being frequently involved in nosocomial outbreaks that are difficult and very costly to manage (1). A simple and rapid test able to provide sensitive and specific identification of CRE and also the carbapenemase present is critical in any strategy aimed at addressing this problem.

Numerous phenotypic (MIC determination, disc diffusion, selective culture media, acidimetric) and genotypic (PCR amplification, microarrays, DNA sequencing) methods have been used in the laboratory isolation, diagnosis, and confirmation of CRE (2). Recently, two novel immunochromatographic lateral flow assays (Coris BioConcept, Gembloux, Belgium) were developed for the specific detection of OXA-48 (OXA-48 K-SeT) and KPC-like (KPC K-SeT) carbapenemase producers. These assays have been evaluated in multiple laboratories, using diverse sets of organisms (*Escherichia coli*, *Klebsiella*, *Enterobacter*, *Serratia*, *Providencia*, *Pseudomonas* spp.) carrying multiple β-lactamase, OXA-48 (OXA-48, -162, -181, -204, -232, -242), and KPC (KPC-2/3/4) allelic variants (3, 4, 5, 6). Both have a reported sensitivity and specificity of 100% compared to the results of molecular detection of carbapenemase genes as the gold standard (3, 4, 5, 6). They have also been shown to be compatible with organisms recovered from most unsupplemented, selective, solid, and liquid culture media currently in use in diagnostic laboratories and with bacteria taken directly from positive blood culture bottles (BacT/Alert; bioMérieux, Macy L'Etoile, France) or culture-positive urinary samples (3, 4). A modification of this system, Resist-3 O.K.N. (Coris BioConcept) designed for the simultaneous detection of OXA-48, KPC, and NDM-like enzymes using a single disposable cartridge, has now been manufactured. Here we assessed the ability of the Resist-3 O.K.N. assay to detect OXA-48, KPC, and NDM coproduction in a collection of 112 nonreplicate well-defined CRE isolates received in our laboratories.

All isolates were carbapenem-resistant *Enterobacteriaceae* (ertapenem MIC, >1 μg/ml) recovered from routine clinical samples submitted to Barts Health NHS Trust or the Antimicrobial Research Laboratory at Queen Mary University of London between January 2011 and December 2016. Identification was performed using the Bruker matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry system (Bruker UK Ltd., Coventry, UK), with carbapenem MICs determined using MicroScan WalkAway Negative Combo 36 panels (Siemens Healthcare Diagnostics, Deerfield, IL) and confirmed with Etest gradient strips (bioMérieux). Multiplex PCRs

Accepted manuscript posted online 1 February 2017

Citation Wareham DW, Abdul Momin MHF. 2017. Rapid detection of carbapenemases in *Enterobacteriaceae*: evaluation of the Resist-3 O.K.N. (OXA-48, KPC, NDM) lateral flow multiplexed assay. J Clin Microbiol 55:1223–1225. https://doi.org/10.1128/JCM.02471-16.

Editor Karen C. Carroll, The Johns Hopkins University School of Medicine

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TABLE 1 Detection of carbapenemase production using the Resist-3 O.K.N. assay

Isolate	Carbapenemase(s) carried (no. of isolates)	Resist-3 O.K.N. result		
		OXA-48-like	KPC-like	NDM-like
Klebsiella pneumoniae	OXA-48 (23)	23	0	0
	OXA-48 + NDM-1 (4)	4	0	4
	OXA-232 (6)	6	0	0
	KPC-2 (8)	0	8	0
	KPC-2 + VIM-1 (1)	0	1	0
	NDM-1 (14)	0	0	14
	VIM-4 (1)	0	0	0
Escherichia coli	OXA-48 (1)	1	0	0
	KPC-2 (3)	0	3	0
	KPC-2 + VIM-1 (1)	0	1	0
	NDM-1 (6)	0	0	6
	NDM-5 (3)	0	0	3
	NDM-7 (2)	0	0	2
	VIM-1 (1)	0	0	0
Enterobacter cloacae	OXA-48 + NDM-1 (2)	2	0	2
	NDM-1 (2)	0	0	2
	KPC-4 (2)	0	2	0
	IMP-1 (1)	0	0	0
	VIM-1 (4)	0	0	0
Enterobacter aerogenes	KPC-2 (2)	0	2	0
	NDM-1 (1)	0	0	1
Citrobacter koseri	KPC-2 + VIM-1 (1)	0	1	0
Providencia stuartii	NDM-1 (1)	0	0	1
	VIM-1 (2)	0	0	0
Morganella morganii	VIM-1 (1)	0	0	0
Proteus mirabilis	VIM-1 (1)	0	0	0

were used to confirm the presence of class A (KPC, BKC, SME, VEB, PER, GES), B (IMP, VIM, NDM, SIM, SPM, DIM, GIM, KHM, FIM, AIM, DIM, TMB, FRI), and D (OXA-like) carbapenemase genes, and all allelic variants identified were confirmed by Sanger sequencing of the entire coding regions (7, 8). The collection consisted of carbapenemase-producing *Klebsiella pneumoniae* ($n = 57$), *E. coli* ($n = 17$), *Enterobacter* spp. ($n = 14$), and *Providencia stuartii* ($n = 3$) and single isolates of *Citrobacter koseri*, *Morganella morganii*, and *Proteus mirabilis* (Table 1). Carbapenemases produced by the isolates were identified as KPC-2/4, NDM-1/5/7, VIM-1/4, IMP-1, OXA-48, and OXA-232. Nine isolates produced 2 carbapenemases, either OXA-48 in combination with NDM-1 ($n = 6$) or KPC-2 in combination with VIM-1 ($n = 3$).

Detection of OXA-48, KPC, and NDM using the Resist-3 O.K.N. cassettes was carried out according to the manufacturer's protocol. Bacteria were grown for 18 h at 37°C on Mueller-Hinton II agar plates (Oxoid), and a single colony was emulsified in 5 drops of the lysis buffer. Cassettes were loaded with 3 drops of lysate and read within 5 min. Six carbapenem-susceptible Gram-negative type strains (*K. pneumoniae* NCTC 9633, *E. coli* NCTC 12241, *Enterobacter cloacae* NCTC 13380, *Enterobacter aerogenes* NCTC 9375, *Pseudomonas aeruginosa* ATCC 27852, *Acinetobacter baumannii* ATCC 19606) were used as negative controls. Eighteen additional *Enterobacteriaceae* isolates (*K. pneumoniae* [$n = 8$] or *Klebsiella oxytoca* [$n = 1$], *E. coli* [$n = 4$], *E. cloacae* [$n = 2$] or *E. aerogenes* [$n = 2$], *Serratia marcescens* [$n = 1$]) with phenotypic resistance to ertapenem but without a known carbapenemase were also used as negative controls.

There was complete agreement between the carbapenemases detected by PCR and the results obtained with Resist-3 O.K.N. (Table 1). The lateral flow device was able to correctly identify and differentiate OXA, KPC, and NDM production among all CRE species and enzyme variants tested, including those carrying more than one carbap-

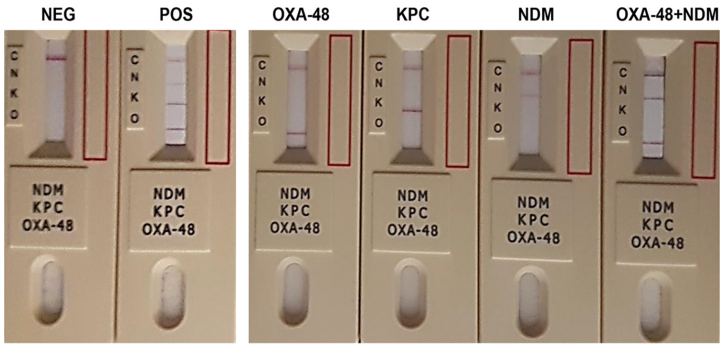

FIG 1 Detection of OXA-48, KPC, and NDM-like carbapenemases in isolates producing single and dual enzymes using the Resist-3 O.K.N. assay. NEG, negative; POS, positive.

enemase. No cross-reactions were observed for strains with carbapenem resistance due to IMP-1 or VIM-1/4 (Table 1) with any of the susceptible type strains or with the 18 CRE that had no OXA, KPC, or NDM carbapenemase detectable by PCR (Fig. 1).

We found the Resist-3 O.K.N. assay to be 100% sensitive and specific in the detection and differentiation of OXA-48, KPC, and NDM-like carbapenemases among CRE recently referred to our laboratory. Although the assay does not currently extend to the detection of VIM or IMP-like metallo-β-lactamases, the ability to detect 3 of the 5 most prevalent and transmissible carbapenemases found worldwide is a significant advantage. The ease of use, speed, and low cost (less than $15) of the cassettes make it attractive as a diagnostic tool for use in the management of CRE outbreaks. Its role either as a primary-screening, confirmatory, or rapid point-of-care test should be assessed further, ideally prospectively in the setting of a polyclonal nosocomial CRE outbreak.

ACKNOWLEDGMENTS

Resist-3 O.K.N. assays were supplied free of charge for evaluation by BioConnections (Knypersley, UK). No other specific funding was used to undertake this study, as it was performed as part of our routine activities.

We declare no conflict of interest and have no association with Coris BioConcept.

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v) Direct detection of carbapenem resistance determinants in clinical specimens using immunochromatographic lateral flow devices

Journal of Antimicrobial Chemotherapy

J Antimicrob Chemother
doi:10.1093/jac/dky095

Direct detection of carbapenem resistance determinants in clinical specimens using immunochromatographic lateral flow devices

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Sir,

Accurate, rapid, affordable diagnostic tests are critically important in tackling the problem of antimicrobial-resistant infections. Assays that can be used for pathogen detection and drug susceptibility, close to the point of care (POC), have the most potential to influence clinical decisions, antimicrobial prescribing, stewardship and patient outcomes.[1,2] Considerable effort has been invested in the development of assays for the rapid detection of bacterial sepsis and antimicrobial resistance determinants. This includes genotypic (DNA amplification/sequencing) and phenotypic assays targeted towards bacterial strains such as the ESKAPE (*Enterobacter*, *Staphylococcus*, *Klebsiella*, *Acinetobacter*, *Pseudomonas*, *Enterococcus* spp.) group of organisms, but also for specific resistances to key antimicrobial therapies.[3]

The need to detect resistance to carbapenems (ertapenem, imipenem, meropenem) has received considerable attention.[4] Once considered drugs of last resort in the treatment of Enterobacteriaceae (*Escherichia coli*, *Klebsiella pneumoniae*), their efficacy is now compromised by the global spread of carbapenemases (KPC, NDM, VIM, OXA). Carbapenemase genes are usually carried on MDR plasmids that encode enzymes able to degrade or destroy carbapenems and also many other β-lactam antibiotics.[5]

There are myriad methods for the detection of carbapenem resistance genes and for carbapenem-resistant Enterobacteriaceae (CRE). Recently a number of immunochromatographic lateral flow cassettes have been developed (CORIS BioConcept, Gembloux, Belgium) that enable 5 min detection of KPC, NDM, VIM and OXA-48-like carbapenemases directly from cultured bacterial colonies.[6–10] Versions of the assay have been evaluated in simplex (KPC *K*-SeT®, OXA-48 *K*-SeT®) and multiplex (RESIST-3 O.K.N.) forms across different laboratories, with 100% sensitivity and specificity reported when compared with molecular methods of detection.[6–10] In lower limit of detection (LLD) studies the minimum bacterial load required in a sample is quoted as low as 10^4–10^5 cfu/mL.

Experience to date suggests that this technology could be well suited and further adapted for use directly with clinical samples as a POC test. Here we assessed the performance of the RESIST-3 O.K.N. card for the direct, simultaneous detection of KPC, NDM and OXA-48-like carbapenemases in positive blood culture bottles.

Thirty carbapenem-resistant (meropenem MIC >4 mg/L) enterobacterial isolates (*K. pneumoniae* $n = 16$, *E. coli* $n = 8$, *Enterobacter cloacae* $n = 4$, *Citrobacter koseri* $n = 2$) known to produce KPC-2 ($n = 8$), OXA-48/181/232 ($n = 14$), NDM-1/5/7 ($n = 12$) and VIM ($n = 2$) β-lactamases (singly or in combination) were used in the evaluation. Isolates were grown overnight at 37°C in 3 mL of Trypticase soy broth (TSB; Oxoid, Basingstoke, UK), then serially diluted in PBS to a final concentration of 10^2 cfu/mL. Inocula were quantified by viable bacterial counts plated using the Miles and Misra technique on Mueller–Hinton II (MH II) agar. Inocula of 10^2 cfu were aliquoted into Aerobic (FA Plus™) and Anaerobic (SN™) BacT/ALERT blood culture bottles (bioMérieux) followed by the addition of 10 mL of sterile heparinized horse blood (Oxoid). Bottles were incubated aerobically on the BacT/ALERT system for 18 h. Aliquots (0.1 and 0.5 mL) were removed from bottles once flagged positive (<10 h) and centrifuged at 12 000 rpm for 1 min (Biofuge, Heraeus). After discarding the supernatant, 10 drops of lysis buffer (LY-A) were added to the deposit. Three drops of this suspension were added to wells of RESIST-3 O.K.N. cassettes and results read within 15 min. Bottles were subcultured to selective mSuperCARBA agar (bioMérieux) to ensure viable growth of each CRE in both blood culture bottles and that each still produced the target carbapenemase.

All BacT/ALERT FA Plus™ and SN™ bottles spiked with isolates known to produce OXA-48-like or KPC-2 carbapenemases were positive following direct inoculation of the RESIST-3 O.K.N. cards; no additional bands were observed with either of the isolates co-producing VIM. None of the isolates producing NDM carbapenemases gave positive bands on cards inoculated with preparations made from blood culture bottles, despite each strain being strongly positive when taken directly from a TSB culture. To investigate any possible inhibitory effects of the BacT/ALERT blood culture base media on the growth of NDM carbapenemase producers, the experiment with NDM-producing strains was repeated using bottles supplemented additionally with 1 mg/L meropenem and 0.1 M $ZnSO_4$. These were added to control for any growth effects due to sequestration of antimicrobials or divalent cations by components of the culture media or horse blood.

We found the RESIST-3 O.K.N. triplex card to be compatible with the BacT/ALERT blood culture media and highly sensitive in the detection of KPC and OXA-48-like carbapenemases in Enterobacteriaceae. This is in accordance with recent reports that used single immunochromatographic assays (OXA-48 *K*-Set®) for the direct detection of OXA-370 and another blood culture system (VersaTREK®; ThermoFisher, Waltham, MA, USA).[10] We observed

only very weak bands for NDM, in stark contrast to those for OXA-48 and KPC, when applying a mixed KPC/OX-48/NDM culture. Furthermore, the effects of the volume of blood culture media and bottle used in incubation influenced the result. Weak positive NDM bands were obtained using 100 μL from BacT/ALERT FA Plus™ bottles whilst tests using cultures from BacT/ALERT SN™-inoculated bottles were negative with 100 μL of culture and only weakly positive on increasing the material to 500 μL. This was despite supplementation of bottles with meropenem and $ZnSO_4$ to promote growth and limit Zn starvation, which could be due to components of the bacterial culture media or addition of horse blood. Increasing the biomass further (>1.5 mL) risks making the procedure impractical for diagnostic laboratories as the volume exceeds the capacity of most microcentrifuges. Direct detection of NDM from positive blood culture sets will likely require an alternative protocol or further enrichment of the inoculum before it can be recommended for diagnostic use in the detection of NDM.

Rapid identification of KPC- and OXA-48-like-mediated carbapenem resistance from positive blood culture appears to be possible using the RESIST-3 O.K.N. cassettes. This requires minimal technical expertise and only access to a semi-automated blood culture system, common to most diagnostic laboratories. Adaptation of the test as a true POC test may be best pursued by applying it as a tool to either aid patient screening for carriage of CRE or in the diagnosis or empirical treatment of urinary tract infections. Successful identification of OXA-48 directly from pre-incubated (2 h) spiked stool samples has recently been reported with high sensitivity and a lower limit of detection of 10^2 cfu/mL.[10] The same could be expected with urine samples, for which the critical limit of 10^5 cfu/mL bacteria is widely used as a diagnostic criterion to inform treatment.

Further evaluation of rapid immunochromatographic assays for the detection of carbapenem-resistant infections should be undertaken prospectively and in parallel with prevailing tests (e.g. PCR) to assess their utility and economic viability in the management of CRE carriage and infection using real-time patient samples in a setting with a high prevalence of CRE infections. This, along with a cost–benefit analysis, will promote their most effective and diligent use as part of any strategy aimed at combatting antimicrobial resistance.

Acknowledgements

RESIST-3 O.K.N. assays were supplied free of charge for evaluation by CORIS BioConcept.

Funding

This study was performed as part of our routine activities.

Transparency declarations

None to declare. The authors have no association with CORIS BioConcept.

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