THE OUTCOMES OF CARBAPENEM RESISTANT *ENTEROBACTERIACEAE* (CRE) INFECTED PATIENTS AND THEIR ASSOCIATION WITH ANTIBIOTIC THERAPY

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DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF PATHOLOGY (MICROBIOLOGY)

UNIVERSITI SAINS MALAYSIA

2018

ACKNOWLEDGEMENTS

Bismillahirrahmanirrahim

I thank Allah S.W.T The Most Gracious and The Most Merciful for giving me strength to complete this dissertation as part of partial fulfilment of Master of Pathology (Microbiology). Indeed, without his help and will, nothing is accomplished.

My sincere thanks and deepest appreciation to my supervisor, Assoc. Prof. Dr. Zaidah Abdul Rahman and my co-supervisor Dr. Nurahan Maning for their guidance, support and advices throughout completion of this dissertation.

My appreciation also goes to all my colleagues especially Dr Mahirah Kamil Puat and staffs in the Department of Medical Microbiology and Parasitolgy, Hospital Universiti Sains Malaysia, specifically En. Mohamad and Pn. Amanina Aminuddin for their technical assistance and continuous support.

Not forgetting, my deepest gratitude to my family especially my husband, Dr Yusni Hisham Yusof and my children, Khadijah Khalilah, Aisyah Amani, Muhammad Muaz, Umar Ubaidillah and Sofia Sumayyah for endless love, support and encouragement, and my dearest parents and in-laws for their patience and understanding.

Finally, I owe gratitude to all those who have helped me directly or indirectly throughout completing this dissertation. No word can suffice to say how much I appreciate all the help that I have received and only Allah S.W.T will rewarded their good deeds.

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ABBREVIATIONS

APAC	Asia Pacific
ATCC	American Type Culture Collection
AST	Antibiotic Susceptibility Testing
CAUTI	Catheter Associated Urinary Tract Infection
CRE	Carbapenem Resistant *Enterobactericeae*
CSF	Cerebral spinal fluid
CP-CRE	Carbapenamase Producer- Carbapenem Resistant *Enterobacteriaceae*
CDC	Centres for Disease Control
CLABSI	Central Line Associated Bloodstream Infecion
CLSI	Clinical and Laboratory Standards Institute
CMS	Colistimethate
CRKP	Carbapenem Resistant *Klebsiella pneumoniae*
DNA	Deoxyribonucleic Acid
ESBL	Extended Spectrum of Beta-Lactamases
ETT	Endotracheal tube
EDTA	Ethylene Diamine Tetra Acetic Acid
FDA	Food and Drug Administration
GES	Guaiana-Extended Spectrum
GIM	German-imipinemase
HAI	Health care associated infection
HAP	Hospital Acquired pneumonia
HUSM	Hospital Universiti Sains Malaysia
ICU	Intensive Care Unit
IMP	Imipinemase Metallo-beta-lactamase

KPC	*Klebsiella pneumoniae* Carbapenemase
LPS	Lipopolysaccharide
MBC	Minimum bactericidal concentration
MBL	Metallo-Beta-Lactamases
MIC	Minimum Inhibitory Concentration
MHA	Muller-Hinton Agar
MHT	Modified Hodge Test
NDM-1	New Delhi Metallo-beta-lactamase-1
NCCLS	National Committee for Clinical Laboratory Standard
OXA	Oxacilinase
PBP	Penicillin Binding Protein
SME	Serratia Marcescens Enzyme
SSI	Surgical site infection
STI	Soft tissue infection
UTI	Urinary Tract Infection
VAP	Ventilator Associated Pneumonia
VIM	Verona Integron-encoded Metallo-beta-lactamase

ABSTRACT

THE OUTCOMES OF CARBAPENEM RESISTANT *ENTEROBACTERIACEAE* (CRE) INFECTED PATIENTS AND THEIR ASSOCIATION WITH ANTIBIOTIC THERAPY

Introduction

Carbapenem Resistant *Enterobacteriaceae* (CRE) especially Carbapenem producing CRE (CP-CRE) has emerged as a global threat which commonly associated with hospital acquired infection including blood stream infection, pneumonia, surgical site infection and urinary tract infection. Overall, isolation of CRE from any site whether this represents clinical infection or not was associated with poor outcomes. Despite their increasing burden, the most optimal treatment for CRE infections was largely unknown. Therefore, the aim of this study is to determine the outcomes of CRE infected patients and their association with antibiotic therapy.

Methods

This was a retrospective study conducted in Hospital Universiti Sains Malaysia from January 2013 till March 2017. The list of CRE cases selected from Medical Microbiology & Parasitology laboratory. Patient's records were reviewed for demographics and clinical characteristics and those that meet the inclusion and exclusion criteria were included in this study. Archived isolates were further tested for MIC testing for meropenem, imipenem, ertapenem, doripenem and polymyxin B by using E test method. The results were interpreted according to CLSI guidelines. Data were analysed using SPSS Statistic version 22.

Results

A total of 57 CRE infected patients and started on targeted antimicrobial therapy was included in the study. The most common infection related to CRE infection was pneumonia (29/57, 50.9%) followed by blood stream infection (15/57, 26.3%) and urinary tract infection (7/57, 12.3%) with *Klebsiella pneuomoniae* as a major CRE pathogen (54/57, 94.7%). Most of the patients were located in a medical ward and ICU, accounting for 42.1% (24/57) and 33.3% (19/57) respectively of the subjects. Majority of patients had underlying disease and the most common disease was chronic kidney disease (38.6%, 22/57) followed by diabetes mellitus (36.8%, 21/57). The all-30-day mortality in this study was 43.9% (42/57). Based on in vitro susceptibility testing on carbapenems, imipenem has the highest sensitivity rate (9/57, 15.8%) followed by meropenem (7/57, 12.3%) according to CLSI breakpoint 2012. All isolates were susceptible to polymyxin B. Most of the subjects in this study received monotherapy (33/57, 57.9%) compared to combined therapy (24/57, 42.1%). We found that no significant association between patient's outcome with monotherapy or combined therapy ($p = 0.113$) and also with the type of antimicrobial received. However, the 30-day mortality in combined therapy group much higher (13/23, 56.5%) if compare to monotherapy group (12/34, 35.3%). Out of 45 repeated samples cases, 82.2% (37/45) had achieved microbiological clearance. We found that no significant association between meropenem MIC levels with microbiological clearance ($p = 0.641$).

Conclusion

Overall, based on in vitro susceptibility testing polymyxin B is considered to be the most active in vitro agents against CRE. However, imipenem and meropenem still have a role in treating CRE infection especially as a combination therapy. Even though no significant association was found between antibiotic therapy with the outcome but the 30-day mortality in combined therapy group much higher than monotherapy group. This could be bias as combined therapy was started among severe infection and critically ill patients. A larger sample size and prospective study (randomised control trial) may be needed to prevent bias in term of patient's selection on starting type of treatment regime.

ABSTRAK

KESAN JANGKITAN CARBAPENEM RESISTANT *ENTEROBACTERIACEAE* (CRE) KE ATAS PESAKIT DAN KAITAN DENGAN RAWATAN ANTIBIOTIK.

Pengenalan

CRE terutamanya CP-CRE, telah muncul sebagai ancaman global yang biasanya dikaitkan dengan jangkitan semasa pesakit berada di pusat rawatan termasuklah jangkitan saluran darah, pneumonia, jangkitan pada luka pembedahan dan jangkitan saluran kencing. Secara umumnya, isolasi CRE sama ada di sebabkan jangkitan atau tidak boleh membawa kesan yang buruk kepada pesakit. Di samping peningkatan kes jangkitan, rawatan yang optima untuk CRE secara umumnya tidak diketahui. Oleh yang demikian, kajian ini dibuat bertujuan mengkaji kesan jangkitan CRE terhadap pesakit dan hubungannya dengan terapi antibiotik.

Tatacara kajian

Kajian retrospektif ini dijalankan di Hospital Universiti Malaysia dari Januari 2013 sehingga March 2017. CRE positif telah di kenal pasti daripada Makmal Mikrobiologi Perubatan & Parasitologi. Rekod pesakit di semak, maklumat di catat dan semua kes yang memenuhi syarat di masukkan ke dalam kajian. CRE yang di kenalpasti kemudian diuji untuk ujian tahap MIC dengan menggunakan teknik E test terhadap antibiotik meropenem, imipenem, ertapenem, doripenem dan polymyxin B. Keputusan MIC di terjemah mengikut panduan CLSI. Data dianalisa menggunakan perisian SPSS Statistic versi 22.

Keputusan

Keseluruhan terdapat 57 pesakit jangkitan CRE yang menerima rawatan antibiotik. Jenis jangkitan CRE yang paling tinggi adalah pneumonia (29/57, 50.9%) diikuti jangkitan saluran darah (15/57, 26.3%) dan jangkitan saluran kencing (7/57, 12.3%) dengan *Klebsiella pneumoniae* sebagai penyebab utama (54/57, 94.7%). Kebanyakan pesakit adalah daripada wad perubatan dan unit rawatan rapi yang masing-masing adalah 42.1% (24/57) and 33.3% (19/57). Sejumlah besar pesakit mempunyai penyakit yang sedia ada dan kebanyakannya adalah penyakit buah pinggang kronik (38.6%, 22/57) diikuti dengan kencing manis (36.8%, 21/57). Peratus kematian dalam masa 30 hari selepas diagnosa jangkitan dibuat adalah 43.9% (42/57). Berdasarkan kepada ujian sensitiviti in vitro terhadap ubat-ubat carbapenem, imipenem mempunyai tahap peratusan sensitiviti yang paling tinggi (9/57, 15.8%) diikuti dengan meropenem (7/57, 12.3%) berdasarkan panduan CLSI 2012. Semua isolasi di dapati sensitif kepada polymyxin B. Kebanyakan pesakit di dalam kajian ini menerima rawatan satu jenis antibiotik sahaja (33/57, 57.9%) berbanding rawatan kombinasi antibiotik (24/57, 42.1%). Kami dapati tiada kaitan signifikan antara kesan jangkitan ke atas pesakit dengan rawatan satu jenis antibiotik atau rawatan kombinasi antibiotik ($p = 0.113$) dan juga jenis antibiotik yang diterima. Walaubagaimanapun, didapati kadar kematian dalam masa 30 hari selepas diagnosa jangkitan dibuat lebih tinggi bagi kumpulan pesakit yang menerima rawatan kombinasi (13/23, 56.5%) berbanding dengan kumpulan yang hanya menerima satu jenis antibiotik sahaja (12/34, 35.3%). Daripada 45 kes yang mempunyai ulangan pengambilan sampel, 82.2% (37/45) telah mencapai kebebasan kuman. Walaubagaimanapun, kami dapati tiada kaitan yang signifikan antara tahap MIC meropenem dengan kebebasan kuman dari sampel ulangan ($p = 0.641$).

Kesimpulan

Secara keseluruhan, berdasarkan kepada ujian sensitiviti in vitro, polymyxin B merupakan antibiotik yang paling aktif terhadap CRE. Walaubagaimanapun, imipenem dan meropenem masih mempunyai peranan dalam merawat jangkitan CRE terutamanya sebagai salah satu rawatan kombinasi. Walaupun tiada kaitan signifikan antara rawatan antibiotik dan kesan jangkitan ke atas pesakit, didapati kumpulan rawatan kombinasi mempunyai kadar kematian dalam masa 30 hari diagnosa jangkitan lebih tinggi berbanding kumpulan yang menerima satu jenis antibiotik sahaja. Ini mungkin disebabkan oleh rawatan kombinasi kebiasaannya diberikan kepada pesakit yang mempunyai jangkitan yang teruk dan kritikal. Sampel saiz yang lebih besar dan kajian prospektif (randomised control trial) mungkin diperlukan bagi mengelakkan kecenderungan pemilihan pesakit terutamanya untuk menerima jenis rejim rawatan.

CHAPTER 1 INTRODUCTION

1.1 Introduction to *Enterobacteriaceae*

1.1.1 Characteristics and taxonomy

Enterobacteriaceae is among the most frequently encountered pathogenic bacteria in clinical samples (Framer, 1999). It is a Gram negative bacilli, non-spore forming, facultative anaerobes that ferment glucose and others sugars, reduce nitrate to nitrite, form catalase but do not produce oxidase except for *Plesiomonas*. Most are motile except for *Klebsiella* spp and *Shigella* spp and the motility is by having peritrichious flagella. For taxonomy of *Enterobacteriacea*, it is classified in the domain Bacteria, phylum Proteobacteria, class Gamma proteobacteria, order Enterobacteriales and family *Enterobacteriacea*. Taxonomy is a dynamic and on-going process whereby new species and genera will continue to be added especially in the advent of 16s rRNA sequencing that can help in identifying many clinical and environmental isolates up to species level that unidentifiable by conventional method. To date there are 53 genera and over 170 named species of bacterial family of *Enterobacteriacea* with 29 of them are considered as medically important that known to cause infection in humans (England, 2015). Those medically important *Enterobacteriacea* members are *Citrobacter, Edwardsiella, Enterobacter, Escherichia, Hafnia, Klebsiella, Morganella, Pantoea, Plesiomonas, Proteus, Providencia, Salmonella, Serratia, Shigella* and *Yersinia*.

1.1.2 Epidemiology

Enterobacteriaceae can be found and widely distributed in the variety of environment sources including in soil and water. They mainly inhabit the lower gastrointestinal tract of humans and various animals, thus these organisms are often referred as enterics. However, these bacteria also have been found to colonized extraintestinal including oropharyngeal and vagina (Filius et al., 2005, Altoparlak et al., 2004). The severity of underlying disease, mechanical ventilation and the presence of nasogastric feeding tubes were associated with oropharyngeal Gram-negative bacteria colonization (Korona-Glowniak et al., 2012, Johanson et al., 1969).

Most of medically important *Enterobacteriaceae* can cause a wide variety of infections in both healthy people and those with pre-existing illness either in the community or hospital setting. Colonized patients have a higher risk of hospital acquired o nosocomial infection than patients who are not colonized. Generally, those pathogenic *Enterobacteriaceae* can be classified into 3 groups:

i) Coliforms that can rapidly ferment lactose eg *E.coli* and *Klebsiella spp*

ii) Non-coliform which do not ferment lactose eg *Proteus spp*

iii) True pathogen, eg *Salmonella spp* and *Shigella spp*.

Both coliform and non-coliform are opportunistic pathogens with the infecting bacteria can be either exogenous or endogenous. Majority of nosocomial infections appear to arise from endogenous flora. However, coliform bacteria other than *E.coli* are frequently found in tap water or even in distilled or deionized water. They may persist or actively multiply in water associated with respiratory therapy or hemodialysis equipment that can lead to hospital outbreak (Marilyn,1996). Members of

Enterobacteriaceae are the most common Gram negative isolates in microbiology laboratories and can be isolated from numerous clinical specimen including blood, sterile body fluid and others non sterile sample including pus, urine or samples from respiratory tract.

1.1.3 Pathogenicity factors

Generally, the virulence factors of the pathogenic micro-organism depending on the ability of these organisms to enter, establish and multiplication in the host, avoidance of host defence mechanisms, causing tissue damage and exit. These properties are usually depending on the structural of the organism itself, the ability of the organism to produce toxins and the presence of genes that encoded a special virulence factors especially antimicrobial resistance genes (Finlay, 1997).

Structurally, members of *Enterobacteriaceae* are rod in shaped measuring about 1 to 3 μm in length and 0.5 μm in diameter with the presence of surface appendages including pili and flagella. They have both inner and outer phospholipid membranes, which enclose a periplasmic space that contains the peptidoglycan cell wall. The outer membrane is comprise of asymmetrical lipid bilayer with the inner part composed of mainly phospholipids and the outer part composed of lipopolysaccharide (LPS).

Pili and the outer membrane protein have the adhesive properties which aid these bacteria to enter the host. Type 1 pili or also known as type 1 fimbriae are ubiquitous in *Enterobacteriaceae*. It is composed of a rigid rod formed by repeating subunits of the FimA protein with the tips of these pili contain a short fiber composed of FimH and FimG. The FimH which is type 1 fibrial adhesin will bind to mannose residues in

glycoproteins and glycolipids on host cell surfaces that result in adherence and later bacterial invasion (Hung, et al 2002; Ofek, et al 1977).

While the LPS which consist of lipid A, a core phosphorylated oligosaccharide with the repeating oligosaccharide side chains is a very potent virulence factors for the members of *Enterobacteriaceae*. Lipid A or also known as endotoxin is biologically active portion of LPS has the extraordinary potency to induce the innate immune response via signalling Toll like receptors 4 (TLR4) that lead to release of pro-inflammatory cytokines including tumor necrosis factor, chemokines, interlukin-6 and major histocompability complex receptors. The host response to LPS is the important factor in determining outcome gram negative bacterial infections (Miller, et. al). Other than endotoxin, many of the *Enterobacteriaceae* members able to produce toxins that capable to induce lysis of the host cells that cause tissue damage known as hemolysins (Marilyn, 1996) Besides that, these organisms also have the capability to produce additional surface polysaccharide, including enterobacterial common antigen, colonic acid and an envelope of surface polysaccharide known as capsule (Whitfield, 1999). In certain genera the capsules can be quite luxuriant causing highly mucoid colonial morphology such as *Klebsiella* and *Enterobacter*. These capsule provide the ability of these bacteria to avoid phagocytosis and killing by human serum. The K1 capsule produced by many *E.coli* strains has been implicated with bacterial survival while passing the blood brain barrier which leads to meningitis (Scholl et al., 2005). Other than that the capsule of *Klebsiella pneumoniae* are important for colonization of the urinary tract (Struve and Krogfelt, 2003) and the K54 capsule in *E.coli* is important for systemic infections (Russo and Singh, 1993).

The other important pathogenicity factor in *Enterobacteriaceae* is by having a several highly efficient systems that scavenge iron from the host in order to proliferate and survived within the host. The proliferative capability of many invasive pathogens is limited by the bioavailability of iron as an essential element for their growth (Parrow et al., 2013). These systems are control by a ubiquitous regulator protein that activates gene transcription at low iron concentration. Most of these organisms have produced low molecular weight iron-chelating molecules known as siderophores and certains strains have the capability to bind and transport the heme (Mandell, 2015).

As known, the *Enterobacteriaceae* family have genomic structure consists of a single circular chromosome which may include multiple plasmids of various sizes in the cytoplasm. These plasmids which are an extrachromosomal autonomously replicating DNA element play major roles in pathogenesis of the infection. Even though they are not considered as virulence factors per se, the encoded genes in the plasmids may contribute to the presence of virulence factors in certain bacteria. In addition, most of these plasmids are very promiscuous with regard of their ability to transfer the encoded genes between the others genera. The most concern now is the emergence and dissemination of broad host range plasmids containing antimicrobial resistance genes among the *Enterobacteriaceae* members that lead to global spread of multidrug resistant organism (Schultsz and Geerlings, 2012).

1.1.4 Infection related to *Enterobacteriaceae*

Enterobacteriaceae can cause a wide variety of infections includes community acquired infection and hospital acquired infection which can be occur as sporadic infection or outbreaks. As most of the *Enterobacteriaceae* members inhabit the human's

gastrointestinal tract, the intraabdominal infection is primarily occurred due to the invasion of these bacteria through the mucous tissues or via the breaks of intestinal wall such in perforated appendix. Some of *Enterobacteriaceae* members can be transmitted by consumed contaminated food or water for example *Salmonella*, *Shigella* and *E.coli* (Enterotoxigenic *E.coli* or Enterohaemorrhagic *E.coli*). The extraintestinal infections can be occurred as the *Enterobacteriaceae* have the extended niche especially in those with these bacteria colonization. It is also known that these bacteria can be transmitted by person to person which an important transmission route in health care associated infections (HAI) which also can leads to outbreaks. The coliforms and *Proteus* spp. are the most commons *Enterobacteriaceae* members that cause community acquired infection. Those bacteria related to community acquired infection are usually having a known virulence factors. *E.coli* is among the species frequently isolated from clinical specimens known to cause infections including cholecystitis, cholangitis, urinary tract infection and bacteraemia. *E.coli* was found as a leading cause of community acquired urinary tract infection (Dias Neto et al., 2003). The pathogenic strains of *E.coli* differ from commensal organisms by which they produce virulence factors specific for each pathotype. Those related to urinary tract infection having a specific virulence factors include P-fimbriae and α-hemolysins (Bien et al., 2012). The *E.coli* 0157:H7 or also known as enterohaemorrhagic *E.coli* produce Shiga toxins can cause varying severity of the disease including watery diarrhoea, bloody diarrhoea, haemorrhagic colitis, haemolytic uraemic syndrome (HUS) and death (Nguyen and Sperandio, 2012). According to one prospective surveillance study from eight Asian countries, *K. pneumoniae* was the second most common pathogen (15.4%) causing community acquired pneumonia (Song et al., 2008) and it

was associated with significant high mortality rate (29.7%) (Lin et al., 2015). *P. Mirabilis* usually related to complicated UTIs, such as patients with functional or anatomical abnormalities of urinary tract, especially those with urolithiasis as it has the capability to produce urease that promote further urolithiasis (Chen et al., 2012).

Healthcare-associated infections (HAIs) were a major problem in hospitals Worldwide. Based on CDC 2016, HAI was defined as the infection fitted with the NHSN site-specific infection criterion and the date of event occurred on or after the 3rd calendar day of admission to an inpatient location where day of admission is consider as day one. The *Enterobacteriaceae* was found to be the most common isolates (28%) involved in HAIs and overall, 45% of these bacterial isolates were multidrug resistant (Morgan et al., 2010). According to CDC 2016, the HAI was further divided as central line-associated blood stream infection (CLABSI), catheter- associated urinary tract infection (CAUTI), surgical site infection (SSI) and ventilator-associated pneumonia (VAP).

1.1.5 Treatment

Generally, the antimicrobial therapies that can be used to treat *Enterobacteriaceae* infections are fluoroquinolones, beta-lactams and aminoglycosides. The beta-lactam antibiotics which inhibit cell wall synthesis are widely used to treat infection that caused by *Enterobactericeae* members. The beta-lactams agents with antibacterial activity against enteric bacteria include amoxicillin, ampicillin, piperacillin, ticarcillin. However, in the presence of strain that can produce beta-lactamase enzymes limit of these beta-lactams agent usage as it can lead to treatment failure. Thus, they are

commonly used in combination with beta-lactamase inhibitors such as clavulanic acid, sulbactam or tazobactam.

According to the Ambler classification there are four molecular classes of β-lactamases namely, A, B, C and D. The genes that encode beta-lactammases enzyme AmpC type which an Ambler class C is present in *Serratia, Citrobacter* and *Enterobacter* (MacDougall, 2011). It can rapidly hydrolyze penicillins, cephalosporins, and monobactams but not inhibited by the beta-lactamase inhibitors. Treatment of *Enterobacter* infections with third-generation cephalosporins may select for mutant strains associated with hyperproduction of AmpC beta-lactamase that cause about 5% to 20% of these bacteria developed resistant during therapy (MacDougall, 2011). Approximately 33% of bloodstream isolates have been shown to contain *E. cloacae* that produce ESBL as well as AmpC beta-lactamases that make these strain not only resistant to third-generation but also to fourth-generation cephalosporins (Szabo D, 2005). Thus, the use of these agents in *Enterobacter spp* infection should be avoided for the treatment of serious infections.

The other important beta-lactamase enzyme was extended spectrum beta-lactamases (ESBLs) which an Ambler class A and D. The spread of ESBLs in health care setting which also has been found in community-acquired infections causing difficulty in treating *Enterobacteriaceae* infections (Valverde and Cantón, 2004). These β-lactamases enzyme caused bacterial resistance to the penicillins, cephalosporins including the extended spectrum and aztreonam (but not to cephamycins or carbapenems). However they were inhibited by beta-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam (Bush, 2011). The most common ESBLs

are SHV- and TEM- with the CTX-M were increasingly detected worldwide (Paterson , 2005). TEM-1, TEM-2, and SHV-1 were conferring resistance to ampicillin, amoxicillin, and other penicillins, as well as to early but still sensitive to 3rd generation cephalosporin. However, some of *Enterobacteriaceae* members especially those commonly causing hospital acquired infection like *Klebsiella pneumoniae* began to produce mutated versions of these beta-lactamases that made them resistant to third-generation cephalosporins and to the monobactam aztreonam. According to the National Nosocomial Infections Surveillance (NNIS) System report, in 2003, about 20.6% of *K.pneumoniae,* 31.1% of *Enterobacter* spp and 5.8% of *E.coli* isolated from patients in ICU were nonsusceptible to third generation cephalosporin (CDC, 2004). In a study of patients with ESBL-producing *K.pneumoniae* bacteremia, 54% of patients receiving treatment with a susceptible cephalosporinas determined by in vitro methods, experienced clinical failure (Paterson, 2001). Subgroup analysis from a randomized, evaluator-blind trial comparing cefepime with imipenem in patients with nosocomial pneumonia showed that 100% of patients receiving imipenem for pneumonia caused by an ESBL producer experienced a positive clinical response compared with only 69% of patients treated with cefepime (Zanetti, 2003). A prospective, observational study of patients with *K.pneumoniae* bacteremia reported an all cause14-day mortality rate of 3.7% with carbapenem alone, compared with rates of 36.3% and 44.4% with quinolone and noncarbapenem beta-lactam monotherapy, respectively (Paterson, 2004). Thus, carbapenems currently considered to be the most preferred agents for treatment of serious infections caused by *Enterobacter* spp and ESBL-producing *Enterobacteriaceae*. These drugs were highly stable to beta-lactamase hydrolysis, and porin penetration is facilitated by their general size and structure.

1.2 Carbapenem Resistant *Enterobacteriaceae* (CRE)

1.2.1 Definition

The previous Centers for Disease Control and Prevention (CDC) CRE definition based on 2012 CRE toolkit is an *Enterobacteriaceae* that nonsusceptible to imipenem, meropenem, or doripenem and also resistant to all third generation cephalosporins. However, in the development of molecular method, it was found that this definition might miss some carbapenamase producing CRE (CP-CRE). CDC began to identify CRE that were positive for the KPC gene and were resistant to ertapenem that not included in previous definition but still susceptible to the other carbapenems (Arnold et al., 2011). Other than that, CRE producing OXA-48-type carbapenemases might not exhibit resistance to third-generation cephalosporins which might be missed by the previous definition (Singh-Moodley and Perovic, 2016). The pre-2015 CDC CRE surveillance definition was found misclassified nearly 13% of carbapenem-nonsusceptible *Klebsiella* spp. isolates and 21% of KPC-producing *Klebsiella* spp. isolates as non–carbapenemase producing (Nora et al., 2015).

The ability to detect CP-CRE is an important component of outbreak investigations as it's possess a more stable and transferable form of resistance and this resistance can spread through either clonal expansion or transfer of carbapenemase genes to naive bacteria (Nordmann P, 2011). In an attempt to further increase the ability to identify CRE that produce carbapenemases, CDC in January 2015 has modified the CRE definition to *Enterobacteriaceae* which was resistant to imipenem, meropenem, doripenem, or ertapenem or documentation of carbapenemase production from the isolates. Ertapenem which included in the new definition is the

most sensitive indicator in detecting KPC-producing strain which has moderately high positive predictive value (79%) with a very high specificity (99.2%) (McGettigan et al., 2009). In some *Enterobacteriaceae* which intrinsically elevated minimum inhibitory concentration (MICs) to imipenem including *Proteus spp.*, *Morganella spp.* and *Providencia Spp* (CLSI and Wayne, 2012) the imipenem resistance alone does not consider as CRE. The other carbapenems which are meropenem, doripenem, and ertapenem should be used for these organisms to determine if these organisms meet the CRE definition.

There were several methods available for detecting carbapenamases with the acceptable methods by CDC including polymerase chain reaction (PCR), Carba-NP test and mettalo-β-lactamase testing such as Modified Hodge Test (MHT). The MHT was widely used in clinical laboratories as it was simple and inexpensive with documented good sensitivity between 93% to 98% for many carbapenemases, including VIM, IMP, and OXA-48-like enzymes except NDM (Vasoo and Lolans, 2013). A new CDC 2015 CRE definition which included a MHT was able to reduce the false positive result from 55% to 12% especially involving the less likely organism to produce carbapenamases such as *Enterobacter* spp (Nora et al., 2015)

As the above phenotypic definition lacks specificity for CP-CRE, the CDC had encouraged clinical and public health laboratories which have the capacity to perform molecular method to test carbapenem resistance mechanism in order to detect the presence of carbapenemases especially KPC, NDM and OXA-48-type carbapenemases enzyme.

1.2.2 Epidemiology

CRE especially CP-CRE has emerged as a global threat with the carbapenemases in *Enterobacteriaceae* have been reported increasingly in the past 10 years (Thaden et al., 2014, Nordmann, 2011). The spread was quite worrisome as it not constrained to a particular geographical location anymore with the current ease of travel and global migration. *Klebsiella pneumoniae* carbapenemases (KPC) have been reported in the United States and then worldwide, with a marked endemicity at least in the United States (Cerqueira et al., 2017). Metalloenzymes (Verona integron-encoded metallo-β-lactamase, IMP) also have been reported worldwide, with a higher prevalence in Mediterranean countries. Carbapenemases of the oxacillinase-48 (OXA) type have been identified mostly in North Africa and West Europe (Nordmann et al., 2011a). Recently, a New Delhi metallo-beta-lactamase (NDM) carbapenemases which originally identified in Sweden in 2008 have been spread worldwide rapidly (Dortet et al., 2014).

In Asia, the prevalence rate of CRE was still low with the rates of resistance to impenem and meropenem in *Enterobacteriaceae* are 0.8% and 1.0% respectively in a study period of 2001 to 2012 but it shows increasing in trend (Xu et al., 2015). However, this data only was provided by 19 countries out of 49 Asia countries with the Malaysia CRE prevalence rate was reported about 1% to 5%. The most common CRE isolated in Asia was *Klebsiella* spp. (39.26%) followed by *E.coli* (21.97%), *Serratia* spp. (19.80%), *Enterobacter* spp. (12.97%), *Proteus* spp. (3.95%) and *Citrobacter* spp. (2.0%). *K. pneumoniae* was the most prevalent species in various conducted study in many regions including in US (Guh et al., 2015, Thaden et al., 2014), Asia pacific

region; Taiwan (Tang et al., 2016) and Singapore (Ling et al., 2015). However, the bacterial distribution of CRE isolates may vary every region thus surveillance investigation should be perform to establish its own epidemiological characteristics.

Based on unpublished data from National Alert Organism Surveillance 2016, overall the incidence rate of CRE in Malaysia was increased in trend from year 2013 to 2015. However, the rate was static from year 2015 to 2016 with the incidence rate of 0.05 of total isolates per 100 admissions even though the frequency of CRE cases have been reported in 2016 increasing in trend (unpublished data 2016, MOH). Most of CRE was recovered from blood sample (26.4%) followed by urine (21.2%), rectal swab (20.1%) and tracheal aspirate (8.4%) with the third most common CRE isolated were *K. pneumoniae* (76.4%), *E. coli* (10.4%) and *Enterobacter* spp. (5.6%). About 46.3% of isolates were related to true infection which was quite close with others study (Thaden et al., 2014) with the 59.9% causing blood stream infection, 13.5% UTI, 7.7% pneumonia, 4.8% SSI and others. Carbapenamases gene detection of the total isolates revealed that the NDM-1 was the most common responsible gene which was about 78% followed by OXA-48 (6.14%), OXA-181 (3.46%) and the others rare genes that were detected including KPC, IMP and VIM. It was found that about 4% of the total isolates in 2016 were resistant to polymixin. In the study area within 2013 to 2015 period, it was found that *Klebsiella pneumoniae* was the predominant species (95%) with the genotype determination shows about 82.4% were positive for New Delhi metallo-β-lactamase 1 (NDM-1) gene (bla_{NDM1}) (Zaidah et al., 2017).

Most of the CRE infections are related to healthcare setting which was about 70 to 90% of the cases (Thaden et al., 2014, Tang et al., 2016). Those patients were acquired infection after 48 hour of hospital admission or with the presence of previous hospitalization, surgery, dialysis, or residence in a long-term care facility in the 12 months preceding identification or with the presence of an invasive device. There were several risk factors have been identified associated with acquisition of CRE infections in various study, including exposure to antibiotics, healthcare exposure, presence of indwelling devices, use of mechanical ventilator, and with the presence of co morbidities especially diabetes mellitus, chronic renal failure and immunodeficiency patients (Ling et al., 2015, Wang et al., 2016). However, it has been reported about 30% of CRE infections were acquired from community settings in Taiwan (Tang et al., 2016) and 6% in the south eastern United States (Thaden et al., 2014). The most common community acquired infection related to CRE was urinary tract infection followed by pneumonia which mostly occurred in elderly (Tang et al., 2016).

1.2.3 Mechanisms of resistance.

There were two main mechanisms of carbapenems resistant in CRE which by the production of β-lactamases (cephalosporinase ; AMP-C β-lactamase and ESBL) in combination with decreased permeability due to porin loss or by the production of a carbapenem-hydrolyzing β-lactamase (carbapenamase) that able to hydrolyze the carbapenems itself (Nordmann et al., 2011a). Porins were hydrophilic protein channels that located at the outer membranes of *Enterobacteriaceae* which allow essential nutrients uptakes for bacterial survival. The presence of these porins also allows the transport of antibiotics across the outer membrane of these bacteria into the periplasmic space. In *K.pneumoniae,* there were two major porins involved with this function which

OmpK35 and OmpK36 that homologous to OmpF and OmpC in *E.coli* (Tsai et al., 2011). The absence or modification of these porins will lead to decrease the permeability of these bacteria to the antibiotics. The combination of AMP-C β-lactamases or ESBL with the porin loss usually associated with low level of resistant of carbapenems and commonly affected ertapenem more than meropenem or imipenem (Domenech-Sanchez et al., 2003) .

The most concern mechanism of resistant in CRE was a consequence of acquisition of carbapenemase genes as it usually associated with high level of resistant to carbapenems and has been spread worldwide (Queenan and Bush, 2007). These carbapenamases resistant genes resides in the mobile genetic elements including transposons, integrons or plasmid causing potential spread even to the naive bacteria. There were large variety of carbapenemases have been identified in *Enterobactericeae* that were classified in 3 classes of Ambler molecular classification based on amino acid homology which Ambler class A, B and D β-lactamases. They were different in terms of active site of hydrolysing beta lactams whereby class A and D required a serine residue at their active site, whereas class B need zinc for their activities. Details on each class were discussed below.

1.2.3.1 Class A Carbapennemases

There were three major families of class A serine carbapenemases include the NMC (not metalloenzyme carbapenemase)/IMI (imipenem-hydrolyzing β-lactamase), SME (*Serratia marcescens* enzyme), and KPC enzymes. The other rare carbapenamase that previously regard as ESBL is GES (Guiana extended spectrum). These carbapenamases can be chromosome encoded including NmcA, Sme and IMI-1or plasmid encoded including KPC, IMI-2 and GES. All have the ability to hydrolyze a broad variety of β-

lactams including carbapenems, cephalosporins, penicillins, and aztreonam but they were inhibited by clavulanate and tazobactam. KPCs were the most common class A carbapenamase that was first identified in 1996 in the United States (Yigit et al., 2001) then was spread globally in a few years. Although the KPC β-lactamases are predominantly found in *K.pneumoniae*, there have been reports of these enzymes in other species including *Enterobacter* spp. and in *Salmonella* spp. (Bratu, 2005, Miriagou and Whichard., 2003). KPC carbapenemases hydrolyzed all classes of β-lactams especially penicillins and early cephalosporin but 10-fold-less efficiently to hydrolize cefotaxime and aztreonam (Queenan and Bush, 2007). The level of resistance to carbapenems of KPC producers may vary markedly with the ertapenem was the lowest activity (Nordmann et al., 2011a). The important of KPC family was it has the greatest potential for spread due to its location on plasmids, especially since it was most frequently found in *K.pneumoniae*, an organism notorious for its ability to accumulate and transfer resistance determinants.

1.2.3.2 Class B Metallo-B- Lactamases

The most common class B enzymes involved in CPE including Verona integron-encoded metallo-beta-lactamase (VIM), imipinemase metallo-beta-lactamase (IMP) and New Delhi metallo-beta-lactamase (NDM). The first acquired MBL, IMP-1, was reported in *Serratia marcescens* in Japan 1994 (Osano et al., 1994). These enzymes hydrolyze all β-lactams except aztreonam and their activity was inhibited by ethylene diamine tetra acetic acid (EDTA) but not by clavulinic acid. Resistance levels to carbapenems in MBL producers may vary. Recently, the NDM-1 β-lactamase enzymes give a major concern as it has been reported to occur in various bacterial species all

over the world since it was first reported in 2009 (Yong et al., 2009). Although most of the NDM-producing strains identified were *Enterobacteriaceae*, this carbapenemase has also been reported from *Acinetobacter* spp. and more rarely from *P. Aeruginosa* (Dortet et al., 2014). The widespread dissemination of blaNDM-1 was mainly due to promiscuous plasmids and clonal outbreaks. Compared with other carbapenamases, NDM-1 has deeply disconcerting for public health worldwide as it was not only easily to spread but may cause pan drug resistance as plasmids carrying the $bla_{\text{NDM-1}}$ gene can harbor a high number of resistance genes associated with other carbapenemase genes (OXA-48 types, VIM types), plasmid-mediated cephalosporinase genes, ESBL genes, aminoglycoside resistance genes (16S RNA methylases), macrolide resistance genes (esterase), rifampin (rifampin modifying enzymes) and sulfamethoxazole resistance genes (Kumarasamy et al., 2010, Nordmann et al., 2011b).

1.2.3.3 Class D Enzymes of the OXA Type

Class D β-lactamases, also known as OXA-type enzymes or oxacillinases, were represented by more than 350 genetically diverse enzymes that widely disseminated in Gram-negative bacteria. They were not inhibited by clavulanic acid, tazobactam, and sulbactam but inhibited in vitro by sodium chloride (NaCl). They were broadly classified into narrow- and extended-spectrum enzymes based upon the conferred resistance profile against β-lactam antibiotics. Class D carbapenemases, also known as carbapenem-hydrolyzing class D β-lactamases (CHDLs) (Poirel et al., 2010), represent a further expansion of the substrate profile of class D enzymes to include carbapenem antibiotics. CHDLs have been subdivided into several subgroups based on their amino acid sequence identity and those enzymes belonging to the OXA-23,

OXA-24/40, OXA-48, OXA-51, OXA-58, and OXA-143 subgroups of a major clinical importance due to their wide dissemination in bacterial pathogens. The majority of these carbapenemases, have been identified in various *Acinetobacter* isolates, predominantly in *Acinetobacter baumannii* except for OXA-48 (Evans et al., 2013). OXA-48 first identified from carbapenem-resistant *K. pneumoniae* isolates in Turkey, 2001 (Poirel et al., 2004) and this enzyme has spread among other members of the *Enterobacteriaceae* family including *E.coli* and *Enterobacter spp.* (Poirel et al., 2012b). This enzyme hydrolyses penicillins at a high level and carbapenems at a low level, sparing broad- spectrum cephalosporins especially ceftazidime and cefepime but was not susceptible to β-lactamase inhibitors. When combining permeability defects or with the presence of ESBL, OXA-48-like producers may exhibit a high level of resistance to carbapenems(Poirel et al., 2004). The OXA-48 enzyme was a plasmid encoded ($bla_{\text{OXA-48}}$ gene) and it was not associated with an integron, in contrast to most of the oxacillinase genes. Molecular investigations showed that plasmid pOXA-48a was identified in all OXA-48-producing isolates recovered from many countries (Poirel et al.,2012a).

1.2.4 Laboratory diagnosis

The detection of CRE especially CP-CRE is very important for infection control measure along with patient's management itself. The confirmation of *Enterobactericeae* family should be done first by morphological characteristic of colonies, gram stain and biochemical testing. In difficult identification of the species an automated system may be required such as VITEK 2 System, Microscan and others. Then, those isolates that were suspected of CRE based on antibiotic susceptibility testing (disc diffusion method) towards carbapenems will proceed to

confirmatory test. Confirmation of CRE based on CDC definition has been discussed before either by knowing the MICs level of all carbapenems, phenotypic tests to demonstrate the presence of carbapenamases enzymes or by molecular method.

1.2.4.1 Culture method

Enterobacteriaceae can be grown on various basic and selective media such as nutrient agar, MacConkey agar and blood agar. The isolates should be incubated in an incubator at 35˚C for 20 to 24 hours. Their colonies can be differentiate on MacConkey agar based on their lactose fermenter properties. Certain *Enterobactericeae* family can produce a special colonies pigment which can help in identification of this organism such as *Serratia marcescens* that produced red pigment.

1.2.4.2 Biochemical Tests

The basic biochemical test including triple sugar iron reaction, motility, indole, citrate, urease, methyl red and voges-prosakaur allows the differentiation of the various members of *Enterobacteriacea*. In difficult identification, an extended biochemical testing may be required either by using manual identification products such as API 20E or automated systems such as Vitek2 System (GN card).

1.2.4.3 Antibiotic Susceptibility Testing (AST)

The detection of CRE in clinical laboratory was based on susceptibility testing results obtained either via automated systems, liquid media, e test and disk diffusion

tests (Nordmann et al., 2012a). AST is in vitro testing of bacterial cultures with antibiotics which were used to determine susceptibility of bacteria to a specific antibiotic therapy. Generally, the AST can be done by dilution, diffusion or combination method which known as antimicrobial gradient method or Epsilometer test (E test).

The optimal interpretation of susceptibility test requires knowledge of the pharmacokinetics of the drugs in humans and the ability of particular drug in eradicating bacteria at various body sites. It should be referred to an expert source such as the CLSI. The CLSI zone size and MIC interpretive criteria are established by analysis of 3 kinds of data : (1) microbiologic data, including a comparison of MICs and zone sizes on a large number of bacterial strains, including those with known mechanisms of resistance that have been defined either phenotypically or genotypically; (2) pharmacokinetic and pharmacodynamic data; and (3) clinical studies results (including comparisons of MIC and zone diameter with microbiological eradication and clinical efficacy) obtained during studies prior to FDA approval and marketing of an antibiotic (Wayne, 2008). It provide break points for both MIC values and disk diffusion zone diameters for all relevant antibiotics most of the bacterial genera which give the clinical interpretation of susceptibility to susceptible, intermediate or resistant. A “susceptible” result indicates that the organism should respond to therapy with that antibiotic using the dosage recommended normally for that type of infection and species. Conversely, an organism with a MIC or zone size interpreted as “resistant” should not be inhibited by the concentrations of the antibiotic achieved with the dosages normally used with that drug. An “intermediate” result indicates that a microorganism falls into a range of susceptibility in which the MIC approaches or exceeds the level of

antibiotic that can ordinarily be achieved and for which clinical response is likely to be less than with a susceptible strain. Most of the clinical laboratory used CDC definition for the diagnosis of CRE by using the break point of carbapenems from CLSI guideline. The revised interpretative criteria for carbapenems were published by Clinical and Laboratory Standards Institute in CLSI M-100-S20-U, June 2010 lowered the carbapenems breakpoint significantly with addition of doripenem breakpoint to permit better detection of carbapenem-resistant isolates. Details of the carbapenems break point were presented in the Table 1.

Table 1 Carbapenems breakpoints according to CLSI 2012 (M100-S26).

Carbapenems	**S**	**I**	**R**
Doripenem	≤1	2	≥4
Ertapenem	≤0.5	1	≥2
Imipenem	≤1	2	≥4
Meropenem	≤1	2	≥4

1.2.4.3.1 **Dilution method**

Dilution susceptibility testing methods were used to determine the minimal concentration of antimicrobial to inhibit growth of the microorganism or also known as minimal inhibitory concentration (MIC). This can be achieved by dilution

of antimicrobial in either agar or broth media. Antimicrobials were tested in log_2 serial dilutions (two fold). The macrobroth or tube-dilution method was one of the earliest AST methods (Ericsson and Sherris, 1971). This procedure involved preparing two-fold dilutions of antibiotics in a liquid growth medium dispensed in test tubes with the antibiotic containing tubes were inoculated with a standardized bacterial suspension. The tubes were examined for visible bacterial growth as evidenced by turbidity after overnight incubation at 35˚C. The MIC expressed as the lowest dilution, which inhibited growth judged by lack of turbidity in the tube.

The main advantage of the 'broth dilution' method for the MIC determination lies in the fact that it can readily be converted to determine the minimum bactericidal concentration (MBC) as well. This method has been simplified by the used of small, disposable and plastic "microdilution" trays thus, it was namely as microbroth dilution method. The agar dilution technique has advantage in terms of the ability to test several organisms on each plate. The presence of bacterial colonies on the plates after incubation indicates growth (Wiegand et al., 2008). The MIC in this technique was the antibiotic concentration of the first plate showing ≥ 99% inhibition. The major disadvantages of this method were due to labour intensive, time consuming and have a higher cost.

1.2.4.3.2 **Diffusion method**

Diffusion tests or also known as disc diffusion method was widely used to determine the susceptibility of organisms isolated from clinical specimens as it was simple, practical and has been well standardized. There were two method of disc diffusion test

which the Kirby-Bauer and Stokes' methods with the Kirby-Bauer method being recommended by the Clinical Laboratory Standards Institute (CLSI). The test was performed by inoculated a standardized bacterial inoculum to the Mueller-Hinton agar plate followed by placement of antimicrobial disks. The disks used for a disk diffusion assay contain a standardized known amount of an antimicrobial agent, which diffuse into agar when in contact with the agar surface. The zones of growth inhibition around each of the antibiotic disks are measured to the nearest millimetre after incubated under standardized conditions. The diameter of the zone is related to the susceptibility of the isolate and to the diffusion rate of the drug through the agar medium. The zone diameters of each drug are interpreted using the criteria published by the CLSI which provide a "qualitative" rather than MIC that categorized as susceptible, intermediate, or resistant. Many variables can influence the zone sizes such as depth of agar, inoculums density and the size or molecular weight of tested antibiotics that can affected the diffusion process which can lead to unreliable results. In view of those limitations; when equivocal results are obtained or in prolonged serious infection e.g. bacterial endocarditis, the quantitation of antibiotic action vis-a-vis the pathogen needs to be more precise by doing the test that able to determine the MIC of the antibiotic to the organisms concerned.

The disk diffusion breakpoints identified a much higher proportion of the carbapenemase producers than the MIC breakpoints thus, it was a useful tool in screening for CP-CRE especially for KPC-producing and VIM-producing *K. pneumoniae* (Vading et al., 2011).

1.2.4.3.3 **Epsilometer test (E test)**

E test also known as the epsilometer test was an antimicrobial gradient diffusion method uses the principle of establishment of an antimicrobial concentration gradient in an agar medium as a means of determining susceptibility. The E test was a quantitative method for antimicrobial susceptibility testing applies both the dilution of antibiotic and diffusion of antibiotic into the medium. A predefined stable antimicrobial gradient was present on a thin inert carrier strip and once this E test strip applied onto an inoculated agar plate, there was an immediate release of the drug. Following incubation, a symmetrical inhibition ellipse will be produced. The intersection of the inhibitory zone edge and the calibrated carrier strip indicates the MIC value over a wide concentration range (>10 dilutions) with inherent precision and accuracy. Generally, E test results have correlated well with MICs generated by broth or agar dilution methods (Rennie et al., 2012, Baker et al., 1991).

1.2.4.4 Automated systems

There are 4 automated instruments presently cleared by the FDA for use in the United States with three of these can generate rapid (3.5 to16 hours) susceptibility test results including MicroScan WalkAway, BD Phoenix Automated Microbiology System and Vitek 2 System while the fourth is an overnight system which was Sensititre ARIS 2X (Trek Diagnostic Systems). This instrumentation can provide a standardize reading of end points and often produce susceptibility test results in a shorter period than manual readings as it has a sensitive optical detection systems that allow detection of subtle changes in bacterial growth. It have been shown that providing rapid susceptibility test results can lead to more timely changes to appropriate antimicrobial therapy, substantial