Mechanisms of colistin resistance and the antimicrobial effects of antibiotic and adjuvant combination on colistin-resistant *Klebsiella pneumoniae*

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กลไกการดื้อยาโคลิสตินและผลการต้านเชื้อจากการใช้ยาต้านจุลชีพและสารเสริมฤทธิ์ ร่วมกันต่อเชื้อ *Klebsiella pneumoniae* ที่ดื้อยาโคลิสติน

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เอย์ มยา ซิทู เซียน : กลไกการดื้อยาโคลิสตินและผลการต้านเชื้อจากการใช้ยาต้านจุลชีพและสารเสริมฤทธิ์ ร่วมกันต่อเชื้อ *Klebsiella pneumoniae* ที่ดื้อยาโคลิสติน. (Mechanisms of colistin resistance and the antimicrobial effects of antibiotic and adjuvant combination on colistin-resistant *Klebsiella pneumoniae*) อ.ที่ปรึกษาหลัก : ธนิษฐา ฉัตรสุวรรณ, อ.ที่ปรึกษาร่วม : อัษฎาศ์ ลีฬหวนิชกุล

ความชุกของเชื้อ *Klebsiella pneumoniae* ที่ดื้อยาโคลิสติน (ColRkp) เพิ่มสูงขึ้นทั่วโลก การศึกษานี้มีวัตถุประสงค์เพื่อศึกษากลไกการดื้อยาโคลิสตินของเชื้อ ColRkp ที่แยกได้จากผู้ป่วยในประเทศไทย และศึกษาระดับการแสดงออกของปัจจัยก่อโรคที่สัมพันธ์กับเชื้อ ColRkp ร วมถึงประเมินการเสริมฤทธิ์กันของยาต้านจุลชีพและสารเสริมฤทธิ์ต่อเชื้อ ColRkp ทั้งในหลอดทดลองและสัตว์ทดลอง โดยทำการศึกษาในเชื้อ *K. pneumoniae* ที่ดื้อยาคาร์บาพีเนม จำนวน 165 สายพันธุ์ จากโรงพยาบาลจุฬาลงกรณ์ ระหว่างปี 2559 ถึง 2564 พบความชุกของการดื้อยาโคลิสตินร้อยละ 28.5 (47 สายพันธุ์) ซึ่งทั้งหมดเป็นเชื้อดื้อยาแทบทุกขนาน (XDR) และเชื้อดื้อยาทุกขนาน (PDR) โดยเชื้อ ColRkp พบมีการกลายพันธุ์ของยีน *mgrB*, *pmrB* และ *phoQ* ซึ่งอยู่บนโครโมโซมของเชื้อร้อยละ 91.5 และพบยีนดื้อยาโคลิสตินที่อยู่บนพลาสมิด คือ ยีน *mcr-1.1*, *mcr-8.1* และ *mcr-8.2* ในเชื้อร้อยละ 8.5 ซึ่งเป็นเชื้อที่มีเฉพาะยีน *mcr* หรือมียีน *mcr* ร่วมกับการกลายพันธุ์ของโปรตีน PmrB ที่ R256G นอกจากนี้ยังพบว่ายีน *mgrB* มีการกลายพันธุ์ที่เป็นการแทรกหรือการหายไปหรือการแทนที่ของนิวคลีโอไทด์ในเชื้อ ColRkp ร้อยละ 85.1 โดยมีความสัมพันธ์กับการเพิ่มการแสดงออกของ *phoPQ* และ *pmrK* ซึ่งเกี่ยวข้องกับการเติมหมู่ Ara4N ให้กับ lipopolysaccharide ที่เป็นกลไกสำคัญในการดื้อยาโคลิสติน เนื่องจากยีน *mgrB*, *pmrAB* และ *phoPQ* เป็นยีนที่เกี่ยวข้องกับปัจจัยก่อโรคของเชื้อ การศึกษานี้พบว่าเชื้อดื้อยาโคลิสตินที่ดื้อยาแทบทุกขนาน (XDR ColRkp) มีการสร้างไบโอฟิล์มเพิ่มขึ้นอย่างมีนัยสำคัญ นอกจากนี้เชื้อ XDR ColRkp ยังมีการเปลี่ยนแปลงการแสดงออกของยีนที่สร้างปัจจัยก่อโรคอย่างมีนัยสำคัญ การศึกษาการใช้ยาต้านจุลชีพและสารเสริมฤทธิ์ร่วมกันแบบใหม่ คือ ยาโคลิสตินร่วมกับ EDTA พบการเสริมฤทธิ์กันในเชื้อ XDR และ PDR ColRkp ที่เจริญทั้งในแบบเซลล์อิสระและไบโอฟิล์ม ในหลอดทดลอง และพบว่าการใช้ยาโคลิสตินร่วมกับ EDTA มีประสิทธิภาพในการรักษาโดยสามารถกำจัดเชื้อ ColRkp ที่สร้างไบโอฟิล์มและก่อโรคติดเชื้อที่สัมพันธ์กับสายสวน รวมถึงทำให้ความเสี่ยงในการติดเชื้อซ้ำหมดไปทั้งในสัตว์ทดลองและในหลอดทดลอง นอกจากนี้การใช้ยาโคลิสตินร่วมกับ EDTA ยังมีประสิทธิภาพในการรักษาและมีความปลอดภัย โดยลดปริมาณเชื้อในอวัยวะภายใน ลด creatinine ในซีรั่มและเพิ่มอัตราการรอดชีวิตในสัตว์ทดลอง งานวิจัยนี้เป็นการศึกษาทั้งในหลอดทดลองและสัตว์ทดลองเป็นครั้งแรก ซึ่งแสดงให้เห็นถึงแนวทางในการรักษาเพื่อกำจัดเชื้อดื้อยาโคลิสตินให้เป็นผลสำเร็จ โดยการใช้ยาสูตรผสมแบบใหม่นี้คือ ยาโคลิสตินร่วมกับ EDTA ในการรักษาโรคติดเชื้อ ColRkp ที่สร้างไบโอฟิล์มและก่อโรคติดเชื้อที่สัมพันธ์กับสายสวน

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Aye Mya Sithu Shein : Mechanisms of colistin resistance and the antimicrobial effects of antibiotic and adjuvant combination on colistin-resistant *Klebsiella pneumoniae*. Advisor: Dr TANITTHA CHATSUWAN, Ph.D. Co-advisor: Assoc. Prof. ASADA LEELAHAVANICHKUL, M.D., Ph.D.

The prevalence of colistin-resistant *Klebsiella pneumoniae* (ColRkp) has progressively increased globally. The purposes of this study were to characterize the molecular mechanisms responsible for colistin resistance among ColRkp clinical isolates in Thailand, to determine the expression levels of virulence factors associated with ColRkp clinical isolates, and to evaluate *in vitro* and *in vivo* synergistic activities of novel combination of antibiotic and adjuvant against ColRkp clinical isolates. A total of 165 carbapenem-resistant *K. pneumoniae* clinical isolates were obtained from King Chulalongkorn Memorial Hospital between 2016 and 2021. We discovered a rising trend of ColRkp displaying extensively drug-resistant (XDR) and pandrug-resistant (PDR) characteristics, with a prevalence of 28.5% (n=47). Both chromosomal *mgrB*, *pmrB*, or *phoQ* genes mutations (91.5%) and plasmid-mediated *mcr*-1.1, *mcr*-8.1, or *mcr*-8.2, alone or in combination with R256G PmrB (8.5%), were responsible for colistin resistance in these ColRkp isolates. Several independent insertions, deletions, or substitutions in *mgrB* (85.1%) associated with increased expressions of Ara4N-related *phoPQ* and *pmrK* transcripts were observed to be crucial in establishing colistin resistance in our isolates. Since *mgrB, pmrAB*, and *phoPQ* are involved in supporting bacterial virulence, we observed a significant association between XDR ColRkp and increased biofilm production (p<0.0001). Moreover, significantly altered bacterial virulence factors expressions were found to be associated with XDR ColRkp clinical isolates. A novel colistin-EDTA combination exhibited potent synergistic activity in both planktonic and mature biofilms of all tested XDR and PDR ColRkp isolates *in vitro*. A combination of colistin and EDTA also exhibited significant therapeutic effectiveness in eradicating ColRkp catheter-related biofilm infections and eliminating the risk of recurrence both *in vitro* and *in vivo*. Furthermore, colistin-EDTA combination demonstrated its significant therapeutic efficacy and safety in decreasing bacterial loads in internal organs, reducing serum creatinine, and enhancing animals survival *in vivo*. This is the first *in vitro* and *in vivo* study to highlight that a novel colistin-EDTA combination is a promising therapeutic strategy for successfully overcoming colistin resistance in ColRkp catheter-related biofilm infections.

Field of Study:	Medical Microbiology	Student's Signature .....
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Aye Mya Sithu Shein

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

ABBREVIATIONS

AMC - Amoxicillin-clavulanic acid

AMK - Amikacin

AMP - Ampicillin

Ara4N - Amino-4-deoxy-L-arabinose

ATCC - American Type Culture Collection

bla - β-lactamase gene

bp - Base pair

°C - Degree Celsius

CAZ - Ceftazidime

CCCP - Carbonyl cyanide *m*-chlorophenyl hydrazone

CDC - Centers for Disease Control and Prevention

CFU - Colony forming unit

CIP - Ciprofloxacin

CLSI - Clinical and Laboratory Standards Institute

CLSM - Confocal laser scanning microscope

COL - Colistin

CV assay - Crystal violet assay

ColRkp - Colistin-resistant *K. pneumoniae*

CRkp - Carbapenem-resistant *K. pneumoniae*

Dab - α,γ-diamino butyric acid

DNA - Deoxynucleic acid

dNTP - Deoxynucleotide triphosphate

DOR - Doripenem

E - Glutamic acid

EDTA - Ethylenediaminetetraacetic acid

et al - Et alii

ETP - Ertapenem

EUCAST - European Committee on Antimicrobial Susceptibility Testing

FICI - Fractional inhibitory concentration index

FOS - Fosfomycin

G6P - Glucose-6-phosphate
G - Glycine
IPM - Imipenem
IS - Insertion sequence
K - Lysine
L - Liter
LPS - Lipopolysaccharide
MBEC - Minimum biofilm eradication concentration
MDR - Multidrug-resistant
MEM - Meropenem
mg - Milligram
MIC - Minimum inhibitory concentration
mL - Milliliter
mm - Millimeter
OD - Optical density
OMP - Outer membrane protein
P - Proline
PBS - Phosphate buffer saline
PCR - Polymerase chain reaction
PDR - Pandrug-resistant
PEtN - Phosphoethanolamine
QS - Quorum sensing
R - Arginine
SXT - Trimethoprim-sulfamethoxazole
T - Threonine
TET - Tetracycline
XDR - Extensively drug-resistant
µL - Microliter

CHAPTER I

INTRODUCTION

Klebsiella pneumoniae is a Gram-negative pathogen that predominantly causes serious hospital-acquired infections, such as pneumonia, urinary tract infection, wound or surgical site infection, and septicemia, particularly in individuals with compromised immune systems (1, 2). Furthermore, *K. pneumoniae* is a frequently reported pathogenic microbe involved in the bacterial colonization of vascular catheters causing catheter-related biofilm infections, which commonly occur within 24 hours of catheter use (3-5). The severity of catheter-related biofilm infections is significantly related to the duration of catheterization (3-5). Alarmingly, the occurrence of catheter-related biofilm infections caused by antibiotic-resistant *K. pneumoniae* is on the increasing trend nowadays, posing significant threats for increased morbidity and mortality in clinical settings (3, 4).

Although carbapenems are β-lactams of choice for treating extended spectrum β-lactamase (ESBL)-producing *K. pneumoniae* (6, 7), the accelerated spread of plasmid-mediated carbapenemases has contributed to the establishment of carbapenem-resistant *K. pneumoniae* (CRkp) over the last decades (8). In responses to increasing global CRkp prevalence, a lack of effective therapeutic alternatives, and constraints in novel antibiotics development, clinicians are being encouraged to explore colistin as a feasible treatment approach (7-10).

Colistin is a bactericidal polycationic peptide which triggers bacterial death through outer membrane permeabilization, antiendotoxin effects, osmotic imbalance, oxidative stress and impairment of bacterial respiratory chains (9-13). Nevertheless, colistin-resistant *K. pneumoniae* (ColRkp) has progressively developed in different regions of the world as colistin usage has expanded (12-14). *K. pneumoniae* develops colistin resistance due to decreased electrostatic affinity between colistin and its lipopolysaccharide (LPS) produced by a decrease in LPS net negative charge resulting from amino-4-deoxy-L-arabinose (Ara4N) -related and phosphoethanolamine (PEtN)-related LPS modifications (12, 13, 15). The *pmrHFIJKLM* operon facilitates Ara4N-related LPS alteration, whereas the *pmrCAB* operon supports PEtN-related LPS modification (12, 13). Genetic alterations in *mgrB*, *phoPQ,* and

pmrAB genes upregulate the expressions of *pmrHFIJKLM* and *pmrCAB* operons for triggering LPS alterations as chromosomal-mediated colistin resistance mechanisms (12, 13, 15). Plasmid-mediated colistin resistance mechanisms include presence of *mcr* (mobile colistin resistance) gene with different alleles (*mcr-1* to *mcr-10*) that encode phosphoethanolamine transferase causing PEtN-modified LPS (12, 13).

Furthermore, several studies reported the establishment of hypervirulent ColRkp expressing diverse virulence characteristics (16-19). In *K. pneumoniae*, biofilm development and diverse virulence factors including LPS genes-*uge* and *wabG*, outer membrane porins- *ompK35* and *ompK36*, iron-scavenging siderophores-*ybtS* (yersiniabactin) and *kfu (klebsiella* ferric ions uptake*)*, type 3 adhesin-*mrkD*, and type 2 quorum sensing (QS) regulatory system gene - *luxS* have been implicated in bacterial colonization, invasion, and pathogenicity within the host (2, 20-30). Reportedly, not only PmrAB and PhoPQ support bacterial virulence by regulating virulence gene transcripts, but also *mgrB*-related LPS alterations augment virulence by suppressing early host defenses (31-34), which highlight the importance of exploring the association between colistin resistance and virulence factors that influence bacterial pathogenicity. Due to the convergence of colistin resistance and hypervirulence, clinically untreatable *K. pneumoniae* superbugs may evolve (16-19). This emphasizes an urgent need to discover viable therapeutic strategy to overcome *K. pneumoniae* with colistin resistance which implicated in catheter-related biofilm infections of vascular catheters (16, 18, 35).

Currently, an antibiotic lock technique can be attempted to control catheter-related biofilm infections, which targets intraluminal biofilms lining the lumen of infected catheters by instilling a solution containing high concentrations of single or combined antimicrobial agents (36). Increased occurrences of drug-resistant infections in clinical settings have prompted the evaluation of novel lock solutions that combine different antibiotics with nonantibiotic compounds - adjuvants (7, 36, 37). Colistin combination therapy, when used to treat drug-resistant bacteria, has been shown to significantly lower treatment failure rates and enhance patients survival (38). However, growing concerns about ColRkp in catheter-related biofilm infections and the possibility of bacterial regrowth under antimicrobial selection

pressure (21, 39, 40), prompted us to evaluate a novel antimicrobial lock solution combining colistin with nonantibiotic adjuvant which can strengthen colistin effectiveness by addressing the underlying colistin resistance mechanisms.

Previous studies discovered that ethylenediaminetetraacetic acid (EDTA) can disrupt permeability-associated resistance mechanisms and restore the antibiotic efficacy against resistant bacteria via its metal ions chelation (41, 42). EDTA also augmented the combined antibiotic efficacy in eradicating bacteria biofilms (43-45). Gram-negative bacterial membranes were also permeabilized by another metal chelator-sodium citrate (46) and combination of sodium citrate with antibiotic can prevent the formation of biofilms (47). Recent study showed the efficacy of tramadol against planktonic and mature biofilms of *Candida albicans* through membrane damage and leakage of potassium ions (48). The findings of previous study showed that resveratrol significantly inhibited the biofilms of uropathogenic *Escherichia coli* (49). Addition of DNAase to biofilm leads to cleavage of biofilm matrix component, extracellular DNA and enhance the effects of antibiotics by increasing antibiotic penetration (50). Antibiofilm peptide-DJK-6 enhanced the ability of meropenem to eradicate preformed biofilms and it increased the susceptibilities of bacteria to β-lactam antibiotics (51).

Because the adjuvants are supposed to have no or low antibiotic activity with a comprehensive mechanism of killing (41), bacteria would find it difficult to develop resistance to these adjuvants. It could therefore be hypothesized that combining an antibiotic with the potent adjuvant would increase the antibiotic activities and allow it to become effective against ColRkp associated with catheter-related biofilm infections. To the best of our knowledge, no study has been conducted to evaluate the activities of an antibiotic-adjuvant combination therapy on ColRkp catheter-related biofilm infections. The purposes of this study were to characterize the chromosomal-mediated and plasmid-mediated mechanisms responsible for colistin resistance among ColRkp clinical isolates in Thailand, to determine the expression levels of virulence factors associated with ColRkp clinical isolates and to evaluate both *in vitro* and *in vivo* synergistic activities of novel combination of antibiotic and adjuvant against ColRkp clinical isolates.

CHAPTER II

OBJECTIVES

1. To characterize the molecular mechanisms responsible for colistin resistance among colistin-resistant *K. pneumoniae* clinical isolates in Thailand
2. To determine the expression levels of virulence factors associated with colistin-resistant *K. pneumoniae* clinical isolates
3. To evaluate *in vitro* and *in vivo* synergistic activities of novel combination of antibiotic and adjuvant against colistin-resistant *K. pneumoniae* clinical isolates

CHAPTER III

LITERATURE REVIEW

1. Clinical epidemiology of *K. pneumoniae*

K. pneumoniae is a Gram-negative, encapsulated, non-motile, facultatively anaerobic bacteria (1, 52). In 1875, Edwin Klebs firstly isolated this bacterium from the airways of a patient died from pneumonia, and Carl Friedländer later characterized it in 1882 (1, 53). They have been discovered in a wide range of environments, including soil, surface water, and medical devices respectively (1, 54). Alarmingly, *K. pneumoniae* also colonizes a wide range of human mucosal surfaces, including the gastrointestinal tract and upper respiratory oropharynx, where colonization rates vary significantly between individuals depending on their living environments and exposure risks (1, 54).

According to recent studies, the prevalence of *K. pneumoniae* colonization varies from 18.8% to 87.7% in Asia and 5% to 35% in Western countries (1, 55, 56). The colonization of *K. pneumoniae* can be detected not only in patients, with a carrier rate of 77% in the gastrointestinal tract and 19% in the nasopharynx, but also in non-hospital settings, with a carrier rate of 5% to 38% in feces and 1% to 6% in the nasopharynx (1, 57, 58). A previous study utilized genome sequencing to confirm that, in comparison to non-carriers, there was a subsequent infection of the same *K. pneumoniae* strain which originated from its gastrointestinal tract colonization in the same carrier patient (1, 59, 60). Moreover, according to genomic analysis from previous studies, the gut microbiota is a major source of nosocomial *K. pneumoniae* infections, with self-colonizing strains accounting for 80% of infections (1, 59, 60). The deterioration of host immune defense in individuals triggered by the underlying immunocompromised conditions such as diabetes or malignancies as well as immunomodulatory therapy, is the primary cause of the switch from *K. pneumoniae* colonization to its infection (1, 2, 61, 62).

Eventually *K. pneumoniae* can disseminate from the colonization sites to other areas of the human body, which predominantly results in a wide range of serious untreatable hospital-acquired infections, including pneumonia, urinary tract infection, wound or surgical site infection, and septicemia (1, 2). Furthermore, *K.*

pneumoniae was frequently reported to be implicated in bacterial colonization of vascular catheters, causing catheter-related biofilm infections (4, 40, 63). Catheter-related biofilms frequently develop within 24 hours of catheter usage, and its severity is significantly correlated with the duration of catheterization (3, 5).

2. Increasing problems of antibiotic resistance in *K. pneumoniae*

In the current era of antibiotic resistance, *K. pneumoniae* is one of the most problematic microorganisms involved in antibiotic resistance (2, 64). What makes *K. pneumoniae* even more worrisome is the growing number of antibiotic-resistant strains globally (2, 64). Antimicrobial resistance rates in *K. pneumoniae* have gradually increased over time against the four major antibiotic categories, including cephalosporins, aminoglycosides, fluoroquinolones, and carbapenems, according to the Antimicrobial Resistance Surveillance Report (7). Resistance varies greatly between countries and is endemic to multidrug-resistant (MDR) *K. pneumoniae* due to extended spectrum β-lactamase (ESBL) production, which show resistance to third-generation cephalosporins, fluoroquinolones, and aminoglycosides (7). The MDR *K. pneumoniae* usually harbors plasmids encoding β-lactamases with extended spectrum hydrolytic activities (6).

Carbapenems are the preferred β-lactams of choice for the treatment of infections caused by ESBL-producing *K. pneumoniae* (65). However, the widespread and accelerated spread of plasmid-mediated *K. pneumoniae* carbapenemases including Ambler class A *K. pneumoniae* carbapenemase (bla_{KPC}), class B carbapenemases (bla_{NDM}, bla_{IMP} and bla_{VIM}), and class D oxacillinase-48 carbapenemase (bla_{OXA-48}) among these MDR *K. pneumoniae* has contributed to the establishment of carbapenem resistance in *K. pneumoniae* (CRkp) over the last decade (8). The development of CRkp was related to the high mortality rate of 40% to 70% in critically ill patients (8). With virtually no treatments available, these MDR *K. pneumoniae* have been singled out as an urgent threat to human health (66). As a case in points, drug-resistant *K. pneumoniae* has been added to the World Health Organization's (WHO) list of infections that desperately demand the innovative breakthrough therapies (64, 67). In response to increasing global CRkp prevalence, a

lack of effective therapeutic alternatives, and constraints in novel antibiotic development, clinicians are being encouraged to explore colistin as a feasible treatment approach for overcoming antibiotic resistance issues (7, 9-13).

3. Colistin: last therapeutic strategy for MDR *K. pneumoniae*

3.1. Structure of colistin

Colistin, a bactericidal cationic polypeptide antibiotic, belongs to the polymyxin antibiotic family and it is categorized as polymyxin E (11, 13). Colistin is composed of a mixture of closely-related components, most notably colistin A (polymyxin E1) and colistin B (polymyxin E2), both of which are acylated by (S)-6-methyloctanoic acid and (S)-6-methylheptanoic acid, respectively (11, 13). Each molecule has a cationic polypeptide ring and a lipophilic fatty acid chain (Figure 1) (11, 13). In 1949, Koyama and colleagues in Japan isolated colistin from *Paenibacillus polymyxa* subspecies *colistinus* for the first time (9, 12, 13). The US FDA approved colistin in 1959 as an antibacterial agent against Gram-negative pathogens including *K. pneumoniae* for the treatment of various infections in clinical settings due to its bactericidal action, despite the fact that it was originally used as an intravenous formulation in the 1950s (9, 12, 13). Meanwhile, by the mid-1970s, the therapeutic use of colistin was discontinued due to the documented adverse effects of colistin, particularly nephrotoxicity and neurotoxicity (9, 12, 13). Colistin was then replaced with the newer, more effective aminoglycoside antibiotics (9, 12, 13). Nonetheless, colistin was still used in clinical practice for individuals with pseudomonal lung cystic fibrosis, as well as in topical treatments for ear and eye infections, and in combination with other antibacterial drugs (9, 12, 13). Moreover, colistin has been utilized for decades in agribusiness settings, especially in pig production, as a prophylactic and therapeutic strategy against Gram-negative *Enterobacteriaceae* infections (9, 12, 13, 68, 69).

3.2. Antimicrobial spectrums of colistin

The target site for colistin is the LPS component present in the bacterial outer membrane (9, 12, 13). Colistin is regarded as a narrow-spectrum

antimicrobial agent that exhibits a significant bactericidal activity against Gram-negative *Enterobacteriaceae* including *Escherichia coli*, *Klebsiella* spp., *Enterobacter* spp., *Citrobacter* spp., *Salmonella* spp., and *Shigella* spp., as well as other non-fermentative Gram-negative bacteria including *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Stenotrophomonas maltophilia* respectively (9, 12, 13). Bacteria including *Haemophilus influenzae*, *Legionella pneumophila*, *Aeromonas* spp., and *Bordetella pertussis* are naturally susceptible to colistin (9, 12, 13).

3.3. Mechanisms of actions of colistin

To exhibit its bactericidal effects, colistin promotes outer membrane permeabilities by disrupting the bacterial outer and inner membranes through a "self-promoted uptake" mechanism, in which colistin's amphipathic nature is crucial for its uptake across the bacterial outer membrane barrier (Figure 2 A) (9, 11-13). It is worth noting that colistin possesses a three-fold greater affinity for bacterial LPS than for divalent cations. At physiological pH, the primary amines of the α,γ-diamino butyric acid (Dab) residues in colistin are ionized, resulting in colistin molecules with a net-positive charge (Figure 2 A) (9, 11-13). Electrostatic interactions between the cationic Dab residues of colistin and anionic phosphate groups on the lipid A moiety of LPS in the bacterial outer membrane cause the first fusion of colistin with the bacterial outer membrane (Figure 2 A) (9, 11-13). Colistin then competitively displaces the divalent cations - Mg^{++} and Ca^{++}, which perform as bridges to stabilize LPS on the bacterial membrane (13). Colistin inevitably causes the bacterial outer membrane to destabilize, resulting in increased outer membrane permeabilization, intracellular content release, and bacterial death (Figure 2 A) (13).

Colistin also exhibits anti-endotoxin activity against the lipid A portion of LPS that represents a Gram-negative bacterial endotoxin (Figure 2 B) (13). Binding to and neutralizing the LPS molecules by colistin result in the suppression of shock induction via the release of cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin 8 (IL-8) (Figure 2 B) (13). Another antibacterial mechanism of colistin is the binding of colistin to anionic phospholipid, which causes the inner leaflet of the bacterial outer membrane to fuse with the outer leaflet of the cytoplasmic

membrane, resulting in bacterial cell death due to osmotic imbalance induced by phospholipid loss (Figure 2 C) (13). Colistin also induces bacterial death via hydroxy radical-induced oxidative stress by producing reactive oxygen species (ROS) such as hydroxyl radicals (•OH), superoxide (O_2^-), and hydrogen peroxide (H_2O_2) (Figure 2 D), as well as the impairment of bacterial respiratory chains by inhibiting vital respiratory enzymes, as its additional intracellular targets for eliciting its bactericidal activities (Figure 2 E) (13).

Nevertheless, colistin resistance in *K. pneumoniae* (ColRkp) has been progressively developing in recent years in different regions of the world (70-74). During a case-control study conducted at six Italian hospitals from 2010 to 2014, the prevalence of colistin resistance among CRkp clinical isolates was observed to be increased from <10% to >30%, with a 30-day mortality rate as high as 51% (75). The drivers of colistin resistance include the overuse and misuse of colistin antibiotic in both clinical and agribusiness settings (13, 14). It is consequently followed by the development and spread of resistant bacteria as well as their resistance determinants between or among these clinical and agribusiness sectors around the world (13, 14). Furthermore, prior infections with carbapenem-resistant microorganisms as well as the administration of carbapenems during hospitalization, were identified as substantial risk factors for developing colistin resistance in *K. pneumoniae* (76). Infections caused by *K. pneumoniae* with colistin-resistance and carbapenem-resistance characteristics were linked to the establishment of extensively drug-resistant (XDR) and pandrug-resistant (PDR) *K. pneumoniae* strains, resulting in increased mortalities in affected individuals (75, 77).

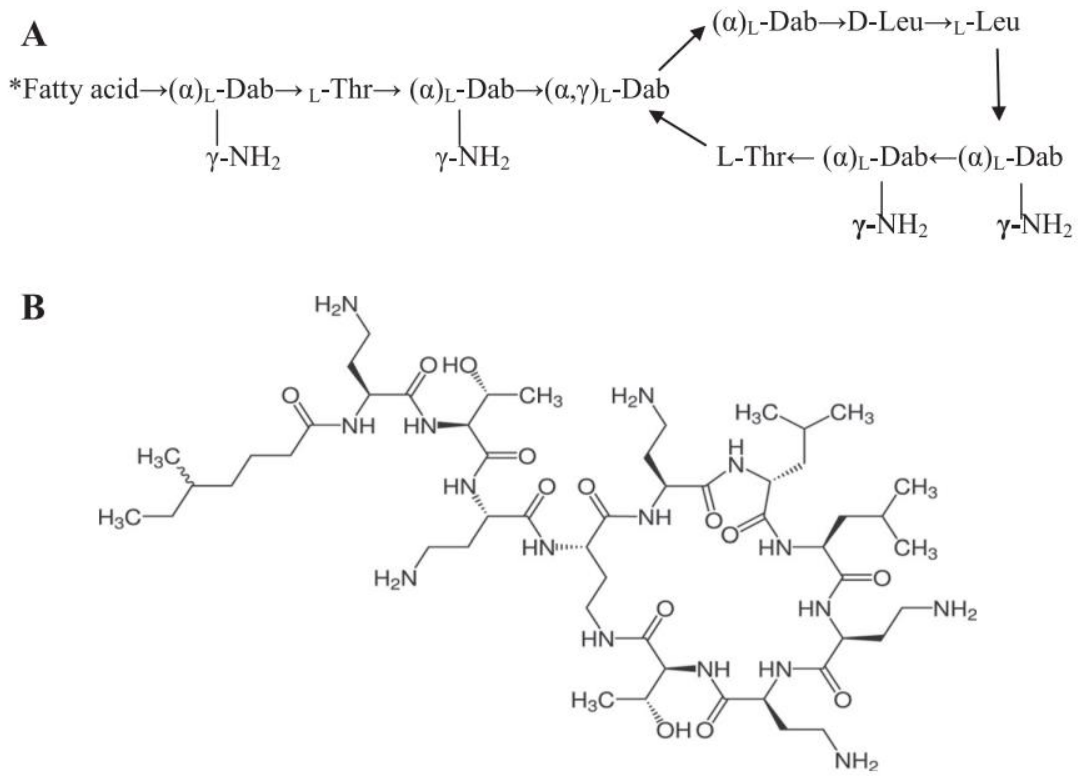


Figure 1. Structure of colistin.

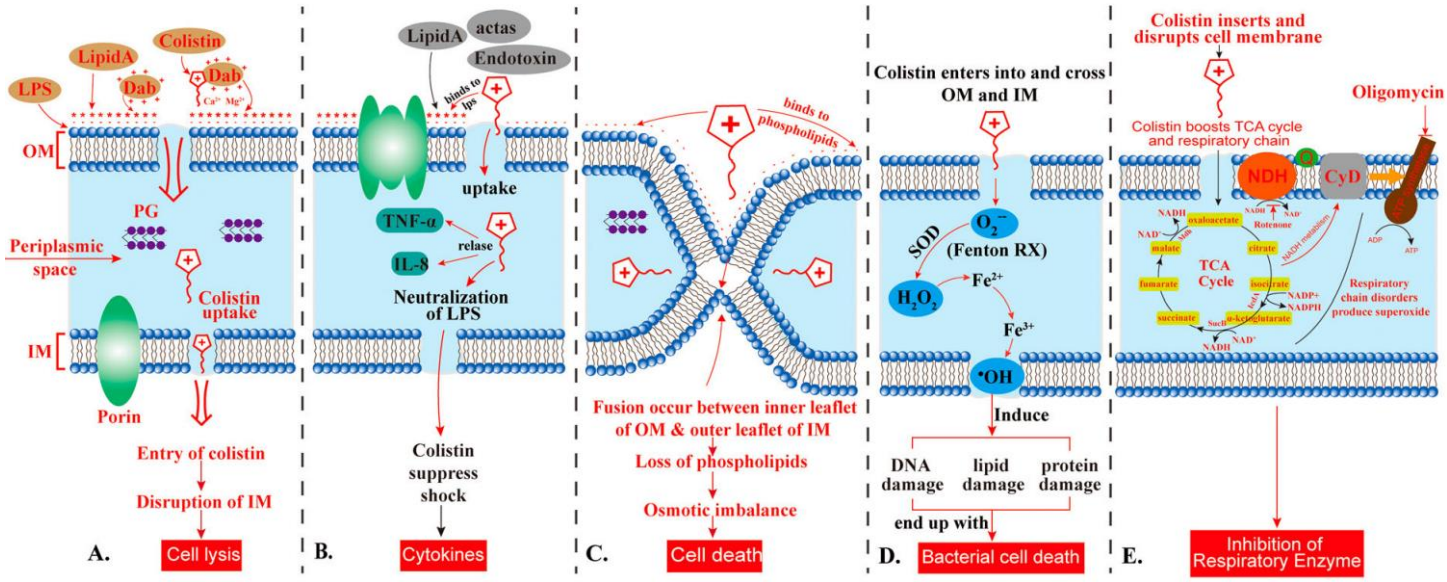


Figure 2. Mechanisms of actions of colistin.

4. Underlying colistin resistance mechanisms in *K. pneumoniae*

Modification of the bacterial outer membrane by adding cationic groups to the LPS for the reduction of net negative charge is the main strategy for displaying colistin resistance in *K. pneumoniae* (12, 13, 15). The existence of efflux pumps overexpression, capsule polysaccharide overproduction, and outer membrane porins mutations that can obscure the colistin binding site, are the additional strategies for establishing colistin resistance in *Enterobacteriaceae* (15). With the exception of *Paenibacillus polymyxa* subspecies *colistinus* that can produce colistinase, there have been no reports of colistin resistance linked to enzymatic inactivation of colistin in *K. pneumoniae* (15).

The primary strategy for generating colistin resistance in *K. pneumoniae* is the replacement of lipid A phosphate moieties in LPS with cationic groups such as Ara4N and PEtN (12, 13, 15). When the lipid A of LPS is modified with Ara4N at 4-phosphate or PEtN at 1-phosphate groups, the negative charge of LPS becomes lowered (12, 13, 15). Consequently, the electrostatic interactions between the cationic colistin and anionic LPS get diminished, thereby decreasing the bactericidal effects of colistin (12, 13, 15). Moreover, the Ara4N modification of LPS is observed to be more efficient than the PEtN modification, as the Ara4N modification reduces the net negative charge of lipid A to zero, while the PEtN modification only reduces it from -1.5 to -1 (78, 79).

4.1. Chromosomal-mediated colistin resistance mechanisms

The chromosomal genes - *phoPQ* and *pmrAB* are the two component systems (TCSs) genes, which function to regulate the expression of other different genes including the genes encoding for LPS modification. The PhoPQ and PmrAB TCSs are composed of both sensor kinases - PhoQ and PmrB, as well as response regulators - PhoP and PmrA, respectively (Figure 3) (12, 13, 15). The PhoQ and PmrB sensor kinases can detect and become activated after triggering with environmental stimulatory signals such as a decrease in cell envelope Mg^{++} and Ca^{++} levels, the presence of cAMP, increased Fe^{+++} concentrations, exposure to Al^{+++}, and low pH, respectively. When PhoQ and PmrB become activated, their activated tyrosine kinase

activities inevitably lead to the phosphorylation of PhoP and PmrA response regulators. The phosphorylated PhoP and PmrA eventually enhance the transcription of the *pmrHFIJKLM* (*arnBCADTEF*) and *pmrCAB* operons, respectively. The *pmrHFIJKLM* operon facilitates the attachment of Ara4N to lipid A, resulting in Ara4N-remodeled LPS, whereas the *pmrC* of *pmrCAB* operon promotes the addition of PEtN to lipid A for PEtN-integrated LPS alterations, which mediate colistin resistance in *K. pneumoniae*. Moreover, phosphorylated PhoP promotes the transcription of connector *pmrD*, which activates *pmrA* by binding to and stabilizing PmrA in its phosphorylated state. Therefore, when PhoPQ TCS is activated, LPS can be modified via two pathways: direct activation of PhoP via PhoQ kinase activity, resulting in Ara4N-related LPS alterations, and indirect activation of PmrA via connector protein-PmrD as PhoPQ-PmrD-PmrAB signal transduction, resulting in PEtN-related LPS alterations. An additional regulatory TCS found in *K. pneumoniae*, is CrrAB which also implicated in the modification of LPS for colistin resistance by activating the PmrAB TCS via CrrC. According to prior studies, the presence of specific mutations in *phoPQ*, *pmrAB* and *crrB* have been linked to upregulated transcriptions of the *pmrHFIJKLM* and *pmrCAB* operons, which leads to chromosomal-mediated colistin resistance in *K. pneumoniae* (80, 81).

The chromosomal *mgrB* is a 144-nucleotide gene that encodes a 47-amino-acid transmembrane protein (12, 13, 15). It functions as a strong negative feedback regulator of the PhoPQ TCS by inhibiting PhoQ kinase activity and suppresses *phoQ* expression to be downregulated (Figure 3). Several insertions, or deletions, or substitutions in chromosomal *mgrB* were observed to induce the expression of PhoPQ TCS with concomitant upregulation of *pmrHFIJKLM* operon, which results in Ara4N-related LPS alterations for mediating colistin resistance in *K. pneumoniae*. Different insertion sequences (IS) belonging to various IS families, including IS*1*-like, IS*3*-like, IS*5*-like, IS*Kpn14*, IS*Kpn26*, IS*Kpn28*, IS*1R*, and IS*L3*, were observed to be inserted at different positions with different orientations within the coding region or the region between *mgrB* start codon and putative promoter region of *mgrB* gene locus (82, 83). These findings imply that *mgrB* acts as a hotspot for insertion sequence integration in *K. pneumoniae* clinical isolates exhibiting colistin

resistance. Previous studies have also discovered that deletion of the *mgrB* gene locus, as well as point mutations in *mgrB* coding sequences causing amino acid substitutions in MgrB, represent the additional mechanisms for chromosomal-mediated colistin resistance in *K. pneumoniae* (84-86). Furthermore, it has been discovered that altered chromosomal *mgrB* activates the PhoPQ signaling pathway which indirectly stimulates the PmrAB TCS via connector PmrD as the PhoPQ-PmrD-PmrAB signal transduction route in *K. pneumoniae* (Figure 3) (12, 13, 15).

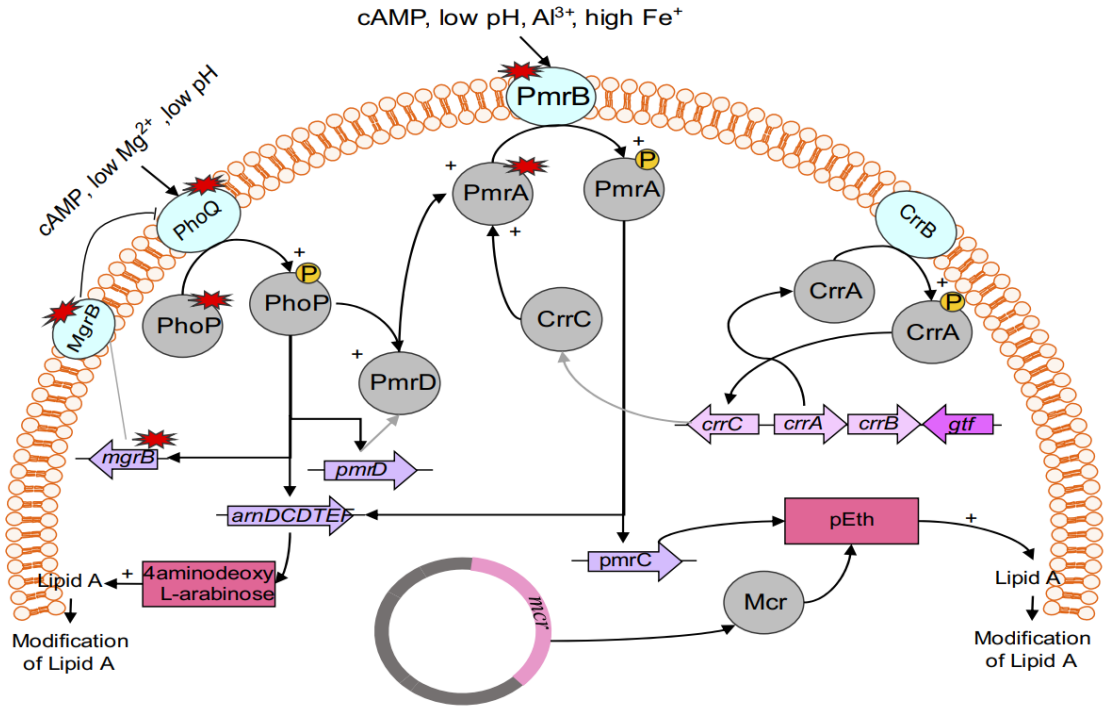


Figure 3. Chromosomal and plasmid-mediated colistin resistance mechanisms in *K. pneumoniae*.

4.2. Plasmid-mediated colistin resistance mechanisms

The existence of phosphoethanolamine transferase designated as mobile colistin resistance gene – *mcr*, which generates PEtN-remodeled LPS, has been implicated as plasmid-mediated colistin resistance in *K. pneumoniae* (Figure 3) (12, 13). Despite the fact that *mcr* genes are considered to have originated in innately resistant environmental bacteria such as *Paenibacillus* species, these *mcr* genes have been observed to be disseminated globally to bacteria of the same or different

genera, including *Enterobacteriaceae*, via horizontal genes transfer through the use of highly transmissible plasmids (12). Following the discovery of *mcr-1* among *Enterobacteriaceae* in China in 2015 (87), the dissemination of *mcr-1* and several *mcr* alleles (*mcr-1* to *mcr-9*) have been identified among different bacteria including *K. pneumoniae*, *E. coli*, *Salmonella* spp., *Shigella* spp., *Enterobacter* spp., *Citrobacter* spp., *Moraxella* spp., and *Raoultella ornithinolytic,* respectively (12, 88).

Currently, numerous variants of *mcr-1* (*mcr-1.0* to mcr-*1.30*), *mcr-2* (*mcr-2.1* to *mcr-2.7*), *mcr*-3 (*mcr-3.1* to *mcr-3.41*), *mcr-4* (*mcr-4.1* to *mcr-4.6*), *mcr-5* (*mcr-5.1* to *mcr-5.4*), *mcr-6* (*mcr-6.1*), *mcr-7* (*mcr-7.1*), *mcr-8* (*mcr-8.1* to *mcr-8.5*), *mcr-9* (*mcr-9.1* to *mcr-9.3*) have been identified worldwide as plasmid-mediated colistin resistance mechanisms triggering PEtN-related LPS modifications (Figure 4) (12). When the protein structures of all nine mcr homologs (*mcr-1* to *mcr-9*) were examined by comparison analysis, it was revealed that the *mcr-3*, *mcr-4*, *mcr-7*, and *mcr-9* genes demonstrate a significant degree of similarity in the structural level (12, 89). A novel *mcr-10* (*mcr-10. 1*) variant with the highest nucleotide (79.69%) and amino acid (82.93%) identities to *mcr-9* was recently discovered in a clinical strain of *Enterobacter roggenkampii* in China (90).

Plasmid-mediated *mcr* genes have been found all over the world, not only in patients and healthy individuals, but also in food chains such as raw meat, retail fruits, and food animals, as well as in the environment, including hospital environments, canal water, wastewater treatment plants; and in animals including wild birds, and vector insects such as housefly (12, 88). Because of the easier transferable abilities of these colistin-resistance *mcr* genes to other colistin-susceptible strains via horizontal gene transfer, the plasmid-mediated colistin resistance has emerged as a significant challenge and global concern that needs to be addressed promptly (12).

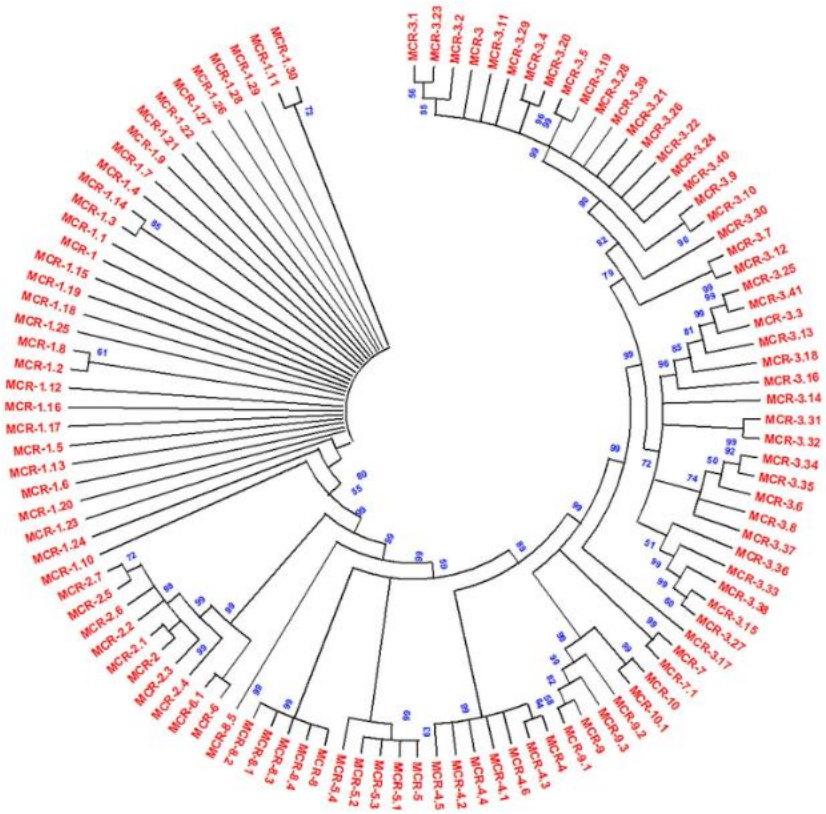


Figure 4. Phylogenetic tree of Mcr variants.

5. Cofounding bacterial virulence factors in *K. pneumoniae*

Furthermore, several studies observed the establishment of hypervirulent ColRkp expressing diverse bacterial virulence characteristics in clinical settings (16-19). Biofilm formation and different bacterial virulence factors have been implicated in bacterial colonization, invasion, and pathogenicity within the host, resulting in chronic, untreatable persistent and recurrent *K. pneumoniae* infections in clinical settings (2, 23, 28).

5.1. Biofilm development in *K. pneumoniae*

Bacteria typically form biofilms and biofilm is an organized bacterial community growing on the lining of epithelial cells and the surface of medical devices (91). Biofilm formation is a multistage developmental process that begins with the stage of initial adhesion where bacteria adhere to host epithelial cells or

medical device surfaces via cell-surface-associated adhesins, followed by the stage of early biofilm development where the adhered bacteria start to replicate and synthesize extracellular polymeric substance (EPS) for reinforcing bacterial adhesion and bacteria embedment inside the EPS matrix. This stage is then followed by the stage of early biofilm development in which bacterial biofilms develop into three dimensional (3D) structures for a multifunctional and protective scaffold, and ends with stage of dispersal, in which bacteria cells in the biofilm matrix leave biofilm for transition into the planktonic phase (Figure 5) (91).

Managing biofilms is extremely challenging due to a variety of factors, including poor antibiotic diffusion caused due to physical barrier effects of biofilms, slow biofilm-embedded bacterial growth at low oxygen tension, expression of biofilm-specific genes, the ability of biofilm-embedded bacteria to interact each other via QS signals, and the existence of persisters inside the biofilms (Figure 6) (29, 91-93). Additionally, when compared to their planktonic counterparts, bacteria residing in biofilms are capable of transferring antibiotic resistance genes encoded in their plasmids at a faster rate. All of these factors help the biofilm-producing bacteria to confer their significant biofilm-related adaptive tolerances to both host defense responses and antimicrobial drugs effects. Minimal biofilm eradication concentration (MBEC) is the lowest concentration of an antimicrobial agent that eradicates all mature biofilms and 99.9% of biofilm-embedded bacteria while reducing bacterial viability by ≤10% when compared to growth controls (94, 95). Bacterial biofilms usually develop adaptive antibiotic tolerance with 10-1000-fold higher MBEC to antibiotics than their planktonic counterparts, resulting in chronic untreatable persistent and recurrent infections (29, 91-93).

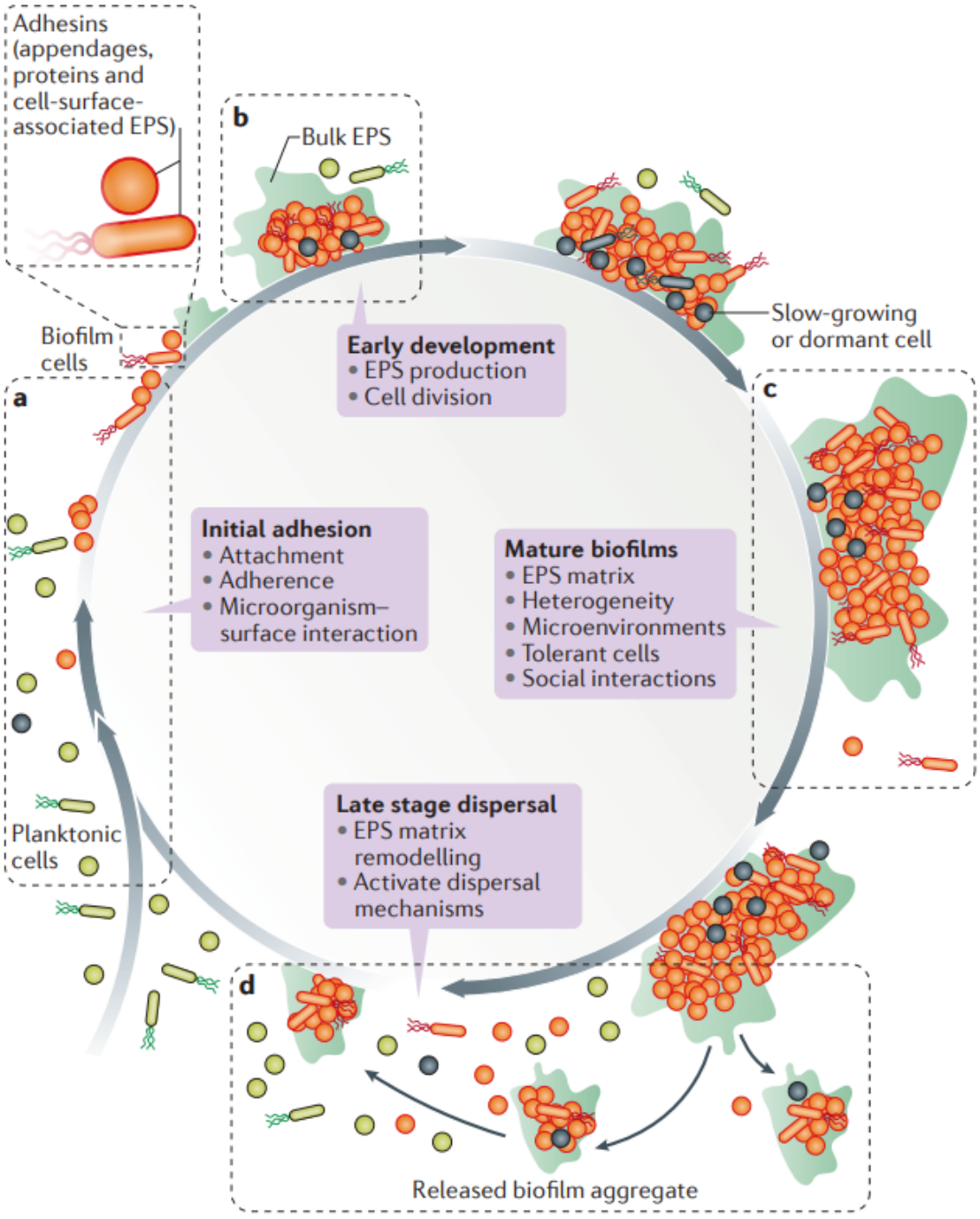


Figure 5. Multistage developmental process of the bacterial biofilms.

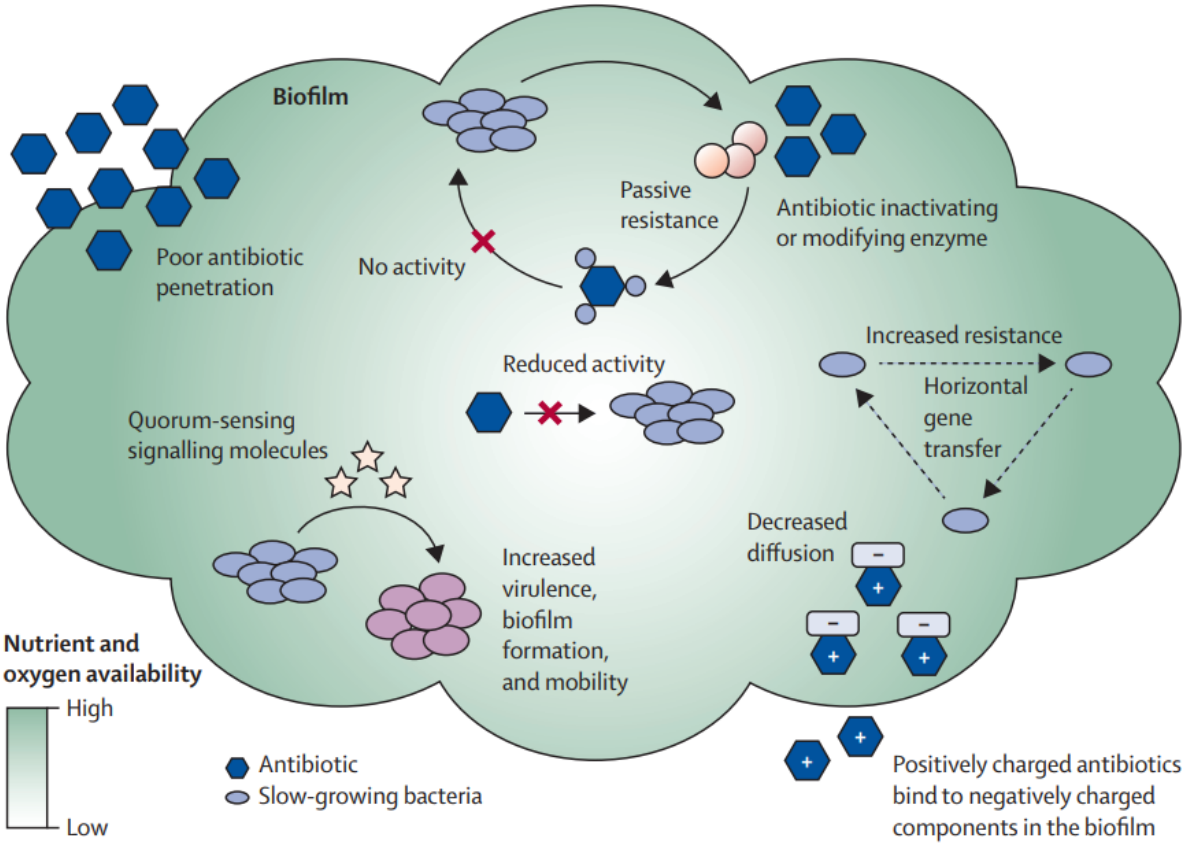


Figure 6. Interactions of the bacteria inside the biofilm.

5.2. Additional bacterial virulence factors in *K. pneumoniae*

Not only does *K. pneumoniae* produce biofilms, but it also utilizes a variety of bacterial virulence strategies to proliferate and defend itself against the host immunological defense responses (2, 28). In *K. pneumoniae*, lipopolysaccharide (LPS), porins, siderophores, and fimbriae have all been implicated as significant bacterial virulence factors for promoting bacterial pathogenicity *in vivo* (Figure 7) (2, 23, 28). Lipopolysaccharide (LPS) is a bacterial endotoxin that is expressed in the outer leaflet of *K. pneumoniae* cell membrane. LPS is made up of an O antigen, a core oligosaccharide, and lipid A. Although lipid A of LPS acts as a potent ligand for the pattern recognition receptor-TLR4 to stimulate host inflammatory cellular responses, *K. pneumoniae* showed a considerable plasticity in its lipid A structure by converting to a 2-hydroxyacyl modification in lipid A via the PhoPQ-regulated or

LpxO-dependent strategies, resulting in a failure of these inflammatory responses activation and thus increasing bacterial virulence *in vivo* (2). Furthermore, LPS contributes to virulence by protecting *K. pneumoniae* from cationic antimicrobial peptides and complement-mediated bacterial death inside the host (2). Important LPS genes such as *uge* (UDP galacturonate 4-epimerase), and *wabG* (GalA transferase), are involved in efficient LPS synthesis for enhancing bacterial pathogenicity inside the host (2). *K. pneumoniae* strains missing these LPS genes are incapable to produce the LPS outer core, thereby significantly lowering their infectivity in murine models of intraperitoneal, pneumonic, and UTI infections (Figure 7) (2, 23).

K. pneumoniae also express OmpK35 and OmpK36 as major porins in their outer membrane (Figure 7) (2). These porins are essential for bacteria survival inside the host as they are involved in transport of iron, nutrients and antibiotic across the membrane (2). Combinations of these porins and LPS maintain the integrity of bacterial outer membrane and act as permeability barrier for inducing intrinsic antibiotic resistance in *K. pneumoniae* (2). OmpK35 forms the larger channel in outer membrane of *K. pneumoniae* and expression of *ompK35* was significantly increased in response to low nutrient level in the environment (2, 25). OmpK36 has been found to have a role in stimulating classical pathway of host complement system for bacterial clearance (2, 25, 30). In a mouse peritonitis model, deficiency of OmpK35 and OmpK36 porins resulted in decreased bacterial virulence of *K. pneumoniae*, but increased antimicrobial resistance (25).

Despite the fact that iron is a limited resource that is essential for bacteria to thrive during its infection, these metal ions are not easily accessible within the host because they are sequestered to lactoferrin as part of the nonspecific host immune response to suppress the growth of these bacteria pathogens (2). On the other hand, *K. pneumoniae* acquires host iron through the secretion of siderophores that have a higher affinity for iron than host iron transport proteins, resulting in iron scavenging from host iron-chelating proteins or the environment for contributing increased bacterial virulence (Figure 7) (2). Hypervirulent *K. pneumoniae* displays iron-scavenging siderophores - *ybtS* and *kfu* genes that promote systemic survival by modulating host immune responses, enhance bacterial dissemination and

regulate the virulence factors production (2, 26, 96). Under iron limited conditions, *ybtS* also encourages biofilm formation (Figure 7) (97).

Type 3 fimbriae in *K. pneumoniae* are helix-like filaments encoded by the *mrkABCD* gene cluster (2, 22). The *mrkD* represents the adhesion subunit located at the tip of the filament, which is implicated in bacterial adhesion to biotic and abiotic surfaces of medical devices such as catheters, resulting in bacterial colonization and enhanced biofilm development (Figure 7) (2, 22). Type 3 fimbriae has also been discovered to play a role in neutrophil oxidative responses stimulation (Figure 7) (2). Additionally, pathogenic bacteria are known to use a QS system to evade host defenses (2). Type 2 QS regulatory system gene - *luxS* facilitates the biofilm development by encouraging cell-to-cell communications (2). This system allows pathogenic bacteria to accumulate to a high cell density before their virulence determinants are expressed, and enables bacteria to produce a variety of virulence factors to disturb host defenses as well as establish infection within the host (2, 27).

Reportedly, not only PmrAB and PhoPQ supported bacterial virulence by regulating virulence gene transcripts, but *mgrB*-related LPS alterations also augmented virulence by suppressing antimicrobial peptides expression as well as early host defense activation without compromising bacterial fitness (31-33). This highlights the importance of exploring the association between colistin resistance and other virulence factors that influence bacterial pathogenicity in *K. pneumoniae* exhibiting colistin resistance. Moreover, as a consequence of converging colistin resistance and hypervirulence, clinically untreatable *K. pneumoniae* superbugs evolve and it further emphasizes the urgent need to discover a viable therapeutic strategy to minimize mortality, morbidity and health-care expenses associated with these infections (16, 18, 35).

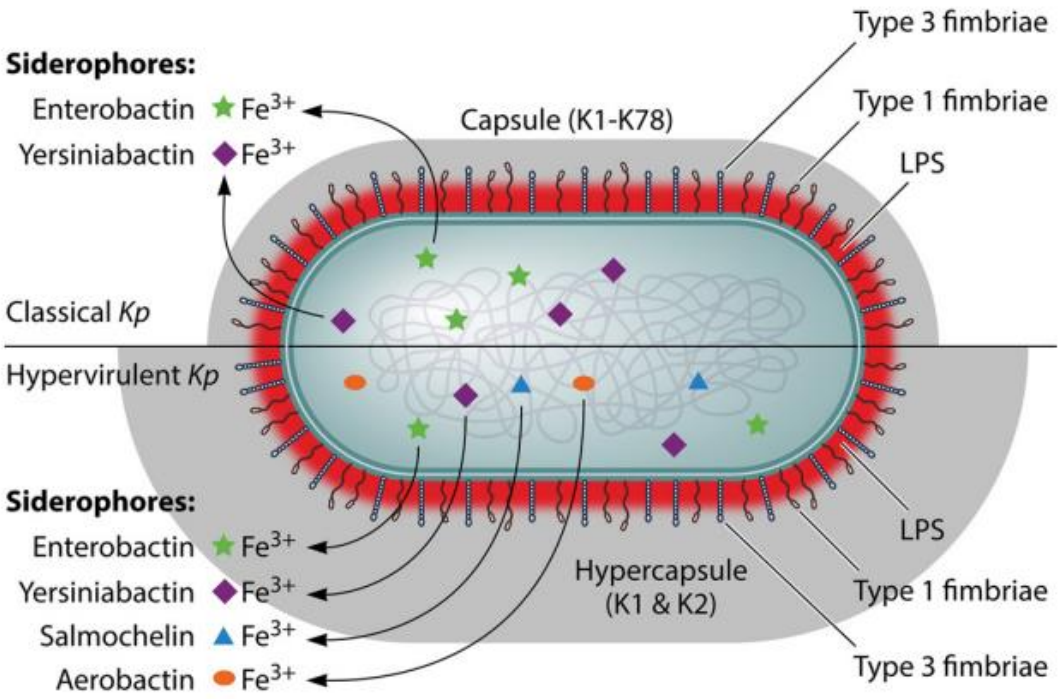


Figure 7. Diverse bacteria virulence factors in *K. pneumoniae*.

6. Increasing problems of ColRkp catheter-related biofilm infections

Adding to the urgency of the problem, ColRkp infections are steadily increasing in intensive care units, notably among patients who rely on vascular catheters for either short-term (<10 days) or long-term (≥30 days), depending on the individuals necessities (3-5, 40). Increasing usage of vascular catheters in these critically ill patients recurrently ends up with catheter-related biofilm infections (3-5, 40). Moreover, these catheters are frequently colonized by ColRkp biofilms, which can lead to development of bloodstream infections during critical phases when the therapeutic options are restricted (3-5, 40).

Alarmingly, the systemic administration of antibiotics was shown to be ineffective in treating bacteremia caused by catheter-related biofilm infections (3, 98). Surgical removal is usually required in most cases, however, obtaining alternate venous access for catheter replacement is incredibly challenging. Furthermore, replacing infected catheters carries the risks of traumatic injuries, decreased catheter

access, and higher healthcare costs (3, 98). In tackling these ColRkp catheter-related biofilm infections, clinicians are confronted with high patients mortality rates and this highlights an emerging issue that needs to be addressed promptly in clinical settings (3, 98).

Currently, an antibiotic lock technique can be attempted to control these complications (36). This procedure targets the intraluminal biofilms lining the lumen of the infected catheters through the instillation of a solution containing high concentrations of single or combined antimicrobial agents (36). This antibiotic lock therapy is recommended to lock inside the catheter lumen for 12-72 hours in order to reduce the risks of catheter-related bloodstream infection associated with long-term catheter usage (36).

7. Combination therapy to overcome drug resistance in *K. pneumoniae*

Combination therapy, which combines two or more antimicrobial drugs during a treatment regimen, is one of the most effective approaches tor combat drug resistance in *K. pneumoniae* (38). According to previous studies, colistin has been demonstrated to exhibit considerable antimicrobial activities against preformed mature biofilms and bacterial burdens of *P. aeruginosa*, *E. coli*, and methicillin-resistant *Staphylococcus aureus* (MRSA) with intrinsic colistin resistance (39, 99). Because colistin exhibits a membrane permeabilization effect that increases the influx of combined antimicrobial drug and produces preferential bactericidal activities against biofilm-embedded bacterial cells or microcolony stalks with lower metabolic activities inside the biofilm matrix, it would be reasonable to use colistin in combination therapy to treat biofilm-producing drug-resistant *K. pneumoniae* clinical isolates (39, 99).

Currently, colistin-based antibiotic combination therapy has been increasingly used as a last-resort treatment for drug-resistant *K. pneumoniae* clinical strains (38, 100-102). Antibiotic combination therapy containing colistin also resulted in significantly lower treatment failure rates with significant improvement in patients survivals (38). Nevertheless, rapid increases in antibiotic resistance rates have been reported when different antibiotics are used as antibiotic combination therapy (103,

104). Moreover, using these antibiotic combination therapies can potentially induce the emergence of XDR and PDR strains as a result of an overall increase in antimicrobial selection pressure caused by increased antibiotic release into the environment from higher antibiotic uses (103, 104). This form of antibiotic-antibiotic combination therapy also lead to the increased risks of antibiotic toxicities, bacterial superinfections, higher costs, and possible antagonistic effects (103, 104).

Rather than combining antibiotic with another antibiotic, pairing antibiotic with non-antibiotic compound - adjuvant as antibiotic-adjuvant combination therapy is a complementary strategy for preserving antibiotic efficacies, addressing drug resistance concerns, and minimizing the adverse consequences of antibiotics (41, 105).

8. Adjuvants in combination therapy

Adjuvants are compounds that have no or little antibiotic activity but they can improve the efficiency of a combined antibiotic by reducing or blocking the respective antibiotic resistance mechanisms (37, 41, 105-108). Adjuvants are also recognized as resistance circuit breakers, or anti-resistance drugs, or anti-virulence drugs, or chemosensitizers, or antibiotic potentiators. Adjuvants used in conjunction with antibiotics help to broaden the antimicrobial spectrum of its combined antibiotic. Adjuvants sensitize the combined antibiotic to become re-effective even against resistant bacteria and contribute to better antibiotic efficiency at lower doses, thereby alleviating potential side effects and toxicity issues of that antibiotic (Figure 8).

The adjuvants are classified into different groups based on the target characteristics of the resistance mechanisms that they oppose (Figure 8) (37, 41, 105-108). Antibiotic augmentation effects of adjuvant are achieved by inhibiting several antibiotic-modifying enzymes including hydrolase and β-lactamases, respectively. Adjuvants also broaden the effects of antibiotics to become re-effective by increasing intracellular accumulation of antibiotics via their activities of increased outer membrane permeabilization or disruption of biofilms or inhibition of efflux pumps. Additionally, adjuvants sensitize the effects of antibiotics by blocking bacterial

virulence, inhibiting bacterial signaling and regulatory pathways as well as strengthening host defenses via its host defense-targeted therapeutic strategies.

FDA-approved clinically validated adjuvants are currently being used with β-lactamase inhibitors - clavulanic acid, tazobactam, sulbactam, and avibactam which prevent enzyme-mediated drug resistance and help to restore antibiotic efficacy (Figure 8) (109-111). Importantly, an overexpression of the efflux pumps for continuous expulsion and decreased uptake of antibiotic, is the another mechanism of antibiotic resistance in *K. pneumoniae*. Therefore, developing an adjuvant therapy targeting these bacterial efflux pumps could assist the combined antibiotic to regain its potency against bacteria exhibiting this resistance mechanisms (Figure 8) (37, 41, 105-108). Peptide analogues such phenylalanine-arginine β-naphthylamide (PaβN), capsaicin, and a variety of synthetic small molecules including aryl-piperazines and pyranopyridnes have been exploited as adjuvants to inhibit the efflux pumps inducing antibiotic resistance (112, 113). Toxin specific inhibitors and bacterial secretion systems inhibitors that specifically address the virulence of target pathogens are also promising adjuvants for antibiotic-adjuvant combination strategy (Figure 8) (41). In addition, previous studies have reported that adjuvants such as meta-bromo-thiolactone, furanone, and savarin can specifically inhibit QS systems of *P. aeruginosa* and *S. aureus* pathogens (114-116). These adjuvants were observed to play a synergistic role in combination with antibiotics in preventing QS signals-related virulence factor expression and biofilms formation, as well as promoting host defenses with minimal impact on resistance (Figure 8) (114-116).

The permeability barrier exerted by the bacterial outer membrane which is occupied by the unique polyanionic LPS molecules stabilized by divalent cations cross-bridging, is the common mechanism for mediating antibiotic resistance in Gram-negative bacteria (78). Adjuvants that remove or displace divalent cations from the bacterial outer membrane can induce these membrane to become more permeable, resulting in boosting antibiotic effectiveness through bypassing permeability-mediated antibiotics resistance mechanisms (Figure 8) (41, 105-108, 117). Delivering antibiotics together with these permeability breaker adjuvants can enhance antibiotic uptake (117). It is also a feasible alternative option for improving antibiotic

efficacy against bacteria that exhibit increased permeability-mediated antibiotic resistance. Additionally, an antimicrobial tolerance conferred by bacterial biofilms is another potential target for adjuvants (41, 105-108, 118). Anti-virulence adjuvants that can inhibit biofilms formation and eradicate mature biofilms are also potential adjuvants to be used in antibiotic-adjuvant combination therapy (118).

Gram-negative bacterial membranes were permeabilized by metal chelator - sodium citrate and combination of sodium citrate with antibiotic can prevent the formation of biofilms (47). Recent study showed the efficacy of tramadol against the planktonic and the mature biofilms of *C. albicans* through membrane damage and leakage of potassium ions (48). Comparative analysis of natural compounds showed that curcumin has inhibitory effects on biofilms and it could be used as adjuvant for the control of biofilm-related infections (119). The findings of previous study showed that resveratrol significantly inhibited the biofilms of uropathogenic *E. coli* (49). Addition of DNAase to biofilm leads to cleavage of biofilm matrix component - extracellular DNA and enhance the effects of antibiotics by increasing the penetration of antibiotics into the biofilms (50). Antibiofilm peptide such as DJK-6 enhanced the ability of meropenem to eradicate preformed biofilms and using this peptide increased the susceptibilities of bacteria to β-lactam antibiotics (51).

EDTA is a well-known metal ion chelator with an established pharmacokinetic safety profile and has been approved by the FDA for intravenous treatment of lead poisoning since 1950 (42). EDTA has recently been identified as an antibiotic resistance breaker (ARB) which are capable of breaking resistance mechanism, most notably the permeability barrier, through its strong metal ions chelation activities (37, 41, 42, 105-108). Despite its low inherent antimicrobial activity, EDTA is commonly adopted as a potent antibiotic adjuvant in combination therapy due to its ability to disrupt permeability-associated resistance mechanisms with the release of LPS by chelating divalent cations from LPS, which allows antibiotics to penetrate to their targets and restores antibiotic activities even against resistant bacteria (Figure 8) (37, 41, 105-108).

EDTA is also recognized as an anti-virulence drug having antibiofilm properties that disrupt the biofilm matrix through its ions chelation activities (37, 41, 42, 105-108). Previous studies discovered that EDTA augmented the effectiveness of existing antibiotics in eradicating mature biofilms produced by Gram-negative bacteria (43-45). It also exhibited preferable bactericidal activities against inner biofilm cells with lower metabolic activities. Because of its antibiofilm and antimicrobial properties, prior studies have revealed that EDTA is a promising adjuvant to be used in combination with antibiotics as a lock treatment to overcome antibiotic resistance in catheter-related biofilm infections (43-45).

The major drawbacks of utilizing these adjuvants with antibiotics, on the other hand, include the necessity for additional comprehensive clinical studies in order to design a successful co-dosing regimen as well as to ascertain whether the combined antibiotic and adjuvant have a compatible pharmacokinetic and pharmacodynamic characteristic for overcoming the potential drug-drug interactions (37, 41, 105-108, 120, 121). Although the clinical use of β-lactamase adjuvants has been validated by extensive studies, the clinical use of other proposed adjuvants has been limited due to a lack of comparable specialized clinical researches. More comprehensive studies dedicated to a better understanding of the efficacy and toxicity profiles will improve the therapeutic significance of these adjuvants to be employed as an alternative antibiotic-adjuvant combination therapy in clinical settings for reversal of antibiotic resistance among Gram-negative pathogens of critical importance, such as *K. pneumoniae* (41, 105-108, 120, 121).

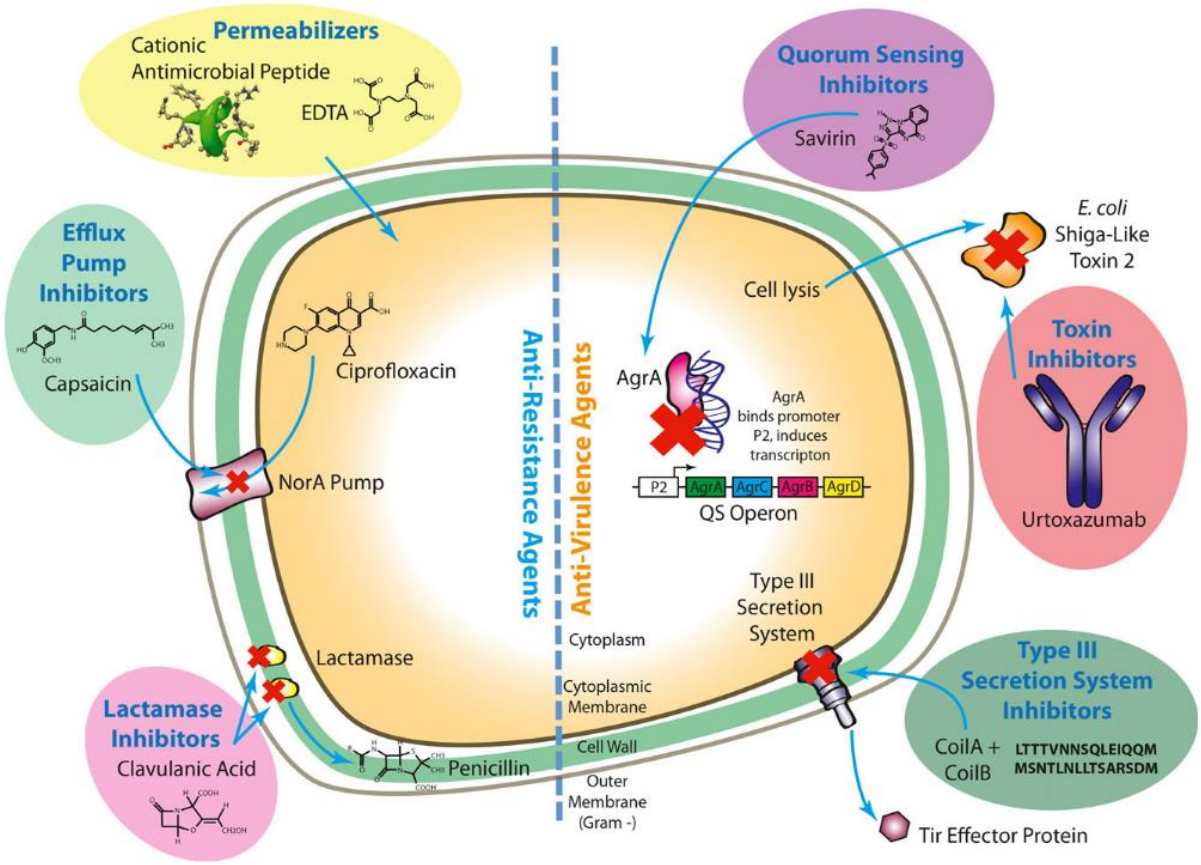


Figure 8. Schematic representation of adjuvants with their mechanisms of actions.

CHAPTER IV

MATERIALS AND METHODS

1. Bacterial strains

1.1. Sample size

K. pneumoniae clinical isolates showing carbapenem resistance (CRkp) were collected from different clinical samples of the patients who were admitted to King Chulalongkorn Memorial Hospital, Bangkok, Thailand, from 2016 to 2021. According to the results of a pilot study analysis, the estimated resistance to colistin among CRkp isolates from King Chulalongkorn Memorial Hospital was 12.08%.

Determination of sample sizes:

$$N = \frac{Z^2 \ PQ}{d^2}$$

P = Prevalence

Q = 1-P

D = Acceptable error = 0.05

$N = 1.96^2 \times 0.12 \times (1-0.12) / 0.05^2$

N = 162 isolates

This study included a total of 165 CRkp clinical isolates.

1.2. Identification of *K. pneumoniae* clinical isolates

A total of 165 CRkp clinical isolates were collected from the patients hospitalized in King Chulalongkorn Memorial Hospital between 2016 and 2021. Gram staining and biochemical tests, such as the triple sugar iron medium test (TSI), motility test, indole test, citrate utilization test, urease test, and growth at 37°C, were used to identify all collected *K. pneumoniae* clinical isolates. The pure *K. pneumoniae* isolates were stored at -80°C in tryptic soy broth containing 20% glycerol.

1.3. Quality control strains for susceptibility determination

For antimicrobial susceptibility determination, *S. aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *E. coli* ATCC 25922, and *P. aeruginosa* ATCC 27853 were utilized as quality control strains in this study (Table 1).

2. Antimicrobial susceptibility testing

All 165 CRkp clinical isolates were evaluated for their antibiotic susceptibilities to different antibacterial agents such as carbapenems (imipenem and meropenem), cephalosporins (ceftazidime), fluoroquinolones (ciprofloxacin), aminoglycosides (amikacin), and fosfomycin by agar dilution method. Susceptibilities to antibiotic - colistin were determined by broth microdilution method according to criteria in EUCAST (European Committee on Antimicrobial Susceptibility Testing) (122) and Clinical and Laboratory Standards Institute (CLSI) guidelines (Table 1) (123). The minimum inhibitory concentrations (MICs) was defined as the lowest concentration of drug showing visible inhibition of bacterial growth. The MICs of imipenem, meropenem, ceftazidime, ciprofloxacin, amikacin, fosfomycin and colistin were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (Table 1) (123). *S. aureus* ATCC25923, *E. faecalis* ATCC 29212, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 were used as quality control strains for quality accuracy of antimicrobial susceptibility determination (Table 2).

2.1. Susceptibility testing by agar dilution

For susceptibility testing with agar dilution method, antimicrobial agents were serially diluted two-fold with sterile deionized distilled water (from 0.007 to 512 mg/L) and mixed thoroughly in Mueller-Hinton agar (MHA) plates (BBL, BD Diagnostic Systems, Sparks, MD). For fosfomycin susceptibility testing, each MHA agar plate was further supplemented with 25 mg/L of glucose-6-phosphate (G6P) (Sigma-Aldrich, USA) as specified by CLSI (123). Pure and fresh culture colonies of tested *K. pneumoniae* clinical isolates and quality control strains (*S. aureus* ATCC25923, *E. faecalis* ATCC 29212, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853) were prepared in sterile normal saline and adjusted with 0.5McFarland turbidity (~$1.5x10^8$ CFU/mL)

which were then diluted 10-fold with sterile normal saline. Each bacterial suspension was applied onto prepared antibiotic-supplemented MHA plates with the final bacterial concentration of approximately 10^4CFU/spot and the plates were incubated at 37^0C for 18-24 hours. The lowest concentration of drug showing visible inhibition of bacterial growth after 18 hour incubation at 37°C was taken as the MIC of the tested drug (Table 1-2).

2.2. Susceptibility testing by broth microdilution

To establish *K. pneumoniae* MIC for colistin by broth microdilution method as specified by CLSI (123), colistin was serially diluted two-fold in cation-adjusted Mueller-Hinton broth (CAMHB) (BBL, BD® Diagnostic Systems, USA) in flat-bottomed 96-well plate (SP Life Sciences), from 0.125 to 2048 mg/L. Pure and fresh culture colonies of tested *K. pneumoniae* clinical isolates and quality control strains (*E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853) were prepared in sterile normal saline and adjusted with 0.5McFarland turbidity (~$1.5x10^8$ CFU/mL) which were then diluted 1: 100 with sterile normal saline. The adjusted bacterial suspension was added into CAMHB with serial diluted colistin as final concentration approximately $1.5x10^5$ CFU/mL and incubated at 37°C for 18-24 hours. The lowest concentration of the drug showing visible inhibition of bacterial growth after 18 hour incubation at 37°C was taken as the MIC of colistin (Table 1-2).

Table 1. Standard MICs for interpretation of *K. pneumoniae*.

Antimicrobial agent	**Interpretative categories and breakpoints of MIC (mg/L)**		
	Susceptible	**Intermediate**	**Resistant**
Imipenem	≤1	2	≥4
Meropenem	≤1	2	≥4
Ceftazidime	≤4	8	≥16
Ciprofloxacin	≤0.25	0.5	≥1
Amikacin	≤16	32	≥64
Fosfomycin	≤64	128	≥256
Colistin	-	≤2	≥4

Table 2. Acceptable ranges for quality control strains used for monitoring the accuracy of MICs.

Antimicrobial agent	**MIC QC ranges (mg/L)**			
	E. coli **ATCC 25922**	***P. aeruginosa*** **ATCC 27853**	***S. aureus*** **ATCC 25923**	***E. faecalis*** **ATCC 29212**
Imipenem	0.06-0.25	1-4	0.016-0.06	0.5-2
Meropenem	0.008-0.06	0.12-1	0.03-0.12	2-8
Ceftazidime	0.06-0.5	1-4	4-16	-
Ciprofloxacin	0.004-0.016	0.12-1	0.12-0.5	0.25-2
Amikacin	0.5-4	1-4	1-4	64-256
Fosfomycin	0.5-2	2-8	0.5-4	32-128
Colistin	0.25-2	0.5-4	-	-

3. Molecular characterization of underlying colistin resistance mechanisms

3.1. Molecular characterization of chromosomal-mediated colistin resistance mechanisms

Genomic DNA from a total of 47 ColRkp clinical isolates and colistin-susceptible *K. pneumoniae* (ColSkp) ATCC 13883 were extracted according to the manufacturer's instructions using a commercial DNA extraction kit (Purelink® genomic DNA micro kit, Invitrogen, USA) . Specific primers for genes implicated in chromosomal-mediated colistin resistance mechanisms, such as *mgrB*, *pmrA*, *pmrB*, *phoP and phoQ*, were used to amplify as reported previously (Table 3) (81, 124).

For *mgrB* gene, the PCR amplification was performed in 0.2mL PCR tube with the total volume of 50µL reaction by adding the following components: 1x*Taq* buffer, 2mM $MgCl_2$, 200µM dNTP, 0.4µM of each primer, 1.25U of *Taq* DNA polymerase, and 2µL of template DNA. The amplification was performed in thermal cycler (ProFlex® PCR system, Applied Biosystems. USA) with an initial of 95ºC for 3 minutes, followed by 30 cycles of 95ºC for 30 seconds, 54ºC for 30 seconds, and 72ºC for 1 minute and a final extension at 72ºC for 10 minutes.

For *pmrA* gene, the PCR amplification was performed in 0.2mL PCR tube with the total volume of 50µL reaction by adding the following components: 1x*Taq* buffer, 2mM $MgCl_2$, 200µM dNTP, 0.4µM of each primer, 1.25U of *Taq* DNA polymerase, and 2µL of template DNA. The amplification was performed in thermal cycler with an initial of 95ºC for 5 minutes, followed by 30 cycles of 95ºC for 1 minute, 52ºC for 30 seconds, and 72ºC for 1 minute and a final extension at 72ºC for 10 minutes.

For *pmrB* gene, the PCR amplification was performed in 0.2mL PCR tube with the total volume of 50µL reaction by adding the following components: 1x*Taq* buffer, 2mM $MgCl_2$, 200µM dNTP, 0.4µM of each primer, 1.25U of *Taq* DNA polymerase, and 2µL of template DNA. The amplification was performed in thermal cycler with an initial of 95ºC for 3 minutes, followed by 30 cycles of 95ºC for 30 seconds, 52ºC for 30 seconds, and 72ºC for 1 minute and a final extension at 72ºC for 10 minutes.

For *phoP* gene, the PCR amplification was performed in 0.2mL PCR tube with the total volume of 50μL reaction by adding the following components: 1x*Taq* buffer, 2mM $MgCl_2$, 200μM dNTP, 0.4μM of each primer, 1.25U of *Taq* DNA polymerase, and 2μL of template DNA. The amplification was performed in thermal cycler with an initial of 95°C for 3 minutes, followed by 30 cycles of 95°C for 30 seconds, 50°C for 30 seconds, and 72°C for 1 minute and a final extension at 72°C for 10 minutes.

For *phoQ* gene, the PCR amplification was performed in 0.2mL PCR tube with the total volume of 50μL reaction by adding the following components: 1x*Taq* buffer, 2mM $MgCl_2$, 200μM dNTP, 0.4μM of each primer, 1.25U of *Taq* DNA polymerase, and 2μL of template DNA. The amplification was performed in thermal cycler with an initial of 95°C for 3 minutes, followed by 30 cycles of 95°C for 30 seconds, 54°C for 30 seconds, and 72°C for 1 minute and a final extension at 72°C for 10 minutes.

The amplified DNA fragments are purified by GenepHlow™ Gel/PCR kit (Geneaid, Taiwan). The purified DNA amplicons were sequenced by using the Bigdye Terminator V 3.1 Cycler sequencing kit by 1st base DNA sequencing service, Singapore. The nucleotide sequences were translated to amino acid sequences by the ExPASy translate tool (http://web.expasy.org/translate/). The nucleotide and amino acid sequences were analyzed by Basic Local Alignment Search Tool (BLAST) from National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). In our study, *K. pneumoniae* MGH 78578, a multi-drug resistant clinical pathogen from human sputum with a complete genome sequence, was chosen as the reference genome based on its highest genomic similarity with our strains. The complete coding regions and amino acid sequences of *mgrB*, *pmrAB* and *phoPQ* of 47 ColRkp clinical isolates and ColSkp ATCC 13883 were compared with reference nucleic sequence of *K. pneumoniae* MGH 78578 (GenBank accession number. CP_000647.1), and reference amino acid sequences of MgrB (GenBank accession number. WP_002911375.1), PmrA (GenBank accession number. WP_23302144.1), PmrB (GenBank accession number. WP_114504106.1), PhoP (GenBank accession number. WP_004150807.1), and PhoQ (GenBank accession

number. WP_004147969.1) using multiple sequence alignment by Florence Corpet (http://multalin.toulhouse. inra.fr/mutalin/multalin. html) to determine the presence of mutations conferring colistin resistance. The PROVEAN tool v. 1. 1. 5 (http://provean.jcvi.org/index.php) was used to predict the effect of amino acid substitutions on protein function (125). PROVEAN score ≤-2.5 was denoted to have a deleterious effect for protein function, and a score > -2.5 was considered to have a neutral effect on protein function.

Table 3. Primers for amplification of chromosomal *mgrB*, *pmrAB* and *phoPQ* genes.

Target gene	**Primer name**	**DNA sequence (5' to 3')**	**References**
mgrB	mgrB ext F	AAGGCGTTCATTCTACCACC	(81, 124)
	mgrB ext R	TTAAGAAGGCCGTGCTATCC	
	EE mgrB F	GGCTATGGCGAGGATAATGAG	
	EE mgrB R	GCTGTGATGTAAGCGTCTGGTG	
	Int mgrB F	CGGTGGGTTTTACTGATAGTCA	
	Int mgrB R	ATAGTGCAAATGCCGCTGA	
pmrA	pmrA ext F	CAT TTC CGC GCA CTG TCT GC	
	pmrA ext R	CAG GTT TCA GTT GCA AAC AG	
pmrB	pmrB F1	GCGAAAAGATTGGCAAATCG	
	pmrB R1	GGAAATGCTGGTGGTCATCTGA	
	pmrB F2	CCCTGAATCAGTTGGTTTC	
	pmrB R2	ATCAATGGGTGCTGACGTT	
phoP	phoP ext F	GAGCTTCAGACTACT ATC GA	
	phoP ext R	GGGAAGATATGCCGCAACAG	
phoQ	phoQ ext F	ATACCCACAGGACGTCATCA	
	phoQ ext R	CAGGTGTCTGACAGGGATTA	

3.2. Molecular characterization of plasmid-mediated colistin resistance mechanisms

To explore the potential role of plasmid-encoded *mcr* genes (*mcr-1* to *mcr-9*) in the development of colistin resistance in our study, the DNA templates of 47 ColRkp clinical isolates were extracted according to the manufacturer's instructions using a commercial DNA extraction kit and stored at -20°C. Then the DNA templates were subjected to multiplex PCR using specific *mcr*-1 to *mcr-9* primers as mentioned in the earlier studies (Table 4) (126, 127).

Multiplex PCR for two reactions was then used to screen for *mcr-1* to *mcr-5* and *mcr-6* to *mcr-9*. The PCR amplification was performed in 0.2mL PCR tube with the total volume of 25µL reaction by adding the following components: 1x*Taq* buffer, 1.5mM $MgCl_2$, 200µM dNTP, 0.2µM of each primer, 1.25U of *Taq* DNA polymerase, and 2µL of boiled DNA. The amplification was performed in thermal cycler with an initial of 94°C for 15 minutes, followed by 30 cycles of 94°C for 50 seconds, 52°C for 90 seconds, and 72°C for 1 minute and a final extension at 72°C for 10 minutes.

The amplified PCR products were separated and analyzed on 1.5% agarose gel in 0.5X Tris-borate-EDTA buffer (TBE; 0.045 M Tris-borate, 0.0001M EDTA pH 8.3±1) and 1X concentration of StainIN™ GREEN Nucleic Acid Stain was added to the solution before pouring the gel into a casting tray. The 6X loading dye buffer was mixed with PCR products and loaded into the gel in electrophoresis chamber containing of 0.5X TBE. Electrophoresis was run for 35 minutes at 100 volts. The agarose gel was then visualized using Gel Documentation System (Bio-Rad), and the predicted size of the amplified PCR product was determined by comparing it to loaded 100 bp plus DNA ladder (Thermo Fisher® Scientific, USA).

Table 4. Primers for amplification of plasmid-encoded *mcr* (*mcr-1* to *mcr-9*) genes.

Target gene	Primer name	DNA sequence (5' to 3')	References
mcr-1	mcr-1-F	AAAGACGCGGTACAAGCAAC	(126, 127)
	mcr-1-R	GCTGAACATGCACGGCACAG	
mcr-2	mcr-2-F	CGACCAAGCCGAGTCTAAGG	
	mcr-2-R	CAACTGCGACCAACACACTT	
mcr-3	mcr-3-F	ACCTCCAGCGTGAGATTGTTCCA	
	mcr-3-R	GCGGTTTCACCAACGACCAGAA	
mcr-4	mcr-4-F	AGAATGCCACTCGTAACCCG	
	mcr-4-R	GCGAGGATCATAGTCTGCCC	
mcr-5	mcr-5-F	CTGTGGCCAGTCATGGATGT	
	mcr-5-R	CGAATGCCCGAGATGACGTA	
mcr-6	mcr-6-F	AGCTATGTCAATCCCGTGAT	
	mcr-6-R	ATTGGCTAGGTTGTCAATC	
mcr-7	mcr-7-F	GCCCTTCTTTTCGTTGTT	
	mcr-7-R	GGTTGGTCTCTTTCTCGT	
mcr-8	mcr-8-F	TCAACAATTCTACAAAGCGTG	
	mcr-8-R	AATGCTGCGCGAATGAAG	
mcr-9	mcr-9-F	TTCCCTTTGTTCTGGTTG	
	mcr-9-R	GCAGGTAATAAGTCGGTC	

4. Determination of carbapenemase and ESBL profiles

4.1. Determination of carbapenemase profiles among ColRkp isolates

A total of 47 ColRkp isolates were determined for the presence of carbapenemase genes including class A (bla_{KPC}), class B (bla_{NDM}, bla_{IMP} and bla_{VIM}), and class D (bla_{OXA-48}) carbapenemases by multiplex PCR using the specific primers as described previously (Table 5) (128, 129).

The DNA templates of 47 ColRkp clinical isolates were prepared by heat-lysis method. A single colony of ColRkp isolate was suspended in 50µL of sterile distilled water and boiled at 100ºC for 10 minutes. The boiling suspension was centrifuged at 13,000 rpm for 10 minutes. The supernatant was used as DNA template and stored at -20ºC.

For amplification of bla_{KPC}, bla_{NDM}, and bla_{OXA-48} genes, the multiplex PCR was performed in final volume of 25µL, containing 1X buffer, 2mM of $MgCl_2$, 0.2mM of each deoxynucleotide triphosphates (dNTPs) (Thermo Fisher Scientific, USA), 0.2 mM OXA-48F and OXA-48R primers, 0.4 mM KPC-F and KPC-R primers, 0.6mM of NDM-F and NDM-R primers, and 1.25U *Taq* polymerase (Thermo Fisher Scientific, USA), and 2µL DNA template. The PCR conditions contained initial denaturation step (94ºC, 10 minutes) followed by 35 cycles of denaturation (94ºC, 30 seconds), annealing (57ºC, 40 seconds) and extension (72ºC, 50 seconds), and a single final extension of 5 minutes at 72ºC.

For amplification of bla_{VIM} and bla_{IMP} genes, the multiplex PCR was performed in final volume of 25µL, 1X buffer, 2mM of $MgCl_2$, 0.2mM of each dNTPs, 0.4 mM of IMP-F and IMP-R, 0.16 mM VIM-F and VIM-R primers, and 0.625U *Taq* polymerase and 2µL DNA template. The PCR conditions contained initial denaturation step (94ºC, 5 minutes) followed by 35 cycles of denaturation (94ºC, 30 seconds), annealing (52ºC, 30 seconds) and extension (72ºC, 50 seconds), and a single final extension of 5 minutes at 72ºC.

The PCR products were analyzed by agarose gel electrophoresis as described in section 3.2.

4.2. Determination of ESBL profiles among ColRkp isolates

All 47 ColRkp isolates were determined for the presence of ESBL genes including bla_{OXA-1}, bla_{TEM}, bla_{SHV}, bla_{CTX-M} and bla_{VEB} ESBL genes by multiplex PCR using the specific primers as described previously (Table 5) (128, 129).

For amplification of bla_{OXA-1}, bla_{TEM}, and bla_{SHV} ESBL genes, the multiplex PCR was performed in final volume of 25µL, 1X buffer, 2 mM of $MgCl_2$, 0.2 mM of each dNTPs, 0.06 mM of OXA-1-F and OXA-1-R, 0.04 mM of TEM-F and TEM-R, and 0.08 mM of SHV-F and SHV-R, 0.5U *Taq* polymerase and 3 µL DNA template obtained by heat-lysis method. The PCR conditions were contained initial denaturation step (94°C, 5 minutes) followed by 30 cycles of denaturation (94°C, 30 seconds), annealing (55°C, 30 seconds) and extension (72°C, 1 minute), and a single final extension of 10 minutes at 72°C.

For amplification of bla_{CTX-M} and bla_{VEB} ESBL genes, the multiplex PCR was performed in final volume of 25µL, 1X buffer, 2mM of $MgCl_2$, 0.2mM of each dNTPs, 0.1mM of CTX-M-F and CTX-M-R, 0.05mM of VEB-F and VEB-R, 0.5U *Taq* polymerase and 3µL DNA template obtained by heat-lysis method. The PCR conditions were contained initial denaturation step (94°C, 5 minutes) followed by 30 cycles of denaturation (94°C, 30 seconds), annealing (55°C, 30 seconds) and extension (72°C, 1 minute), and a single final extension of 10 minutes at 72°C.

The PCR products were analyzed by agarose gel electrophoresis as described in section 3.2.

Table 5. Primers for amplification of carbapenemase and ESBL genes.

Target gene	Primer name	DNA sequence (5' to 3')	References
bla_{KPC}	KPC-F	CGTCTAGTTCTGCTGTCTTG	(128, 129)
	KPC-R	CTTGTCATCCTTGTTAGGCG	
bla_{NDM}	NDM-F	GGTTTGGCGATCTGGTTTTC	
	NDM-R	CGGAATGGCTCATCACGATC	
Bla_{OXA-48}	OXA-48-F	GCGTGGTTAAGGATGAACAC	
	OXA-48-R	CATCAAGTTCAACCCAACCG	
bla_{IMP}	IMP-F	GGAATAGAGTGGCTTAAYTCT	
	IMP-R	CCAAACYACTASGTTATCT	
bla_{VIM}	VIM-F	GATGGTGTTTGGTCGCATA	
	VIM-R	CGAATGCGCAGCACCAG	
bla_{OXA-1}	OXA-1-F	ATATCTCTACTGTTGCATCTCC	
	OXA-1-R	AAACCCTTCAAACCATCC	
bla_{TEM}	TEM-F	ATCAGCAATAAACCAGC	
	TEM-R	CCCCGAAGAACGTTTTC	
bla_{SHV}	SHV-F	AGGATTGACTGCCTTTTTG	
	SHV-R	ATTTGCTGATTTCGCTCG	
bla_{CTX-M}	CTX-M-F	CGCTTTGCGATGTGCAG	
	CTX-M-R	ACCGCGATATCGTTGGT	
bla_{VEB}	VEB-F	CCTTTTGCCTAAAACGTGGA	
	VEB-R	TGCATTTGTTCTTCGTTTGC	

5. Determination of expression levels of LPS modification genes by Quantitative RT-PCR (qRT-PCR)

The expression levels of LPS modification genes including Ara4N-related *pmrK* (part of *pmrHFIJKLM* operon) and *phoPQ* transcripts, connector *pmrD*, and PEtN-related *pmrCAB* transcripts were evaluated by Quantitative RT-PCR (qRT-PCR) using the specific primers as described previously to investigate the association

between their relative expressions and colistin resistance among the representative ColRkp isolates with different underlying colistin resistance mechanisms (Table 6) (130, 131).

The Monarch Total RNA Miniprep Kit (Biolabs, New England) was used to extract total RNA from bacterial cultures grown in Luria-Bertani broth (Merck, Darmstadt, Germany) during the mid-logarithmic growth phase, according to the manufacturer's protocol. RNase-free DNase I (Thermo Fisher Scientific, USA) was used to eliminate genomic DNA contamination from the extracted RNA samples. Nanodrop spectrophotometry (Nanospectrophotometer, USA) was used to determine the quantity of purified RNA. DNase-treated purified RNA was subsequently reverse-transcribed into cDNA using iscript Reverse transcription Supermix for RT-qPCR (BIO-RAD, USA) and stored at -20°C. The QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) was used to perform a real-time PCR expression assay using the Luna® Universal qPCR Master mix (Biolabs, New England) on the generated cDNA of tested ColRkp and ColSkp clinical isolates.

The amplification was performed with an initial of 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. The gene - *rpoD* was utilized as housekeeping gene to normalize the expression levels of LPS modification genes. As a quality control, each qRT-PCR run was undertaken by a blank sample (distilled water) and a no reverse transcriptase control to rule out any DNA contamination. The experiments were repeated in triplicate with three different cultures, each of which was tested in duplicate. The number of cycles required for the fluorescent signal to cross the background signal threshold is denoted by Ct (threshold cycle) and the amount of target nucleic acid in the sample is inversely related to this Ct values. The relative expression level of the genes of interest was computed using the $2^{\Delta\Delta CT}$ method, which involved first normalizing the Ct values by subtracting the control housekeeping gene - *rpoD* and then correlating them to the values obtained for the susceptible isolate.

Table 6. Primers for amplification of *pmrCAB, pmrK, pmrD, phoPQ* genes.

Target gene	Primer name	DNA sequence (5' to 3')	References
pmrK	pmrK int F	AGTATCGGTCAGTGGCTG TT	(130, 131)
	pmrK int R	CCGCTTATCACGAAAGATCC	
phoP	phoP int F	GCGTCACCACCTCAAAGTTC	
	phoP int R	GGCGATATCCGGGAGATGTT	
phoQ	phoQ int F	CTCAAGCGCAGCTATATGGT	
	phoQ int R	TCTTTGGCCAGCGACTCAAT	
pmrD	pmrD int F	GATCGCAGAGATTGAAGCCT	
	pmrD int R	GCGTTGCGGATCTTCAAAGT	
pmrC	pmrC int F	GCGTGATGAATATCCTCACCA	
	pmrC int R	CACGCCAAAGTTCCAGATGA	
pmrA	pmrA int F	GATGAAGACGGGCTGCAT TT	
	pmrA int R	ACCGCTAATGCGATCCTCAA	
pmrB	pmrB int F	TGCCAGCTGATAAGCGTCTT	
	pmrB int R	TTCTGGTTGTTGTGCCCTTC	
rpoD	rpoD-F	TCCGGTGCATATGATTGAGA	
	rpoD-R	ATACGCTCAGCCAGCTCTTC	

6. Determination of *in vitro* biofilm formation of ColRkp clinical isolates

The capacities of 47 ColRkp clinical isolates to form biofilms in 96-well polystyrene-flat-bottomed-microtiter plates were performed to investigate the biofilm-mediated colistin tolerance *in vitro*, as previously described (132). A single colony of ColRkp clinical isolate was inoculated in 2mL of CAMHB in a tube and grown overnight at 37°C in an orbital shaker at 200 rpm for 16 hours. After diluting the overnight culture with fresh CAMHB medium to an optical density (OD) of 0.02 at 600nm (~5×10^7 CFU/mL), 100µL of adjusted culture suspension were loaded in three replicates to flat bottomed-wells of 96-well-polystyrene microtiter plates (SP Life

Sciences), along with non-inoculated CAMHB medium as a negative control. The plates were incubated at 37°C for 24 hours. Following incubation, the contents of each well were gently removed, and each well was carefully washed three times with 100µL of sterile CAMHB using a micropipette to eliminate non-adherent cells. The wells were emptied by flipping the plates after each washing process. The plates were drained in inverted position prior to fixation of biofilms with 0.1% crystal violet for assessment of biofilm volumes developed by ColRkp clinical isolates of this study. All of the experiments were conducted in triplicates and repeated for three times.

6.1. Quantification of biofilms by crystal violet assay

Quantification of biofilms development (133) and their classification (134) were done by crystal violet staining with modifications. The biofilms adhered in each well of 96-well polystyrene microtiter plates was stained for 15 minutes at room temperature with 0.1% crystal violet. Any excess crystal violet was removed using a micropipette after staining, and the microtiter plate was rinsed with sterile water until it was free of any residual stain. After the microplate had been air dried at room temperature, crystal violet stained biofilms were dissolved by incubation with 30% acetic acid at room temperature for 10-15 minutes, and the contents of dissolved biofilms were placed in a new microtiter plate for assessment of optical density at 560nm (OD560nm) using a microtiter-plate-reading spectrophotometer (Varioskan Flash Multimode Reader; Thermo Fisher Scientific). The optical density of uninoculated CAMHB in 30% acetic acid was determined as a negative control of each run. The mean absorbance and standard deviations (SD) of tested ColRkp clinical isolates, as well as the negative control, were computed after optical density measurements. Biofilm producer, *K. pneumoniae* ATCC 13883, was utilized as quality control strain in the study of *in vitro* biofilm-mediated colistin tolerance. All of the experiments were performed in triplicates and repeated for three times. The resulting optical density (OD560nm) readings were averaged and reported as numbers for both of the tested ColRkp isolates and negative control. The cut-off value (ODc) was obtained using three standard deviations (SD) above the average OD of non-

inoculated media (negative control), and ODc value was computed independently for each microtiter plate.

ODc = Average OD of negative control + 3SD of negative control

The final OD value of tested isolate was computed by subtracting the average OD value of test by the ODc value as follows;

OD Test = Average OD of Test - ODc

Based on the calculated results of OD Test, tested 47 ColRkp clinical isolates were classified as no biofilm producer, weak biofilm producer, moderate biofilm producer, or strong biofilm producer as follows:

- No biofilm producer = OD Test ≤ ODc
- Weak biofilm producer = ODc < OD Test ≤ 2ODc
- Moderate biofilm producer = 2ODc < OD Test ≤ 4ODc
- Strong biofilm producer = 4ODc < OD Test

6.2. Confocal laser scanning microscopic analysis

Bacterial supernatants were removed cautiously and treated biofilms were then exposed to the nucleic acid stains that detect bacterial cell membrane integrities, such as green-fluorescent SYTO9 for alive bacterial cells and red-fluorescent propidium iodide (PI) for dead bacterial cells, as directed by the manufacturer of the LIVE/DEAD® BacLight™ Bacterial Viability Kit (Thermo Fisher Scientific, Waltham, MA, USA). The stained biofilms were then assessed under vital conditions with a confocal laser scanning microscope Carl Zeiss LSM 800 (Zeiss, Oberkochen, Germany) for Syto9 excitation at 488-nm laser and detection with 540/75 nm band-pass emission filter, meanwhile PI at 561 nm and 600/40 nm band-pass emission filter (135). The images were created using the Zeiss ZEN Microscope Software version 3.0. For computations of biofilm biomass and biovolume percent inhibition, as well as determination of the viable (green) and dead (red) components of the treated biofilms from acquired z-stacks layered pictures, COMSTAT 2.1 (www.comstat.dk) and the ImageJ software distribution FIJI were used.

7. Determination of expression levels of virulence factors associated with ColRkp clinical isolates

7.1. Determination of presence of virulence factors by PCR

To determine the expression levels of virulence factors associated with ColRkp clinical isolates, the presence of virulence factors (*mrkD*, *ompK35*, *ompK36*, *ybtS*, *kfu*, *wabG*, *uge*, *luxS*) in a total of 47 ColRkp clinical isolates was firstly determined by using the specific primers as reported in the previous studies (Table 7) (136, 137). The genomic DNA from fresh cultures of ColRkp and ColSkp clinical isolates was extracted according to the manufacturer's instructions using a commercial DNA extraction kit (Purelink® genomic DNA micro kit, Invitrogen, USA) and stored at -20°C.

The PCR amplification was performed in 0.2mL PCR tube with the total volume of 50µL reaction by adding the following components: 1x*Taq* buffer, 2mM $MgCl_2$, 200µM dNTP, 0.4µM of each primer, 1.25U of *Taq* DNA polymerase, and 2µL of template DNA. The amplification was performed in thermal cycler with an initial of 95°C for 3 minutes, followed by 30 cycles of 95°C for 30 seconds, 52°C (for *mrkD, luxS*), 60°C (for *ompK35, ompK36*), 51°C (for *ybtS*), 56°C (for *kfu*) , 52°C (for *uge*) each for 30 seconds, and 72°C for 1 minute and a final extension at 72°C for 10 minutes.

The PCR products were analyzed by agarose gel electrophoresis as described in section 3.2.

7.2. Determination of expression levels of virulence factors by qRT-PCR

The expression levels of bacterial virulence factors in XDR ColRkp clinical isolates were then evaluated by Quantitative RT-PCR (qRT-PCR) using specific primers as previously reported (Table 7), (136, 137). The bacterial cultures of both XDR ColRkp and ColSkp clinical isolates were grown in Luria-Bertani broth (Merck, Darmstadt, Germany) during the mid-logarithmic growth phase. The extraction of mRNA and amplification of virulence genes were performed as described in section 5.1. Using the $2^{\Delta\Delta CT}$ method, the relative expressions of virulence factors were computed after normalization with control housekeeping gene - *rpoD* followed by

subsequent normalization against the value obtained for ColSkp isolate to evaluate and compare the fold change differences.

Table 7. Primers for amplification of bacterial virulence factors.

Target genes	Primer name	Primers' sequences (5′ to 3′)	References
mrkD	mrkD-F	AAGCTATCGCTGTACTTCCGGCA	(136, 137)
	mrkD-R	GGCGTTGGCGCTCAGATAGG	
ompK35	ompK35-F	GCAATATTCTGGCAGTGGTGATC	
	ompK35-R	ACCATTTTTCCATAGAAGTCCAGT	
ompK36	ompK36-F	TTAAAGTACTGTCCCTCCTGG	
	ompK36-R	TCAGAGAAGTAGTGCAGACCGTCA	
ybtS	ybtS-F	GACGGAAACAGCACGGTAAA	
	ybtS-R	GAGCATAATAAGGCGAAAGA	
kfu	kfu-F	GGCCTTTGTCCAGAGCTACG	
	kfu-R	GGGTCTGGCGCAGAGTATGC	
wabG	wabG-F	ACCATCGGCCATTTGATAGA	
	wabG-R	CGGACTGGCAGATCCATATC	
uge	uge-F	TCTTCACGCCTTCCTTCACT	
	uge-R	GATCATCCGGTCTCCCTGTA	
luxS	luxS-F	AGTGATGCCGGAACGCGG	
	luxS-R	CGGTGTACCAATCAGGCTC	

8. Effect of adjuvants on planktonic ColRkp clinical isolates

As described in previous studies (138), the proposed adjuvants including EDTA, sodium citrate, tramadol, curcumin, resveratrol, DNAase, and antibiofilm peptides, were screened firstly by broth microdilution to identify the most potent adjuvant that can be used in combination therapy to overcome ColRkp with various colistin resistance mechanisms. These adjuvants are the compounds that target bacteria inherent resistance mechanisms such as the outer membrane permeability barrier, and control bacterial biofilms (41). To establish ColRkp planktonic (independent, free-living bacterial cells) MIC for these adjuvant by broth microdilution method, drugs were serially diluted two-fold in CAMHB (BBL, BD® Diagnostic Systems, USA) in flat-bottomed 96-well plate (from 0.046875 to 48 mg/mL). Pure and fresh culture colonies of tested ColRkp clinical isolates and quality control strains (*E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853) were prepared in sterile normal saline and adjusted with 0.5McFarland turbidity (~$1.5x10^8$ CFU/mL) which were then diluted 1:100 with sterile normal saline. The adjusted bacterial suspension was added into CAMHB with serial diluted adjuvant as final concentration approximately $1.5x10^5$ CFU/mL and incubated at 37^0C for 18-24 hours. The lowest concentration of drug showing visible inhibition of bacterial growth after 18 hours incubation at 37°C was taken as the MIC of the tested adjuvant. The adjuvant that showed the most potent bacterial inhibitory effects was further evaluated to utilize for antibiotic-adjuvant combination therapy.

9. Effects of antibiotics and adjuvants on ColRkp biofilms

9.1. Effect of antibiotics and adjuvants on ColRkp biofilm biovolume

The effects of antibiotics (imipenem, meropenem, ceftazidime, ciprofloxacin, amikacin, fosfomycin and colistin) and adjuvants (EDTA, sodium citrate, tramadol, curcumin, resveratrol, DNAase, and antibiofilm peptides) on biofilm biovolume *in vitro* were evaluated to identify for the most potent antibiotic and adjuvant for eradication of ColRkp mature biofilms, based on the methods described previously (139). Briefly, mature biofilms of 47 ColRkp isolates were allowed to form in to flat bottomed-wells of 96-well-polystyrene microtiter plates (SP Life Sciences)

as described in section 6. Following mature biofilm of ColRkp was developed, the content in each well containing bacterial biofilms was removed and each well was carefully washed three times with 100µL of sterile CAMHB using a micropipette to eliminate non-adherent planktonic cells.

The tested antibiotics and adjuvants stock solutions were prepared and serially diluted twofold in a new 96-well flat-bottomed microtiter plate. Following serial dilution, 50µL of diluted antibiotics and adjuvants were transferred to the microtiter plate containing ColRkp mature biofilms. The final volume in all wells of this microtiter plate was then adjusted to 200µL using CAMHB, with well A1 serving as a control. The mature biofilms of ColRkp isolates were then subjected to different concentrations of antibiotics and adjuvants at 37°C for 24 hours.

Following incubation, the eradication effects of antibiotics and adjuvants were investigated using the crystal violet assay with the assessment of OD560nm using a microtiter-plate-reading spectrophotometer as described in section 6.1 to determine MBEC (minimal biofilm eradication concentration). MBEC is the lowest concentration of drug that eradicates all mature biofilms and 99.9% of biofilm-embedded bacteria while reducing bacterial viability by ≤10% when compared to growth controls (94, 95). All of these experiments were carried out in duplicates and repeated for three times. The percentage of biofilms eradication was computed using the following formula:

$$\text{Percentage of eradication} = \text{OD in control} - \text{OD in treatment} \times \frac{100}{\text{OD in control}}$$

9.2. Effect of antibiotics and adjuvants on ColRkp biofilm cells viability

The efficacies of antibiotics and adjuvants on biofilm cells viabilities *in vitro* were studied, based on the study described earlier (132). The mature biofilms of ColRkp clinical isolates were allowed to form in flat bottomed-wells of 96-well-polystyrene microtiter plates as described in section 6. Following mature biofilm of ColRkp was developed, the content in each well containing ColRkp biofilms was removed and each well was carefully washed three times with 100µL of sterile CAMHB using a micropipette to eliminate non-adherent planktonic cells.

The tested antibiotics and adjuvants stock solutions were prepared and serially diluted twofold in a new 96-well flat-bottomed microtiter plate. Following serial dilution, 50µL of diluted antibiotics and adjuvants were transferred to the microtiter plate of ColRkp preformed mature biofilms. The volume in all wells of this microtiter plate was then adjusted to 200µL using CAMHB, with well A1 serving as a control. The mature biofilms of ColRkp isolates were then subjected to different concentrations of antibiotics and adjuvants at 37ºC for 24 hours.

Following incubation, the contents of each well were gently removed, and each well was carefully washed three times with sterile CAMHB using a micropipette to eliminate non-adherent cells. The plates were incubated in darkness at room temperature for 20 minutes after PrestoBlue (Invitrogen) was applied directly to the wells (10µL/well). Utilizing a microtiter-plate-reading fluorimeter (Varioskan Flash Multimode Reader; Thermo Fisher Scientific), the fluorescence of the contents of each well was measured with excitation 535nm and emission 590nm using two possible reading modes (top from above the plate and bottom from below the plate). All of these experiments were carried out in duplicates and repeated for three times. The percentage of biofilm cell viability after treatment was computed using the following formula:

$$\text{Percentage of cell viability} = \text{OD in control} - \text{OD in treatment} \times \frac{100}{\text{OD in control}}$$

10. Determination of synergistic activity of colistin-EDTA combination on planktonic ColRkp clinical isolates

10.1.Screening of synergistic activity of colistin-EDTA combination by checkerboard assay

Following the identification of the most potent adjuvant, checkerboard synergy assay was used as a screening assay to evaluate the synergistic activity of colistin-EDTA combination against planktonic ColRkp isolates *in vitro*, as described previously (Figure 9) (140). In the checkerboard assay, both antibiotic-colistin and adjuvant-EDTA were combined at different concentrations in a 96-well flat-bottomed microtiter plate by mixing twofold serial dilutions of colistin in a column with twofold serial dilutions of EDTA in a row. Colistin and EDTA stock

solutions were prepared at 8XMIC concentrations, respectively. The colistin stock solution of 50µL was first added to column 12 (A12 to H12), followed by serial dilution with 50µL from column 12 to column 2 (A2 to H2). The adjuvant-EDTA stock solution was added to all wells in row H of another 96-well flat-bottomed microtiter plate, which was then serially diluted from row H to row B. Following the serial dilution, 50µL of diluted EDTA were transferred to the same wells of checkerboard panel of colistin. Following serial dilution, 50µL of diluted EDTA was transferred to the same wells of the microtiter plate containing antibiotic-colistin in the checkerboard panel. The volume in all wells of this microtiter plate was then adjusted to 180µL using CAMHB. The adjusted bacterial inoculum was obtained by suspending pure bacteria colonies in 3mL of sterile normal saline and adjusting the turbidity to 0.5 McFarland, then diluting in 1:100 by using sterile normal saline. The adjusted bacterial inoculum of 20µL was added to all wells of microtiter plate with checkerboard panel of colistin and EDTA with the exception of H12, which was utilized as a negative sterile control by adding CAMHB and the plates were incubated at 37°C for 18-24 hour. All of these checkerboard assay studies were carried out in duplicates.

For the interpretation and determination of the synergistic activity of colistin-EDTA combination, the fractional inhibitory concentration index (FICI) is calculated by the following formula:

$$\text{FIC index} = \frac{\text{MIC (drug A in combination)}}{\text{MIC (drug A alone)}} + \frac{\text{MIC (drug B in combination)}}{\text{MIC (drug B alone)}}$$

The synergistic activities are interpreted as follow:

- Synergy: FIC index ≤ 0.5
- Additive: 0.5 > FIC index ≤ 1
- Indifference: 1 > FIC index ≤ 4
- Antagonism: FIC index > 4

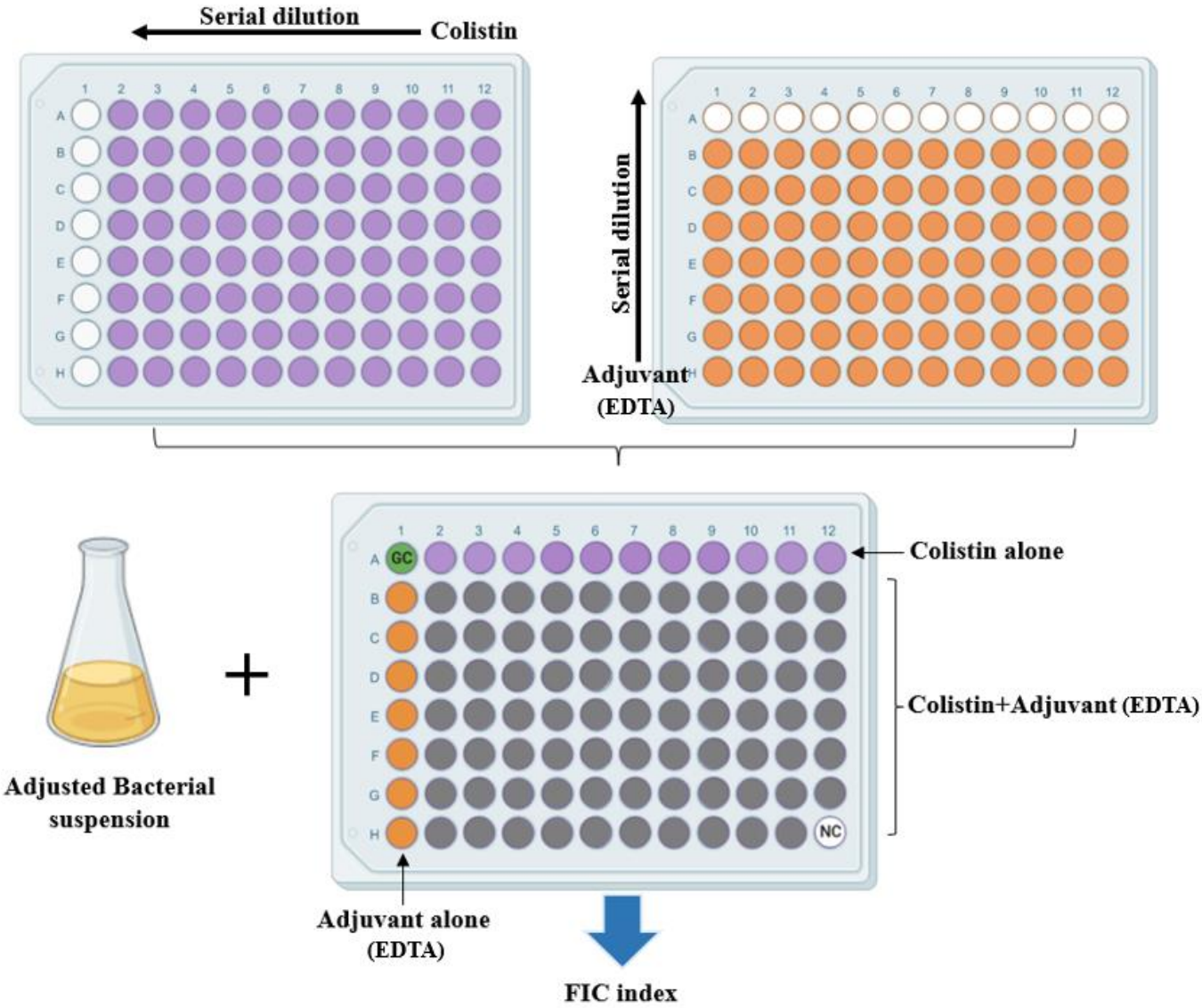


Figure 9. Checkerboard combination panel of colistin and EDTA.

10.2. Confirmation of synergistic activity of colistin-EDTA combination by time-kill assay

Using the previously described time-kill synergy confirmation assay (140), the synergistic activity of colistin-EDTA combination was further confirmed against 11 representative PDR and XDR ColRkp isolates encoded with various underlying chromosomal and plasmid-mediated colistin resistance mechanisms. All PDR and XDR ColRkp isolates were studied under 9 different growth conditions such as no drug (growth control), 1XMIC of colistin, 0.5XMIC of colistin, 1XMIC of EDTA, 0.5XMIC of EDTA, 1XMIC of colistin and 1XMIC of EDTA, 1XMIC of colistin and 0.5XMIC of EDTA, 0.5X MIC of colistin and 1XMIC of EDTA, and 0.5XMIC of colistin and 0.5XMIC of EDTA, respectively. All of the growth conditions were carried out in 125mL Erlenmeyer's flasks with a final total volume of 10mL. The bacterial suspension was made by inoculating a few bacteria colonies into CAMHB, which was then incubated for 2 hours at 37°C with shaking and adjusted the turbidity to 0.5McFarland. The adjusted bacterial inoculum of 100µL was added into each flask of different growth conditions. The flasks were then incubated for 24 hour at 37°C with shaking (120 rpm). The viable cell counts under different growth conditions were determined at different times of 0, 2, 4, 6, 8, 12, and 24 hours by diluting 20µL of each growth condition with 180µL of sterile normal saline to get different dilutions ranging from 10^{-1} to10^{-12} and spotting 100µL (1 spot/10µL) from each dilution on MHA plates and incubating for 18-24 hour at 37°C. After incubation, the number of viable bacterial cells in each growth condition were quantified as CFU/mL and plotted on a semi-log graph. The time-kill assay experiments were performed in triplicates and the lower limit of detection was $2\log_{10}$ (CFU/mL). The synergistic activities are interpreted as follows:

- Synergy was defined as a $\geq 2\log_{10}$ (CFU/mL)-fold decrease in combination compared with the single active drug.
- Indifference was defined as a $2\log_{10}$ (CFU/mL)-fold increase or decrease in combination compared with the single active drug.
- Antagonism was defined as a $\geq 2\log_{10}$ (CFU/mL)-fold increase in combination compared with the single active drug.

- The bactericidal activity was defined as a $3\log_{10}$ (CFU/mL)-fold decrease when compared to the number of viable cells at starting time point.

11. Determination of synergistic activity of colistin-EDTA combination on ColRkp biofilms

11.1. Determination of synergistic activity of colistin-EDTA combination on ColRkp biofilms biovolume

The synergistic activity colistin and EDTA combination on ColRkp biofilm biovolume *in vitro* was evaluated, based on the methods described previously (139). The mature biofilms of 47 ColRkp isolates were allowed to form in flat bottomed-wells of 96-well-polystyrene microtiter plates as described in section 6. Following mature biofilm was developed, the content in each well containing ColRkp biofilms was removed and each well was carefully washed three times with 100µL of sterile CAMHB using a micropipette to eliminate non-adherent planktonic cells.

Both of the drugs such as colistin and EDTA were combined at different concentrations in a new 96-well flat-bottomed microtiter plate by mixing twofold serial dilutions of colistin in a column with twofold serial dilutions of EDTA in a row as described in section 10.1. Following serial dilution, 100µL of serially diluted colistin and EDTA in alone and combination were transferred to the microtiter plate with mature biofilms of ColRkp clinical isolates. The volume in all wells of this biofilm-containing microtiter plate was then adjusted to 200µL using CAMHB, with well A1 serving as a growth control and non-inoculated CAMHB medium in another microtiter plate as a negative control of this experiment. The mature biofilms of ColRkp isolates were then subjected to different concentrations of colistin and EDTA in alone and combination at 37°C, for different exposure times - 6, 12, 24, and 48 hours *in vitro*, respectively.

Following incubation at desired treatment exposure times, the eradication effects of colistin and EDTA in alone and combination were investigated using the crystal violet assay with the assessment of OD560nm using a microtiter-plate-reading spectrophotometer as described in section 6.1 to determine MBEC.

MBEC is the lowest concentration of drug that eradicates all mature biofilms and 99.9% of biofilm-embedded bacteria while reducing bacterial viability by ≤10% when compared to growth controls (94, 95). All of these experiments were carried out in duplicates and repeated for three times. The percentage of biofilms eradicated by colistin and EDTA in alone and combination was computed using the following formula:

$$\text{Percentage of eradication} = \text{OD in control} - \text{OD in treatment} \times \frac{100}{\text{OD in control}}$$

11.2. Determination of synergistic activity of colistin-EDTA combination on ColRkp biofilm cells viability

The synergistic activity of colistin and EDTA combination on ColRkp biofilm cells viabilities *in vitro* was investigated, based on the study described earlier (132). The mature biofilms of ColRkp clinical isolates were allowed to form in flat bottomed-wells of 96-well-polystyrene microtiter plates as described in section 6. Following mature biofilm was developed, the content in each well containing ColRkp biofilms was removed and each well was carefully washed three times with 100µL of sterile CAMHB using a micropipette to eliminate non-adherent planktonic cells.

Both colistin and EDTA were combined at different concentrations in a new 96-well flat-bottomed microtiter plate by mixing twofold serial dilutions of colistin in a column with twofold serial dilutions of EDTA in a row, as described in section 10.1. Following serial dilution, 100µL of serially diluted colistin and EDTA in alone and combination were transferred to the microtiter plate with mature biofilms of ColRkp clinical isolates. The volume in all wells of this biofilm-containing microtiter plate was then adjusted to 200µL using CAMHB, with well A1 serving as a growth control and non-inoculated CAMHB medium in another microtiter plate as a negative control of this experiment. The mature biofilms of ColRkp isolates were then subjected to different concentrations of colistin and EDTA in alone and combination at 37°C, for different exposure time - 6, 12, 24, and 48 hours *in vitro*, respectively.

Following incubation at desired treatment exposure times, the effects of colistin and EDTA on biofilm cell viabilities were determined as described in section 9.2 using PrestoBlue (Invitrogen) to determine MBEC. All of these experiments were carried out in duplicates and repeated for three times. The percentage of biofilm cell viabilities after treating with colistin and EDTA, in alone and combination, were computed using the following formula:

$$\text{Percentage of cell viability} = \text{OD in control} - \text{OD in treatment} \times \frac{100}{\text{OD in control}}$$

12. Establishment of *in vitro* ColRkp catheter-related biofilm infection model

12.1. Catheter pieces preparation

Under the biological safety cabinet, 25-mm catheters (NIPRO) with an inner diameter of 1.45mm were pre-coated with 1.8 mL of 100% fetal bovine serum (Gibco™; Waltham, MA) and vortexed vigorously. Additional 100-200µL of 100% FBS was supplemented to adequately cover all catheter pieces with serum and incubated at 37°C overnight (141).

12.2. Ex-vivo adhesion and development of *in vitro* ColRkp catheter-related biofilm infection model

Each serum-coated catheter was placed in a new 1.5mL microcentrifuge tube and inoculated with cell suspensions of ColRkp at final concentrations of 5×10^7 CFU/mL at 37°C for 90 min to allow for ex-vivo microbial adhesion to catheters. For each experimental set, *in vitro*-infected catheters were processed for assessment of microbial recovery to confirm standardized microbial adherence to catheters prior to implantation. Following *in vitro* microbial adhesion, the prepared catheter pieces were further incubated at 37°C for 7 days for the establishment of catheter-related biofilm model *in vitro* (141).

13. Effects of colistin, EDTA and colistin-EDTA combination on *in vitro* ColRkp catheter-related biofilm model

To evaluate the effects of colistin, EDTA and colistin-EDTA combination on ColRkp catheter-related biofilm model *in vitro*, the catheter pieces were exposed to PBS (control), colistin (1mg/L), EDTA (12mg/mL) and colistin-EDTA (1mg/L+ 12mg/mL) at 37°C for 24 hours. The efficacy of each treatment on ColRkp catheter-related biofilm infection model *in vitro* was assessed using a confocal laser scanning microscope (in terms of biomass, Live/Dead ratio, and biovolume inhibition) as described in section 6.2.

14. Establishment of *in vivo* ColRkp catheter-related biofilm infection

14.1. Animals and suppression of the immune system

The previously published murine model of *in vivo* catheter-related biofilm infection was performed with some modifications (Figure 10) (141). In this study, all *in vivo* experiments used female 6-8-week-old C57BL/6 mice purchased from Nomura Siam International (Pathumwan, Bangkok, Thailand). Before being used, the animals were given a week of rest in the animal facility. Animals were also given free access to food and water and were housed in groups of no more than 2 mice per cage. They were weighed and thoroughly monitored for symptoms of distress during the experiments. The animal study was conducted according to guidelines and protocols approved by the Institutional Animal Care and Use Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand, based on the National Institutes of Health (NIH), USA.

Mice were given 0.25mg/kg/dose dexamethasone subcutaneously once a day for 3 days prior to subcutaneous catheter placement to achieve immunosuppression, and it was maintained throughout the experiment. Antibiotic such as ampicillin sodium powder (0.5g/L), was supplemented to the drinking water to prevent any bacterial contamination of the host, and the antibiotic was discontinued one day before subcutaneous catheter placement in tested mouse (141).

14.2. Catheter pieces preparation

The number of catheter pieces required to be used was calculated before establishing animal model of ColRkp catheter-related biofilm infections. Under the biological safety cabinet, 25-mm catheters (NIPRO) with an inner diameter of 1.45mm were pre-coated with 1.8 mL of 100% fetal bovine serum (Gibco™ ; Waltham, MA) and vortexed vigorously. Additional 100-200µL of 100% FBS was supplemented to adequately cover all catheter pieces with serum and incubated at 37°C overnight (Figure 10) (141).

14.3. Ex-vivo adhesion of ColRkp on FBS-coated catheters

Each serum-coated catheter was placed in a new 1.5mL microcentrifuge tube and inoculated with cell suspensions of ColRkp at final concentrations of 5×10^7CFU/ mL at 37°C for 90 min to allow for ex-vivo microbial adhesion to catheters. For each experimental set, *in vitro*-infected catheters were processed for assessment of microbial recovery to confirm standardized microbial adherence to catheters prior to implantation. Following *in vitro* microbial adhesion, catheter pieces were rinsed with PBS and transferred to a clean microcentrifuge tube (one piece per tube). All were kept on ice until subcutaneously implanted in mice (Figure 10) (141).

14.4. *In vivo* ColRkp catheter-related biofilm infection mouse model

The lower back of the mouse was shaved to remove all animal hairs before being moved to a clean paper tissue. The skin of the mouse was then disinfected with 1% iodine isopropanol or 0.5% chlorhexidine in 70% alcohol and allowed to dry for about 1 minute. Under isoflurane anesthesia, small incision in the skin (about 0.5-1cm) was made one on the left and the right side of the flank of animal. The subcutaneous layer was dissected with a sterile scissor to generate two subcutaneous tunnels, and subcutaneous implantation of catheter was done in each tunnel on the left and right sides of the flank of animal in a horizontal arrangement, under isoflurane anesthesia. The incisions were closed with sutures. Chlorhexidine 0.5% in 70% alcohol was utilized to sanitize the suture wound. Following

subcutaneous implantation of catheters, these catheter pieces were retained in mice for 7 days to develop murine catheter-related biofilm infection model *in vivo* (141). The animals were kept under constant observation until they recovered from anesthesia and thereafter on a daily basis for any signs of distresses. Isoflurane inhalation was used to euthanize these animals, and the collected samples (catheters, blood, heart, tissues around the catheter, lungs, spleen, and kidneys) were aseptically harvested individually (Figure 10) (141, 142).

15. Effects of colistin, EDTA and colistin-EDTA combination on *in vivo* ColRkp catheter-related biofilm infection mouse model

To evaluate the *in vivo* effects of colistin, EDTA and colistin-EDTA combination on murine model of ColRkp catheter-related biofilm infection, animals were divided into control (no therapy) group and experimental (therapy) group with 3 subgroups of different treatments (Figure 10). The ColRkp catheter-related biofilm infections inside the implanted catheters were exposed to PBS (control), colistin (1mg/L), EDTA (12mg/mL) and colistin-EDTA (1mg/L+12mg/mL) for a total of 4 groups with 10 animals in each group. Catheters were removed under aseptic conditions after 24 hour of treatment, and the efficacy of each treatment in different groups was assessed using a confocal laser scanning microscope (in terms of biomass, Live/Dead ratio, and biovolume inhibition) as described in section 6.2.

Also, mice were sacrificed on different days post-infection by cervical dislocation to determine viable cell count (CFU/mL) in treated catheter and internal organs (blood, heart, kidneys, lungs, spleen, and tissues surrounding catheter) after each treatment as described previously (142). The animal organs were removed aseptically and homogenized in 1mL of sterile normal saline. Serial dilutions of the homogenized tissues were made and plated on nutrient agar plates. Plates were incubated at 37°C for 24 hour and bacterial counts (CFU/mL) were determined.

For measurements of serum creatinine levels, cardiac puncture was performed with a G25 needle and non-heparinized syringes to obtain approximately 0.7mL of blood. The blood was immediately decanted into 1.3mL serum gel tubes (Sarstedt, Nürnbrecht, Germany). The serum was separated from the collected blood

by centrifuging the whole blood samples for 3minutes at 9000 rpm, and the separated sera were then utilized with an autoanalyzer to assess known markers of renal dysfunction - creatinine levels (Comas C-501) (143).

In addition, treated animals were monitored regularly for 7 days or until death, whichever occurred first, to determine their survival under different treatments (Figure 10).

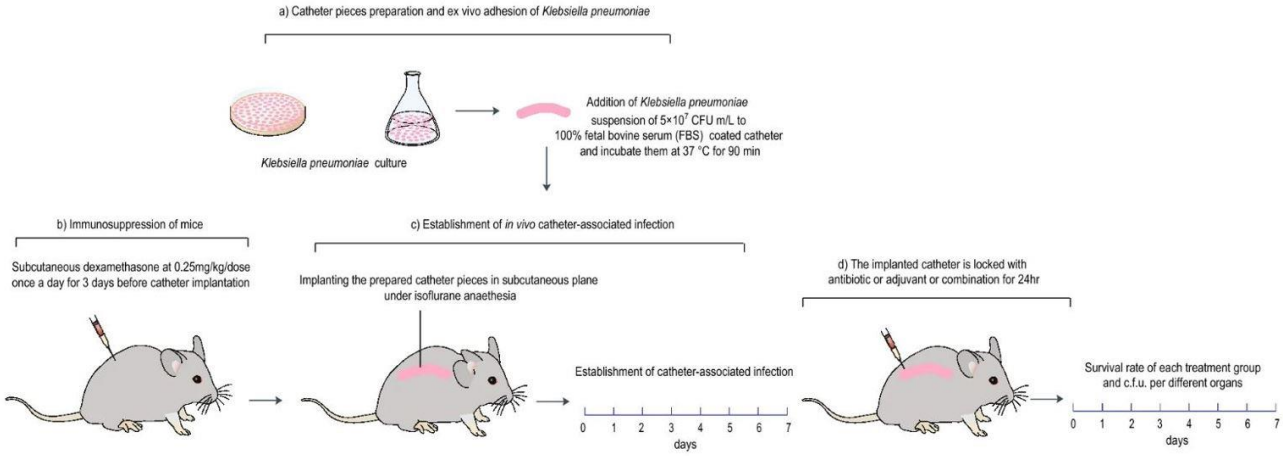


Figure 10. Schematic illustration for study of colistin, EDTA and colistin-EDTA combination on catheter-related biofilm infection mouse model.

16. Effects of single and combination of colistin-EDTA on *in vivo* virulence gene expressions

The effects of single and combination of colistin-EDTA on *in vivo* expressions of different virulence factors (*mrkD*, *ompK35*, *ompK36*, *ybtS*, *kfu*, *wabG*, *uge*, *luxS*) were evaluated by Quantitative RT-PCR (qRT-PCR**)** using specific primers, as previously reported (Table 7) (136, 137). Total mRNA were retrieved from control and experimental groups of *in vivo* ColRkp catheter-related biofilm infections that were challenged with various treatments for 24 hours. The extraction of mRNA and amplifications of virulence genes were performed as described in section 5.1. All samples are analyzed in triplicates. Using the $2^{\Delta\Delta CT}$ method, the relative expressions of bacteria virulence genes for each treatment group were computed after

normalization with control housekeeping gene - *rpoD* followed by subsequent normalization against the value obtained for PBS-treated group to evaluate and compare the fold change differences.

17. Statistical analysis

Statistical analysis was performed using the R statistical package, GraphPad prism (Version 9) and SPSS software (Version 22) (144). The data were compared by either unpaired two-tailed Student's t-test or unpaired two-tailed Mann-Whitney's U test or Chi-square test. All data were presented as the mean±SD. Statistical significance was accepted at *p* values of less than 0.05, 0.01, 0.001 and 0.0001.

CHAPTER V

RESULTS

1. Bacterial strains collected in this study

A total of 165 non-duplicate CRkp clinical isolates were obtained from different clinical samples of patients who were admitted at King Chulalongkorn Memorial Hospital between 2016 and 2021. The age of patients infected with CRkp isolates were ranged from 29 Days to 97 years. These CRkp isolates were isolated from 101 males (61.2%) and 64 females (38.8%). The numbers of CRkp isolates were collected as 7 isolates in 2016 (4.3%), 2 isolates in 2017 (1.2%), 2 isolates in 2018 (1.2%), 8 isolates in 2019 (4.8%), 87 isolates in 2020 (52.7%) and 59 isolates in 2021 (35.8%), respectively.

The majority of CRkp isolates (n=115, 69.7%) were obtained from non-sterile sites, including 65 isolates from urine (39.4%), followed by 38 isolates from sputum (23%), 11 isolates from pus (6.7%), and 1 isolate from urethral swab (0.6%), respectively (Table 8). Among CRkp clinical isolates, a total of 50 isolates (30.3%) were isolated from sterile sites, including 17 isolates from blood (10.3%), followed by 15 isolates from endotracheal aspirate (9.1%), 7 isolates from bile (4.2%), 6 isolates from body fluid (3.6%), 2 isolates from tip catheter (1.2%), 1 isolate from bronchoalveolar lavage (0.6%), 1 isolate from pleural fluid (0.6%), and 1 isolate from intra-abdominal fluid (0.6%), respectively (Table 8).

Table 8. Sources of clinical specimens of 165 CRkp isolates in this study.

Source	Specimen types	No. of isolates (n)	Percentage of total (%)
Sterile sites (n=50/165) (30.3%)	Blood	17	10.3%
	Endotracheal aspirate	15	9.1%
	Bile	7	4.2%
	Body fluid	6	3.6%
	Tip catheter	2	1.2%
	Bronchoalveolar lavage	1	0.6%
	Pleural fluid	1	0.6%
	Intra-abdominal fluid	1	0.6%
Non-sterile sites (n=115/165) (69.7%)	Urine	65	39.4%
	Sputum	38	23.0%
	Pus	11	6.7%
	Urethral swab	1	0.6%
Total		165	100%

2. Antimicrobial susceptibility testing

When the susceptibilities to different antibiotics including colistin, carbapenems (imipenem and meropenem), cephalosporins (ceftazidime), fluoroquinolones (ciprofloxacin), aminoglycosides (amikacin), and fosfomycin were investigated, these CRkp clinical isolates were observed to exhibit different antibiotic susceptibility profiles, showing highest resistance rate to ceftazidime and ciprofloxacin (100%), followed by imipenem (94. 5%), meropenem (90. 3%), fosfomycin (31.5%) and amikacin (23.6%), respectively (Table 9) (Figure 11-16).

The colistin MIC was ranged from 0.125 to >512mg/L, with MIC50 and MIC90 of 0.5 and 64 mg/L respectively (Table 9) (Figure 17). We discovered that 47 isolates (28.5%) showed colistin resistance (ColRkp) which grew rapidly over time from 14.9% (n=7) in 2016, 4.25% (n=2) in 2017, 4.25% (n=2) in 2018, 17% (n=8) in 2019, 23.4% (n=11) in 2020 to 36.2% (n=17) in 2021, respectively (Figure 18). The

majority of ColRkp isolates (n=43, 91.5%) expressed extensively drug-resistance (XDR) characteristics and 8.5% (n=4) exhibited pan-drug-resistance (PDR) profile (Appendix D, Table 15).

Table 9. Susceptibilities of planktonic CRkp clinical isolates to different antibiotics.

Antimicrobial agents	MIC range (mg/L)	MIC50 (mg/L)	MIC90 (mg/L)	Susceptibility interpretation		
				Resistance (n) (%)	Intermediate (n) (%)	Susceptible (n) (%)
Ceftazidime	32->512	>512	>512	165(100%)	-	-
Ciprofloxacin	2->512	512	>512	165(100%)	-	-
Imipenem	0.25->512	64	256	156(94.5%)	3 (1.8%)	6 (3.6%)
Meropenem	0.125->512	128	256	149(90.3%)	4 (2.4%)	12 (7.3%)
Fosfomycin	2->512	64	>512	52 (31.5%)	18 (10.9%)	95 (57.6%)
Amikacin	1->512	16	>512	39 (23.6%)	32 (19.4%)	94 (57.0%)
Colistin	0.125->512	0.5	64	47 (28.5%)	118 (71.50%)	-

MIC - Minimal inhibitory concentrations (mg/L) for planktonic cells.

MC50 - Minimal inhibitory concentrations (mg/L) required to inhibit 50% of isolates tested.

MIC90 - Minimal inhibitory concentrations (mg/L) required to inhibit 90% of isolates tested.

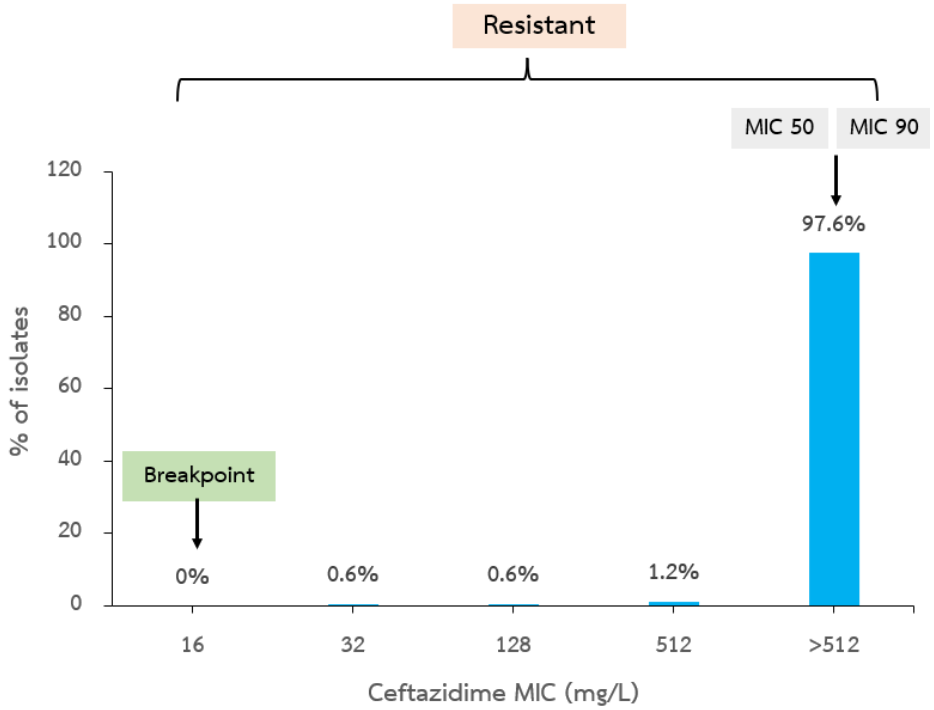


Figure 11. Distribution of ceftazidime MICs among 165 CRkp clinical isolates.

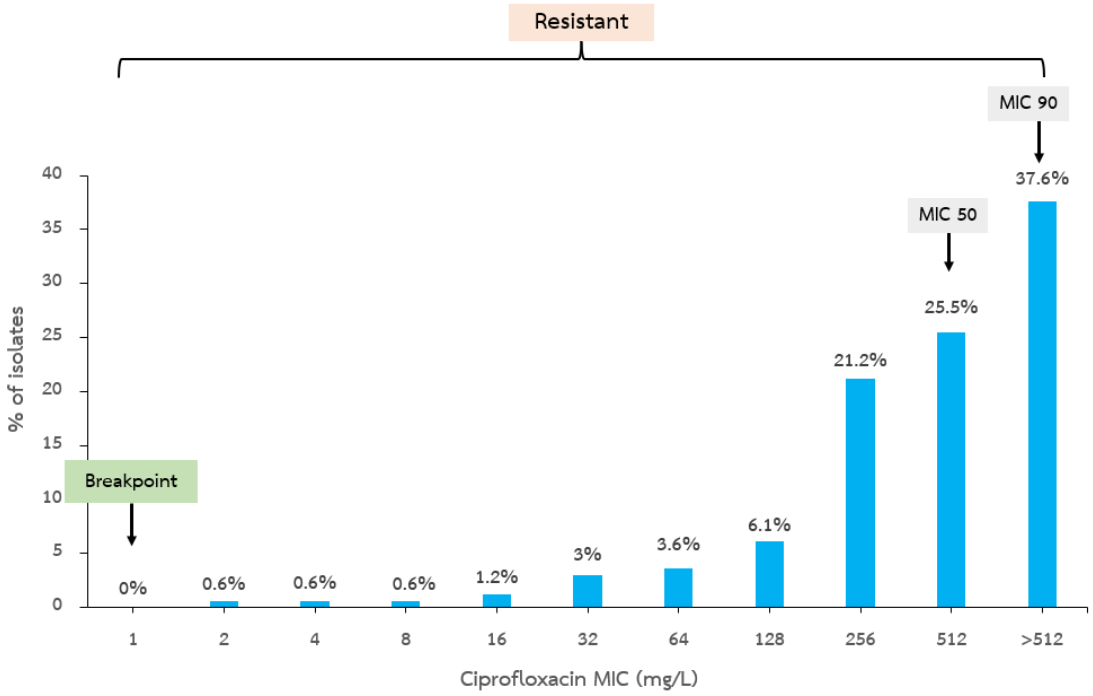


Figure 12. Distribution of ciprofloxacin MICs among 165 CRkp clinical isolates.

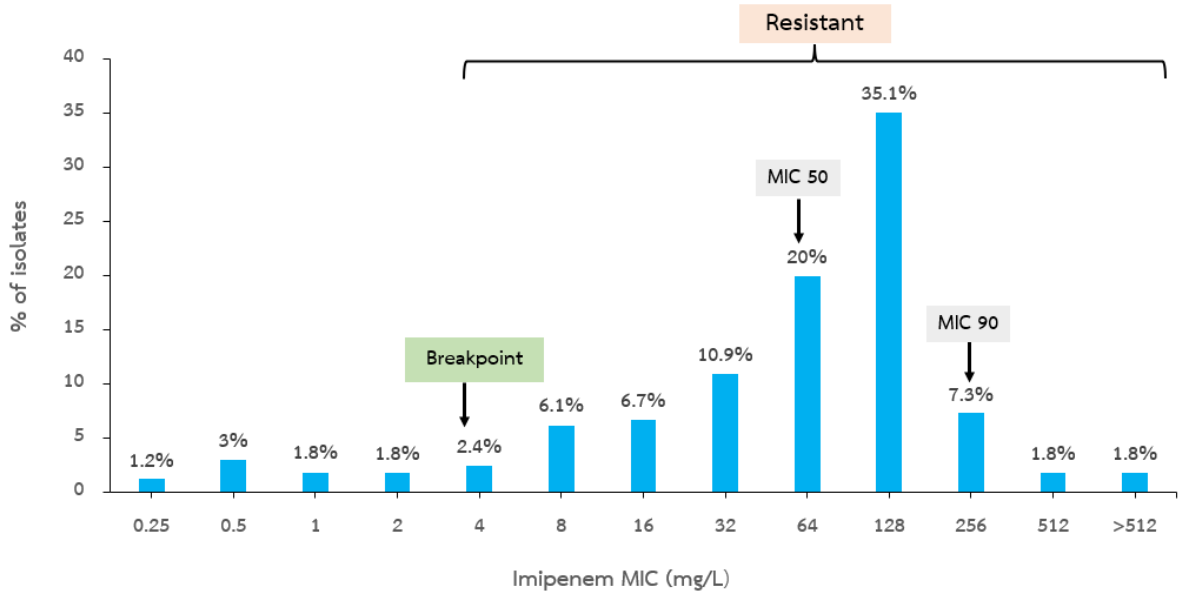


Figure 13. Distribution of imipenem MICs among 165 CRkp clinical isolates.

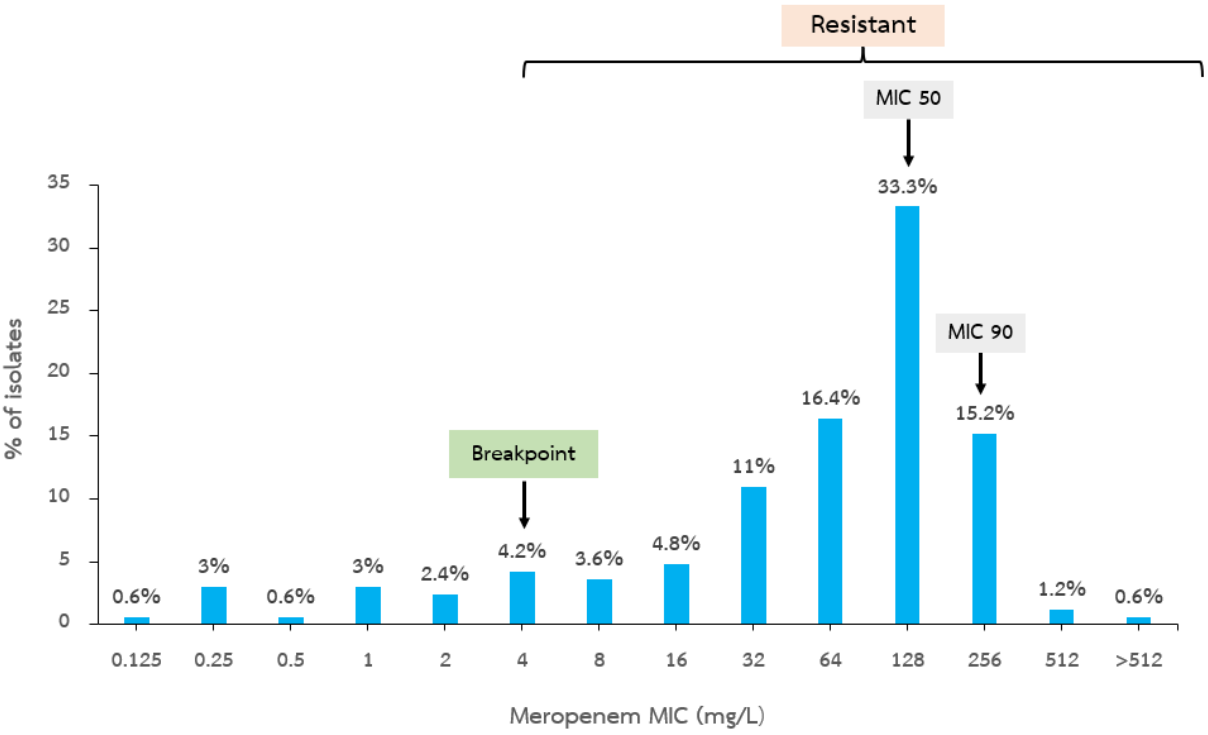


Figure 14. Distribution of meropenem MICs among 165 CRkp clinical isolates.

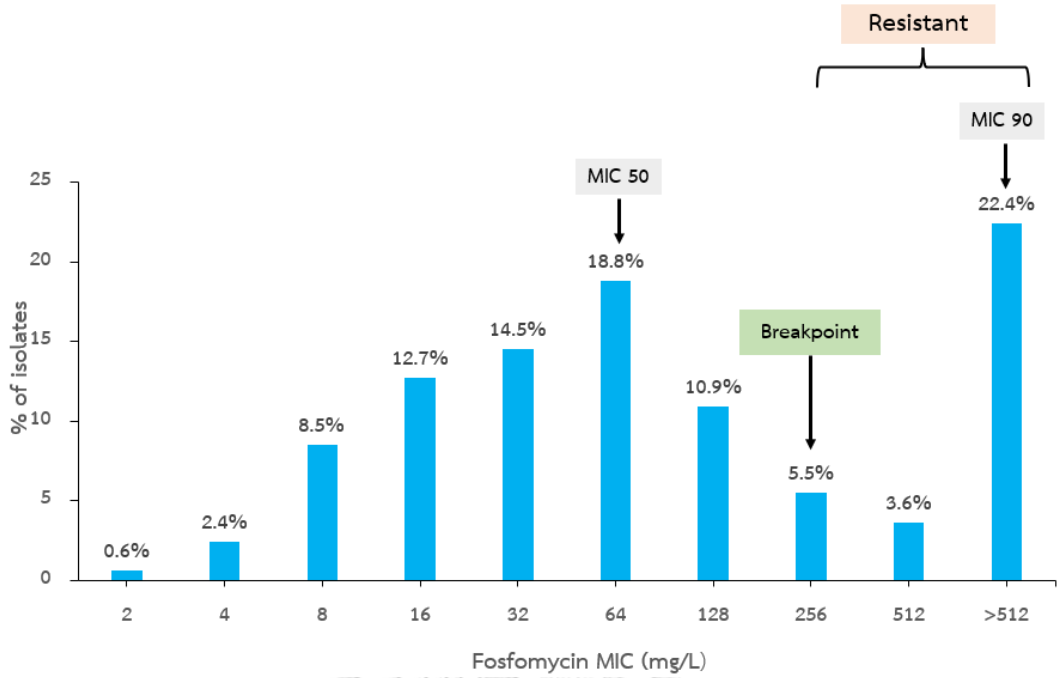


Figure 15. Distribution of fosfomycin MICs among 165 CRkp clinical isolates.

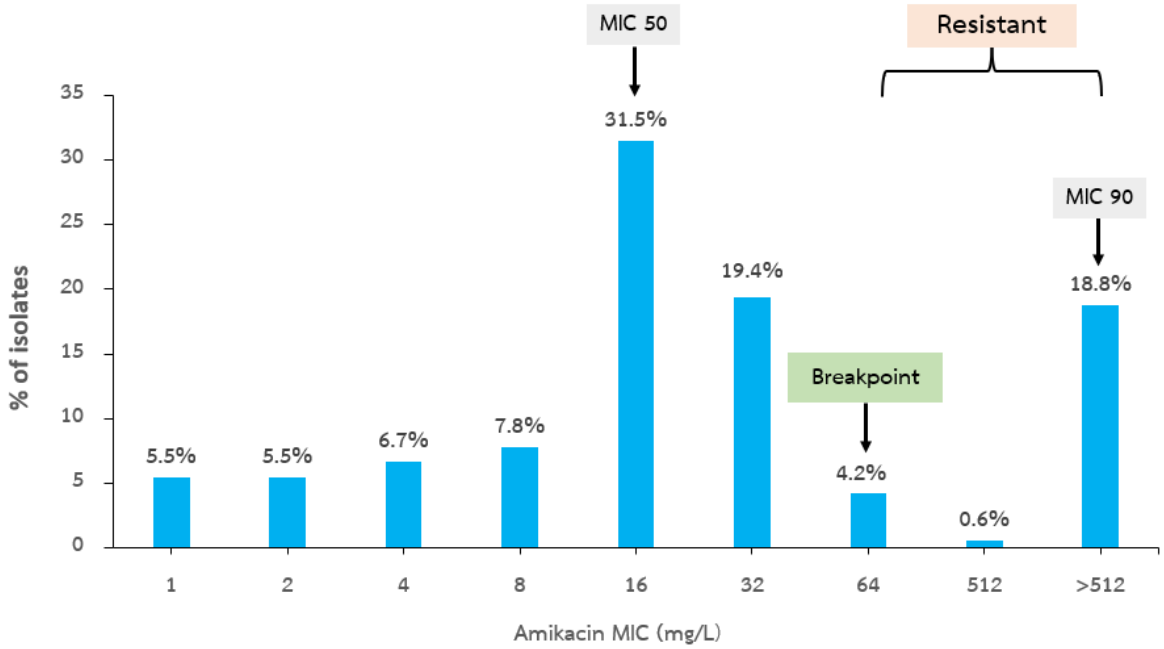


Figure 16. Distribution of amikacin MICs among 165 CRkp clinical isolates.

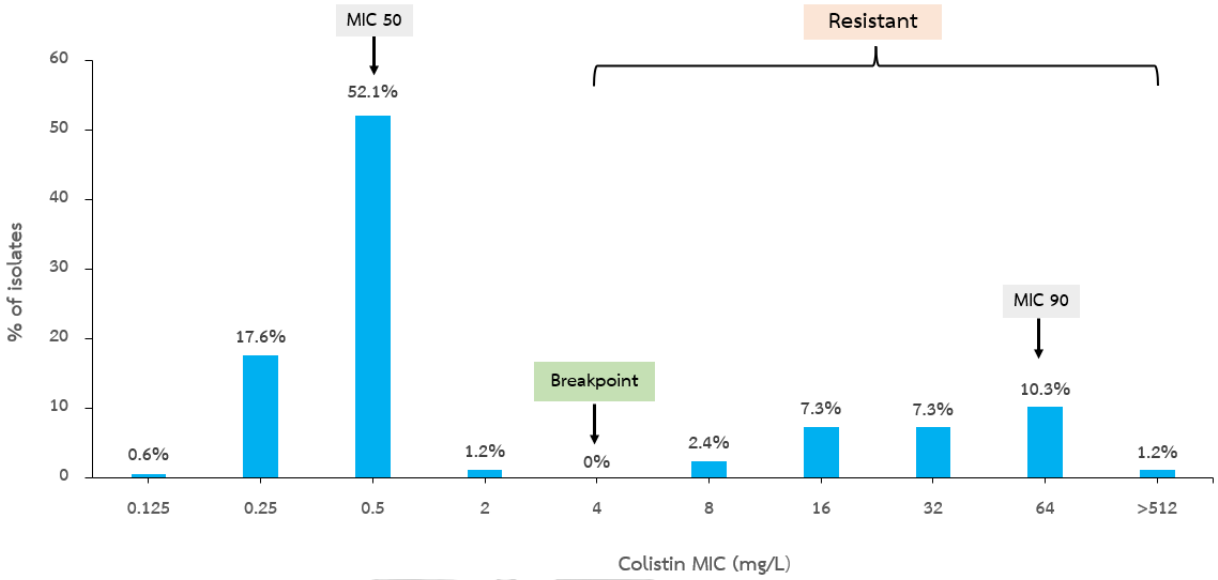


Figure 17. Distribution of colistin MICs among 165 CRkp clinical isolates.

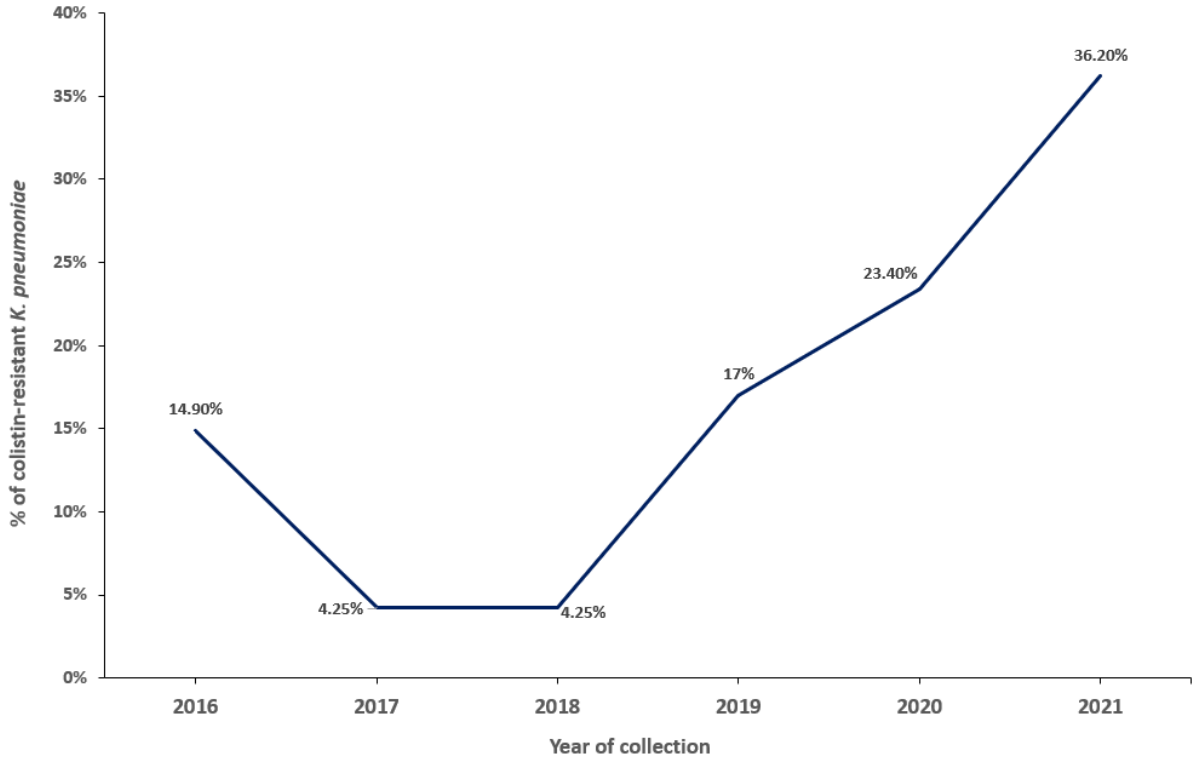


Figure 18. Trend of ColRkp clinical isolates collected between 2016-2021.

3. Molecular characterization of colistin resistance mechanisms

3.1.Molecular characterization of chromosomal-mediated colistin resistance mechanisms

To determine the presence of underlying chromosomal-mediated colistin resistance mechanisms, a total of 47 ColRkp clinical isolates and *K. pneumoniae* ATCC 13883 were selected for DNA sequencing of chromosomal genes including transmembrane regulatory gene - *mgrB*, and TCS genes - *pmrA* (response regulator), *pmrB* (sensor kinase), *phoP* (response regulator), and *phoQ* (sensor kinase), respectively. In our study, *K. pneumoniae* MGH 78578, a multidrug-resistant clinical pathogen from human sputum with a complete genome sequence, was chosen as the reference genome based on its highest genomic similarity with our strains. The complete coding regions of *mgrB*, *pmrAB* and *phoPQ* of 47 ColRkp clinical isolates and *K. pneumoniae* ATCC 13883 were compared with data base sequence of *K. pneumoniae* MGH 78578 (GenBank accession number.CP_000647.1), and reference amino acid sequences of MgrB (GenBank accession number. WP_0029113751, PmrA (GenBank accession number. WP_23302144. 1) , PmrB (GenBank accession number.WP_114504106.1), PhoP (GenBank accession number.WP_004150807.1), and PhoQ (GenBank accession number.WP_004147969.1), respectively.

3.1.1. Insertional integration of *mgrB* by diverse IS was a key mechanism causing colistin resistance

In this study, chromosomal-mediated colistin resistance mechanisms were observed in 91.5% of ColRkp isolates (n=43) (Table 10-11). Among the chromosomal-mediated colistin resistance mechanisms, *mgrB* was the most commonly altered gene (n= 40, 85.1%) (Table 10-11). The chromosomal *mgrB* was observed to be mainly altered by insertional integration with diverse IS elements (n= *29*, 61.7%) (Table 10-11). These IS elements included IS*1*-like (768 bp), IS*kpn14*-like (IS*1*, 768 bp), IS*3*-like (1321 bp), IS*5*-like (1056bp) and IS*Ecp1*-like elements [(IS1*380*) , 1656 bp] , which were inserting with different orientations using their inverted repeats in 10 different sites within *mgrB* coding region and the region

between *mgrB* start codon and putative promoter region, which trigger the alteration of MgrB resulting colistin resistance (Table 10-11) (Figure 19 a-j).

In a total of 23 XDR and PDR ColRkp clinical isolates (48.9%), chromosomal *mgrB* was disrupted by the same 768bp sequences of IS*1* family including IS*1*-like and IS *kpn14* IS elements (Table 10-11) (Figure 19 a-g). According to the ISfinder database, these IS elements were closely linked (99% nucleotide identity) to IS*1* of *E. coli*, with both transposases having 99% amino acid identity. Detailed analysis of their inverted repeats revealed that there were identical direct repeats in the right flank (CCAACTTA) and in the left flank (TAAGTTGG) of the first insertion sequence. Insertion of IS*1*-like elements was observed to be targeted in the *mgrB* coding regions between nucleotides + 55 and +56 position in 6 distinct XDR ColRkp isolates (12.8%) with MIC 8-64mg/L (GenBank Accession number. KJ937472.1) (Figure 19 a), + 71 and +72 position in 5 distinct XDR ColRkp isolates (10.62%) with MIC 16-64mg/L (GenBank Accession number. CP060421.1) (Figure 19 b), between +104 and +105 position in 2 distinct XDR and PDR ColRkp isolates (n=1 each) (4.25%) with MIC >512mg/L (Accession number. MW389562.1) (Figure 19 c), and between + 105 and + 106 position in 1 distinct XDR ColRkp isolate (2.12%) with MIC 32mg/L (GenBank Accession number. CP050360.1) (Figure 19 d), (Table 10-11), respectively. Furthermore, IS*1*-like IS elements were inserted in the region between the start codon and putative promoter region of *mgrB* including the target sites between nucleotides -7 and -8 in 5 XDR ColRkp isolates (10.6%) with MIC 32-64mg/L, when referring to the start codon of *mgrB* as +1 (GenBank Accession number. CP027036.1) (Figure 19 e), (Table 10-11). Insertion of IS *kpn14* elements which belongs to IS*1* family was also observed to be targeted in *mgrB* coding regions between nucleotides + 115 and + 116 position in 2 distinct XDR ColRkp isolates (4.25%) with MIC 16-64 mg/L (Figure 19 f), and between + 117 and + 118 position in 2 distinct XDR ColRkp isolates (4.25%) with MIC 8-32 mg/L (GenBank Accession number. MG930936.1) (Figure 19 g), (Table 10-11) respectively. All of these isolates sequences were analyzed and aligned with data base sequence (Figure 19 a-g), (Table 10-11).

Insertional integration of *mgrB* by other IS elements was also identified in both XDR ColRkp clinical isolates (Table 10-11). Among them, the identical 1321bp

IS*3-like* IS element was observed to target the same location, between nucleotides +121 and +122 of *mgrB* coding region in 2 XDR ColRkp isolates (4.25%) with colistin MIC 16mg/L, (GenBank Accession number. CP053364.1) (Figure 19 h), (Table 10-11). According to the ISfinder database, these IS elements were closely related (99% nucleotide identity) to IS*3* of *E. coli*, with both transposases having 99% amino acid identity. Detailed analysis of their inverted repeats revealed the identical direct repeats in the left and right flanks of the insertion sequence (TGGCCCCTA) (Figure 19 h). All of these sequences were analyzed and aligned with data base sequence (Figure 19 h), (Table 10-11).

Insertion by the same 1056bp IS *903-like* (IS*5*-like) IS elements were detected in 2 distinct XDR ColRkp isolates (4.25%) with MIC 32-64mg/L that targeted the same location between nucleotides +74 and +75 of *mgrB* coding region (GenBank Accession number. MK479294.1) (Table 10-11) (Figure 19 i). According to the ISfinder database, these IS elements were closely related (99% nucleotide identity) to IS*5* of *K. pneumoniae,* with both transposases having 99% amino acid identity. Detailed analysis of their inverted repeats revealed the identical direct repeat in the left and right flanks of the insertion sequence (GGCTTTG) (Figure 19 i). All of these sequences were analyzed and aligned with data base sequence (Table 10-11) (Figure 19 i).

In another 2 distinct XDR ColRkp clinical isolates (4.25%) with MIC 16-32mg/ L, *mgrB* was inserted by the IS *Ecp1-like* elements (IS*1380-like*, 1661 bp) between nucleotides + 124 and + 125 of *mgrB* coding region (GenBank Accession number. KY426739.1) (Figure 19 j), (Table 10-11). According to the ISfinder database, these IS elements were closely related (99% nucleotide identity) to IS*1380* of *E. coli*, with both transposases having 99% amino acid identity. Detailed analysis of their inverted repeats revealed the identical direct repeat in the left and right flanks of the insertion sequence (CCTA) (Figure 19 j). All of these sequences were analyzed and aligned with data base sequence (Figure 19 h-j), (Table 10-11).

3.1.2. Genetic alterations in *mgrB* by point mutations and deletion played roles in mediating colistin resistance.

Different genetic alterations in chromosomal *mgrB*, including deletion (n=5, 10.62%) and point mutations (n=6, 12.75%) were observed in both XDR and PDR ColRkp clinical isolates (Table 10-11).

No amplification product of *mgrB* using the external *mgrB* primers that target for whole *mgrB* gene amplification suggested that there was deletion of *mgrB* locus in a total of 5 distinct XDR (n=4. 8.5%) and PDR (n=1, 2.12%) ColRkp isolates (10.62%), with MIC range of 16-64 mg/L. The absence of *mgrB* in the genome of these isolates was further confirmed by PCR using different *mgrB* primers that target for amplification of external and internal regions of *mgrB* as described previously (Table 10-11).

There were genetic alterations in *mgrB* of 6 distinct XDR ColRkp clinical isolates due to point mutation in initial codon (G3A, GTG>GTA) (n=2, 4.25%) (GenBank Accession number. MH368668.1), and point mutations generating internal stop codon including point mutation at codon 7 generating internal stop codon [A7T, AAA>TAA (Stop)] (n=2, 4.25%) (GenBank Accession number. MH368669.1), as well as point mutation at codon 60 generating internal stop codon [G60A, TGG>TGA (Stop)] (n=2, 4.25%) (GenBank Accession number. CP021859.1), exhibiting 32-64mg/L colistin MIC (Figure 19 k), (Table 10-11). All of these *mgrB* point mutations were analyzed and aligned with data base sequence (Figure 19 k), (Table 10-11).

3.1.3. Point mutations in *pmrB* and *phoP* were involved as additional chromosomal-mediated colistin resistance mechanisms.

Sequence analysis of *pmrB* revealed that there was point mutation in *pmrB* causing amino acid substitution - T157P (A469C, ACC>CCC) in PmrB histidine kinase domain of 2 distinct XDR ColRkp isolates (4.25%) with colistin MIC 16-64mg/L (Figure 19 l), (Table 10-11). Combined presence of R256G substitution in PmrB due to point mutation (C766G, CGC>GGC) with 16-32mg/ L colistin MIC was specified in 2

distinct XDR ColRkp isolates (4.25%) expressing plasmid-mediated either *mcr*-1.1 or *mcr*-8.1 genes (Figure 20 c), (Table 10-11). All of these PmrB substitutions were analyzed and aligned with data base sequence (Figure 19 l, 20 c), (Table 10-11).

Amino acid substitution in PhoP - E82K due to point mutation in *phoP* (G244A, GAA>AAA) was also found in 1 PDR ColRkp isolate (2.12%) with 64mg/ L colistin MIC (Figure 19 m), (Table 10-11). This PhoP substitution was analyzed and aligned with data base sequence (Figure 19 m), (Table 10-11).

Meanwhile, all of these 47 XDR and PDR ColRkp clinical isolates in this study revealed wild type *pmrA* and *phoQ* genes (Table 11).

Table 10. Distributions of underlying colistin resistance mechanisms observed in 47 XDR and PDR ColRkp isolates of this study.

ColRkp isolates	Plasmid-mediated colistin resistance (n=4, 8.5%)		Chromosomal-mediated colistin resistance (n=43, 91.5%)								
	mcr-1-9 genes		*mgrB*-mediated colistin resistance (n=40, 85.1%)							*phoP*-mediated colistin resistance (n=1, 2.12%)	*pmrB*-mediated colistin resistance (n=2, 4.25%)
	mcr alone	*mcr* combined with R256G PmrB	Insertional alterations in *mgrB* (n=29, 61.7%)				Point mutations in *mgrB* (n=6, 12.75%)		Deletion of *mgrB* (n=5, 10.62%)		
			IS *1-like*	IS *3-like*	IS *5-like*	IS *1380-like*	Point mutations in initial codon	Point mutations causing internal stop codon			
XDR ColRkp (n=43)	1 (2.12%)	2 (4.25%)	22 (46.8%)	2 (4.25%)	2 (4.25%)	2 (4.25%)	2 (4.25%)	4 (8.5%)	4 (8.5%)	-	2 (4.25%)
PDR ColRkp (n=4)	1 (2.12%)	-	1 (2.12%)	-	-	-	-	-	1 (2.12%)	1 (2.12%)	-
Total (n=47)	2 (4.25%)	2 (4.25%)	23 (48.9%)	2 (4.25%)	2 (4.25%)	2 (4.25%)	2 (4.25%)	4 (8.5%)	5 (10.62%)	1 (2.12%)	2 (4.25%)

Table 11. Mechanisms of colistin resistance with respective colistin MIC ranges, ESBL profiles, carbapenemase profiles and *in vitro* biofilms formation of 47 XDR and PDR ColRkp isolates.

Mechanisms of colistin resistance	ColRkp isolate	Type of isolate	No of isolates (n) (%)	Colistin MIC range (mg/L)	ESBL profiles	Carbapenemase profiles	Biofilm producer
Altered *mgrB* due to insertion of IS *1-like* between Nucleotide +55 and +56	kp 921	XDR	6 (12.8%)	8-64	TEM,CTX-M	NDM,OXA-48	Strong
	kp 924	XDR			TEM, CTX-M	NDM, OXA-48	Strong
	kp 946	XDR			TEM, CTX-M	OXA-48	Strong
	kp 319	XDR			TEM, CTX-M	OXA-48	Strong
	kp 340	XDR			SHV,TEM, CTX-M	NDM, VIM	Strong
	kp 414	XDR			SHV,TEM, CTX-M	OXA-48	Weak
Altered *mgrB* due to insertion of IS *1-like* between Nucleotide +71 and +72	kp 926	XDR	5 (10.62%)	16-64	TEM, CTX-M	NDM, OXA-48	Strong
	kp 947	XDR			TEM, CTX-M	OXA-48	Strong
	kp 357	XDR			TEM, CTX-M	OXA-48	Moderate
	kp 366	XDR			SHV,TEM, OXA-1, CTX-M	NDM	Strong
	kp 413	XDR			SHV,TEM, CTX-M	NDM	Moderate
Altered *mgrB* due to insertion of IS *1-like* between Nucleotide +104 and +105	kp 122	XDR	1 (2.12%)	>512	TEM, CTX-M	NDM, OXA-48	Weak

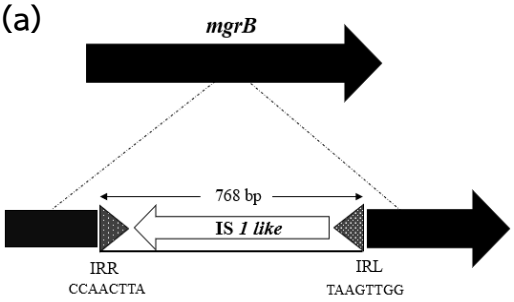
Mechanisms of colistin resistance	ColRkp isolate	Type of isolate	No of isolates (n) (%)	Colistin MIC range (mg/L)	ESBL profiles	Carbapenemase profiles	Biofilm producer
Altered *mgrB* due to insertion of IS *1-like* between Nucleotide +105 and +106	kp 119	XDR	1 (2.12%)	32	OXA, CTX-M	NDM	Strong
Altered *mgrB* due to insertion of IS *1-like* between Nucleotide -7 and -8 (in promoter region, upstream of *mgrB* start codon)	kp 104	XDR	5 (10.6%)	32-64	TEM, CTX-M	NDM, OXA-48	Strong
	kp 114	XDR			SHV,TEM, CTX-M	NDM, OXA-48, VIM	Strong
	kp 301	XDR			SHV,TEM, CTX-M	NDM, OXA-48, VIM	Moderate
	kp 390	XDR			TEM, CTX-M	NDM, OXA-48	Strong
	kp 332	XDR			TEM, CTX-M	NDM, OXA-48	Strong
Altered *mgrB* due to insertion of IS *Kpn14-like* (IS *1-like*) between Nucleotide +115 and +116	kp1225	XDR	2 (4.25%)	16-64	TEM, CTX-M	NDM	Strong
	kp 396	XDR			TEM, CTX-M	NDM	Strong
Altered *mgrB* due to insertion of IS *Kpn14-like* (IS *1-like*) between Nucleotide +117 and +118	kp 80	XDR	2 (4.25%)	8-32	SHV	NDM	Strong
	Kp 383	XDR			SHV	NDM	Strong

Mechanisms of colistin resistance	ColRkp isolate	Type of isolate	No of isolates (n) (%)	Colistin MIC range (mg/L)	ESBL profiles	Carbapenemase profiles	Biofilm producer
Altered *mgrB* due to insertion of IS *3-like* between Nucleotide +121 and +122	kp 944	XDR	2 (4.25%)	16	SHV,TEM, CTX-M	OXA-48	Strong
	kp 353	XDR			TEM, CTX-M	OXA-48	Strong
Altered *mgrB* due to insertion of IS *903-like* (IS *5-like*) between Nucleotide +74 and +75	kp 389	XDR	2 (4.25%)	32-64	SHV,TEM, CTX-M	OXA-48	Strong
	kp 402	XDR			SHV,TEM, CTX-M	OXA-48	Strong
Altered *mgrB* due to insertion of IS *Ecp1-like* (IS *1380*) between Nucleotide +124 and +125	kp 289	XDR	2 (4.25%)	16-32	SHV,OXA-1, TEM	NDM, OXA-48	Weak
	kp 411	XDR			SHV,OXA-1, TEM	NDM, OXA-48	Strong
Genetic alterations in *mgrB* due to point mutation in initial codon-G3A,GTG>GTA and point mutations causing internal stop codon-A7T, AAA>TAA; G60A, TGG>TGA)	kp1189	XDR	6 (12.8%)	32-64	TEM, CTX-M	OXA-48	Strong
	kp 328	XDR			TEM, CTX-M	OXA-48	Strong
	kp 375	XDR			SHV,TEM, CTX-M	NDM, OXA-48, VIM	Strong
	kp 380	XDR			SHV,TEM, CTX-M	NDM, OXA-48, VIM	Strong
	kp 243	XDR			TEM, CTX-M	NDM, OXA-48	Non
	kp 354	XDR			TEM, CTX-M	NDM, OXA-48	Strong

Mechanisms of colistin resistance	ColRkp isolate	Type of isolate	No of isolates (n) (%)	Colistin MIC range (mg/L)	ESBL profiles	Carbapenemase profiles	Biofilm producer
Loss of *mgrB*	kp 202	XDR	4 (8.5%)	16-64	TEM, CTX-M	NDM, OXA-48	Strong
	kp 261	XDR			SHV,TEM, CTX-M	NDM, VIM	Strong
	kp270A	XDR			SHV,TEM, CTX-M	OXA-48	Moderate
	kp 309	XDR			SHV,TEM, CTX-M	NDM, OXA-48, VIM	Moderate
Genetic alteration in *pmrB* due to point mutation (A469C, ACC>CCC) generating proven deleterious PmrB (T157P)	kp 1194	XDR	2 (4.25%)	16-64	OXA-1, CTX-M	OXA-48	Strong
	kp 151	XDR			SHV,TEM, OXA-1, CTX-M	NDM, OXA-48	Strong
mcr-8.2 with wild type *mgrB, pmrAB* and *phoPQ*	kp 1078	XDR	1 (2.12%)	8	SHV,TEM, CTX-M	-	Weak
mcr-8.1 or *1.1* with R256G PmrB due to point mutation in *pmr*B (C766G, CGC>GGC), wild type *mgrB, pmrA* and *phoPQ*	kp 395	XDR	2 (4.25%)	16-32	SHV,TEM, CTX-M	NDM, OXA-48	Strong
	kp 400	XDR			SHV,TEM, OXA-1, CTX-M	NDM	Strong

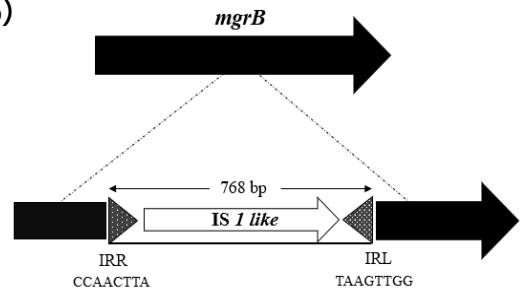
Mechanisms of colistin resistance	ColRkp isolate	Type of isolate	No of isolates (n) (%)	Colistin MIC range (mg/L)	ESBL profiles	Carbapenemase profiles	Biofilm producer
Altered *mgrB* due to insertion of IS *1-like* between Nucleotide +104 and +105	kp 372	PDR	1 (2.12%)	>512	TEM, CTX-M	NDM, OXA-48	Strong
Loss of *mgrB*	kp 264A	PDR	1 (2.12%)	16	SHV,TEM, CTX-M	OXA-48	Non
Point mutation in *phoP* (G244A, GAA>AAA) cause E82K substitution in the response regulator domain of PhoP	kp 248	PDR	1 (2.12%)	64	CTX-M	OXA-48	Strong
mcr-1.1 with wild type *mgrB, pmrAB* and *phoPQ*	kp 291	PDR	1 (2.12%)	8	SHV,TEM, CTX-M	NDM, OXA-48, VIM	Strong

(a)



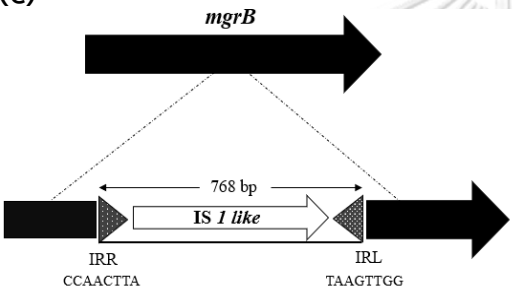
IS *1-like* between Nucleotide +55 and +56 position

(b)



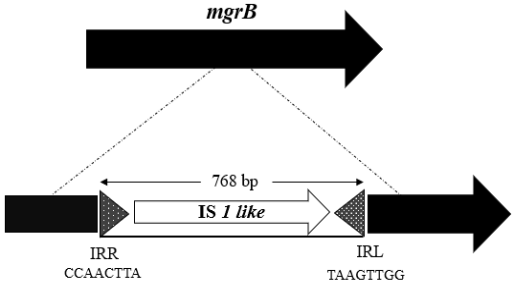
IS *1-like* between Nucleotide +71 and +72 position

(c)



IS *1-like* between Nucleotide +104 and +105 position

(d)



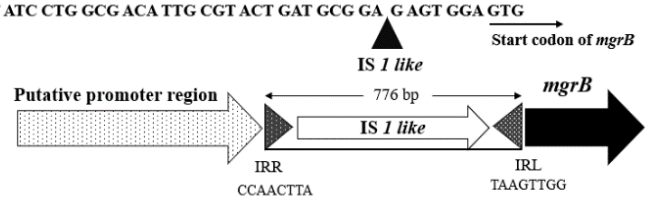
IS *1-like* between Nucleotide +105 and +106 position

(e)

TTA TTT GTA TGA TCC CTG GCG TGA TTT TGA CAC GAA CACG TTTTGA AAC AAG TCG ATG ATT CC TAAACT TAC CTT TCG TAA TAC AG

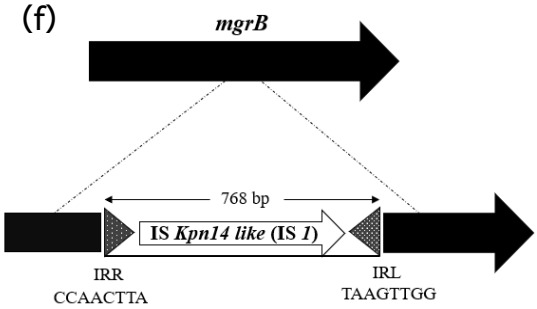
-35 *PROM* -10

TTA GCC GCG GTT TAA GAA GGC CGT GCT ATC CTG GCG ACA TTG CGT ACT GAT GCG GA G AGT GGA GTG



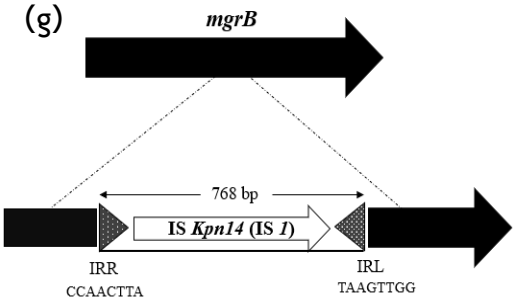
IS *1-like* between Start codon of *mgrB* and Putative promoter region

(f)



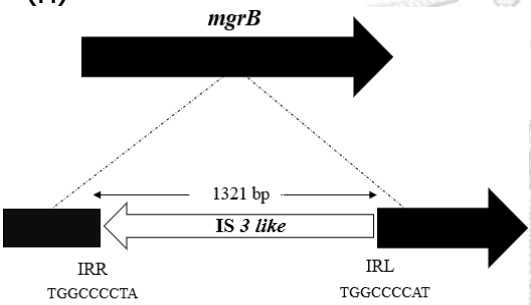
IS *kpn14* between Nucleotide +115 and +116

(g)



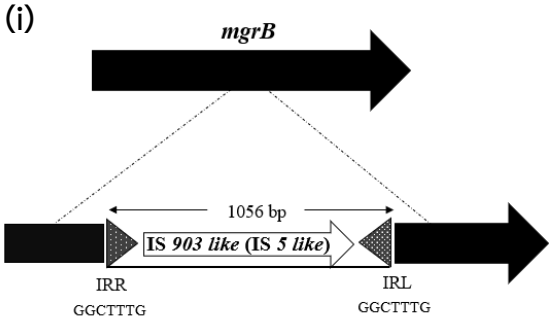
IS *kpn14* between Nucleotide +117 and +118 position

(h)



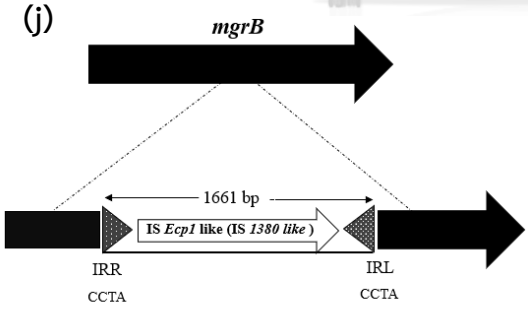
IS *3-like* between Nucleotide +121 and +122 position

(i)



IS *5-like* between Nucleotide +74 and +75 position

(j)



IS *1380-like* between Nucleotide + 124 and +125 position

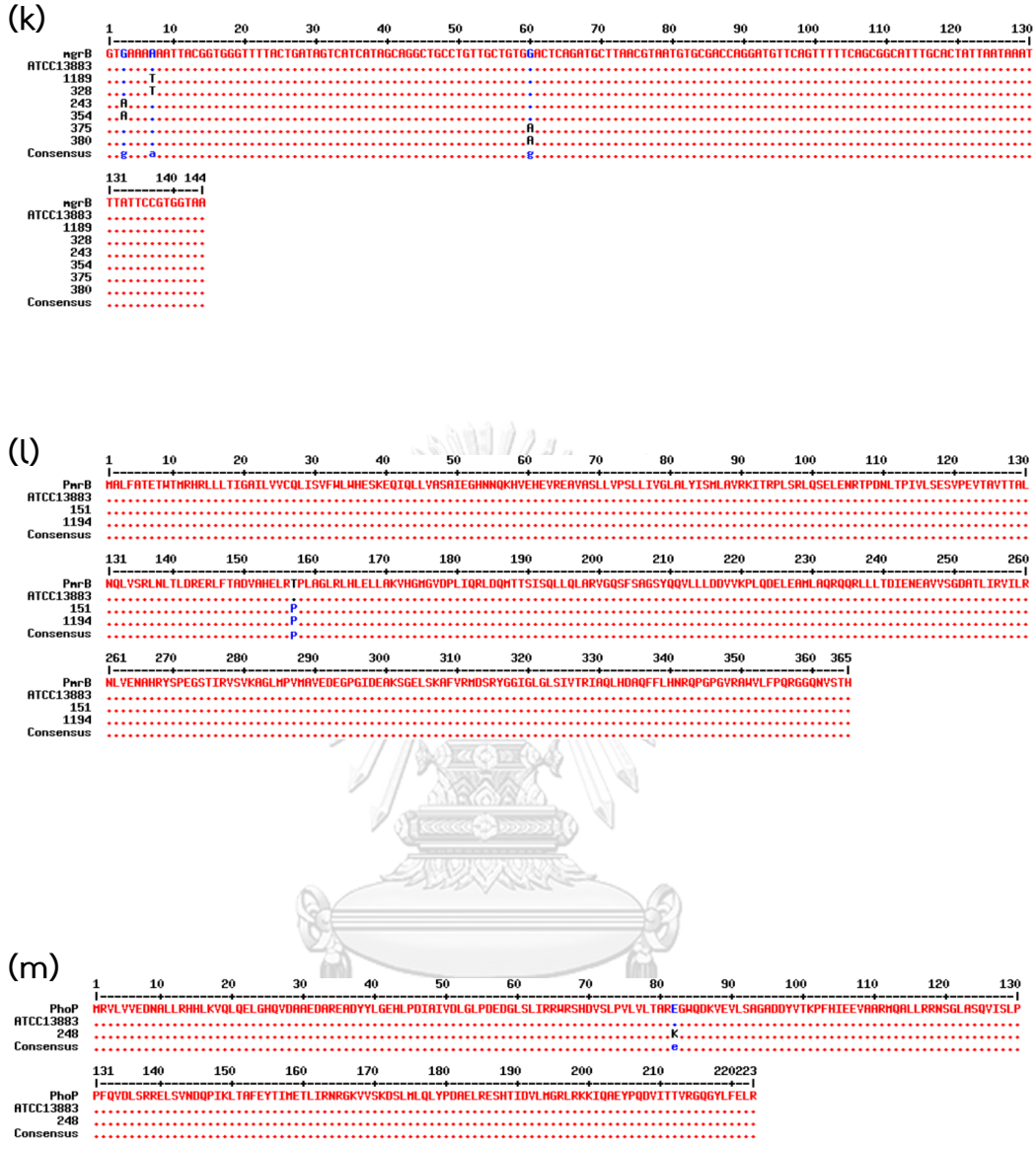


Figure 19. Chromosomal-mediated colistin resistance mechanisms.

3.2. Plasmid-encoded *mcr-1.1*, *mcr-8.1*, or *mcr-8.2* genes were involved as plasmid-mediated colistin resistance mechanisms.

In order to analyze the plasmid-mediated mechanisms accounting for colistin resistance, the presence of plasmid-mediated *mcr*-1 to *mcr*-9 genes were investigated in all 47 ColRkp clinical isolates.

In a total of 4 XDR and PDR ColRkp isolates with plasmid-mediated colistin resistance (8.5%), the presence of *mcr-1.1* or *mcr-8.2* alone having 8mg/L of colistin MIC (n=2, 4.25%) and the combined presence of *mcr-1.1* or *mcr-8.1* with PmrB - R256G (C766G, CGC>GGC) demonstrating 16-32mg/L of colistin MIC (n=2, 4.25%), were observed (Figure 20 a-c), (Table 10-11). To confirm the presence of plasmid-encoded *mcr-1.1*, *mcr-8.1* and *mcr-8.2* phosphoethanolamine transferase in this study, PCR products of these *mcr* genes were sequenced using the primers that can be used for amplification of whole *mcr-1* and *mcr-8* genes as well as analyzing the nucleotides and deduced protein sequences with reference sequences – Mcr-1.1 (WP_049589868. 1), Mcr-8. 1 (WP_114699275. 1) and Mcr-8. 2 (WP_072310976. 1), respectively (Figure 20 a-b), (Table 10-11).

In both XDR and PDR ColRkp isolates with plasmid-mediated colistin resistance, different carbapenemase and ESBL genes were identified (Table 11).

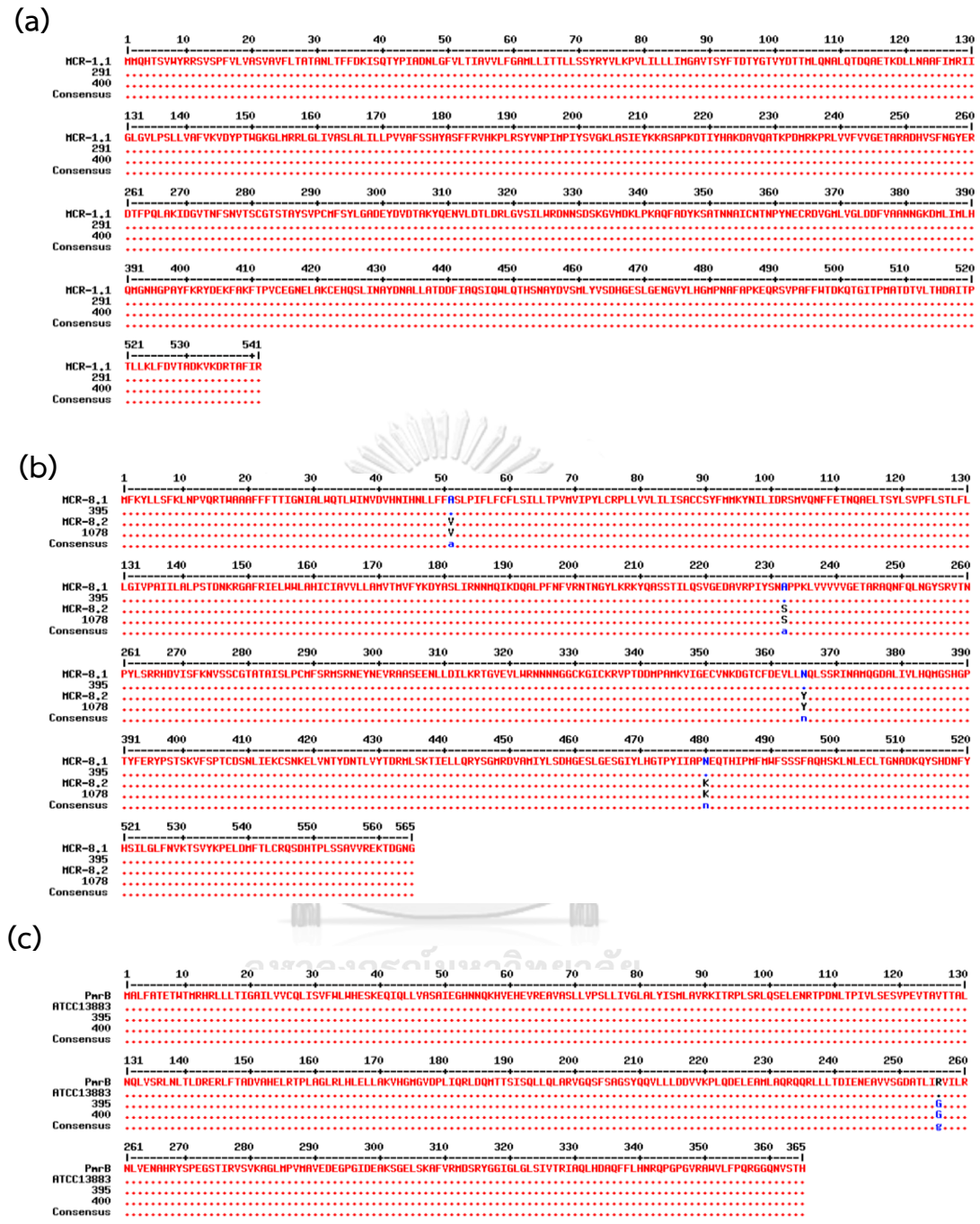
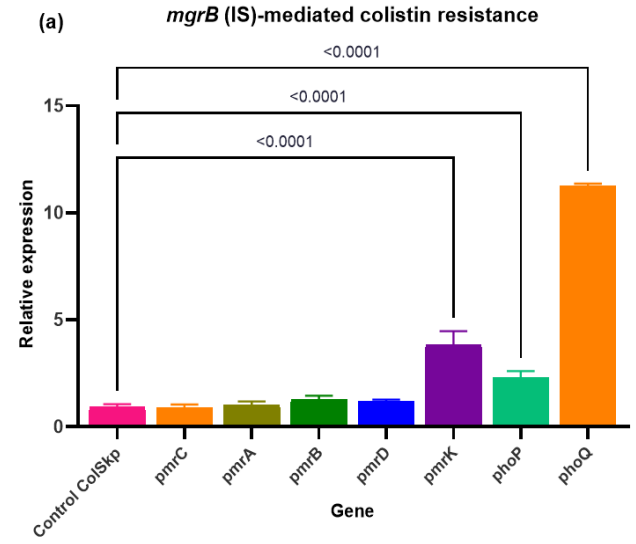
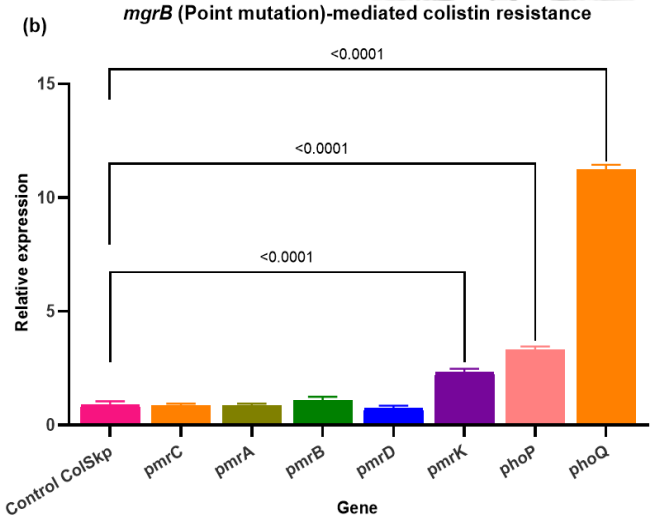


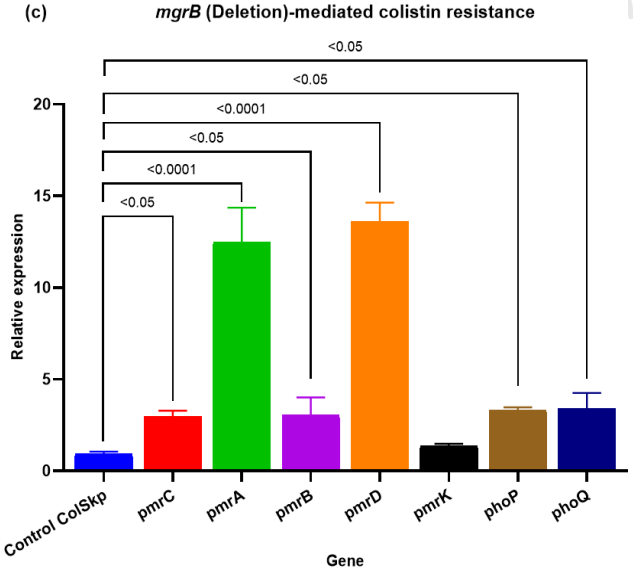
Figure 20. Plasmid-mediated colistin resistance mechanisms.

4. Increased expressions of Ara4N-related *phoPQ* and *pmrK* transcripts were crucial in establishing colistin resistance.

In ColRkp isolates with altered *mgrB* (due to IS integration or point mutation), transcriptional levels of Ara4N-related *phoPQ* and *pmrK* were found to be significantly overexpressed as compared to the levels of ColSkp clinical strains with wild type *mgrB* ($p<0.001$) (Figure 21 a-b). Meanwhile, Ara4N-related *phoPQ*, connector-*pmrD*, and PEtN-related *pmrCAB* transcripts were significantly upregulated in ColRkp with deleted *mgrB* ($p<0.05$) (Figure 21 c). In T157P PmrB-mediated ColRkp strains, expression of PEtN-related *pmrCAB* was significantly enhanced ($p<0.05$) (Figure 21 d). Furthermore, ColRkp isolates with E82K PhoP expressed significantly higher levels of Ara4N-related *phoPQ*, *pmrK* and connector-*pmrD* transcripts ($p<0.05$) (Figure 21 e). Isolates with combined presence of *mcr* gene with R256G PmrB expressed significantly higher level of PEtN-related *pmrCAB* transcripts ($p<0.001$) (Figure 21 f). Although colistin resistance with various underlying colistin resistance mechanisms was shown to be significantly correlated with overexpression of Ara4N-related and PEtN-related LPS modification genes, several independent insertions, deletions, or point mutations in *mgrB* (85.1%) associated with increased expressions of Ara4N-related *phoPQ* and *pmrK* transcripts were observed to be crucial in establishing colistin resistance in our isolates (Figure 21 a-f), (Table 10-11).


(a)
mgrB (IS)-mediated colistin resistance
<0.0001
<0.0001
<0.0001
Relative expression
15
10
5
0
Control ColSkp
pmrC
pmrA
pmrB
pmrD
pmrK
phoP
phoQ
Gene


(b)
mgrB (Point mutation)-mediated colistin resistance
<0.0001
<0.0001
<0.0001
Relative expression
15
10
5
0
Control ColSkp
pmrC
pmrA
pmrB
pmrD
pmrK
phoP
phoQ
Gene


(c)
mgrB (Deletion)-mediated colistin resistance
<0.05
<0.05
<0.0001
<0.05
<0.0001
<0.05
Relative expression
20
15
10
5
0
Control ColSkp
pmrC
pmrA
pmrB
pmrD
pmrK
phoP
phoQ
Gene

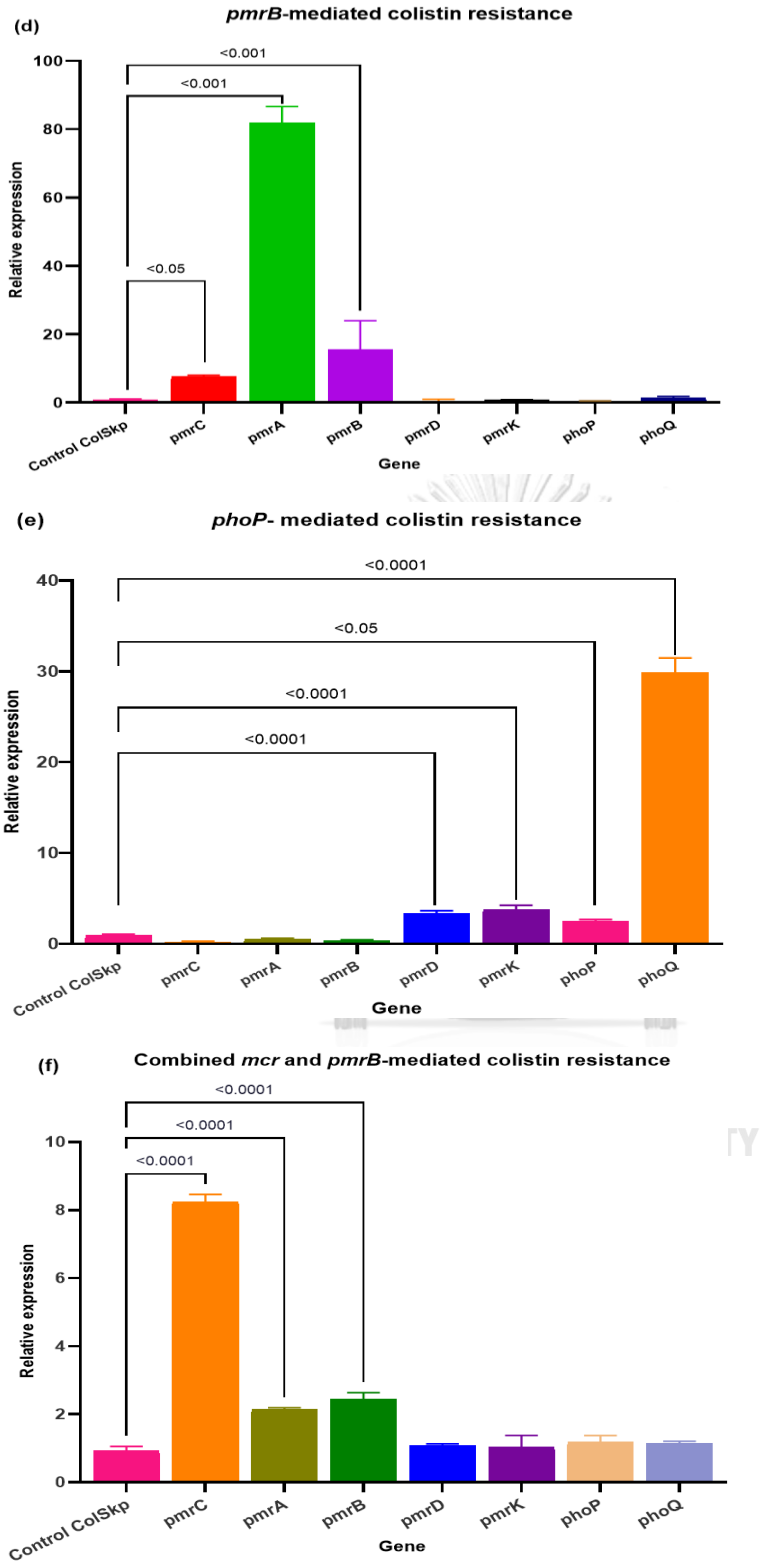


Figure 21. Expressions of LPS modification genes among ColRkp clinical isolates.

5. Biofilm production was significantly higher in ColRkp isolates with XDR characteristics than isolates having PDR characteristics.

Among the ColRkp isolates investigated, 95.7% were able to form biofilms (Figure 22 a), whereas 70.2% of those with XDR patterns produced strong biofilms, 10.6% developed moderate amount of biofilms, 8.5% formed weak biofilms and 2.1% were categorized as non biofilm-producers (Figure 22 b). Meanwhile, 6.4% of ColRkp showing PDR patterns developed strong biofilms and 2.1% were categorized as non biofilm-producers (Figure 22 b). When compared to PDR ColRkp isolates, significantly higher biofilm development capability was discovered in ColRkp isolates showing XDR characteristics (p<0.0001) (Figure 22 b).

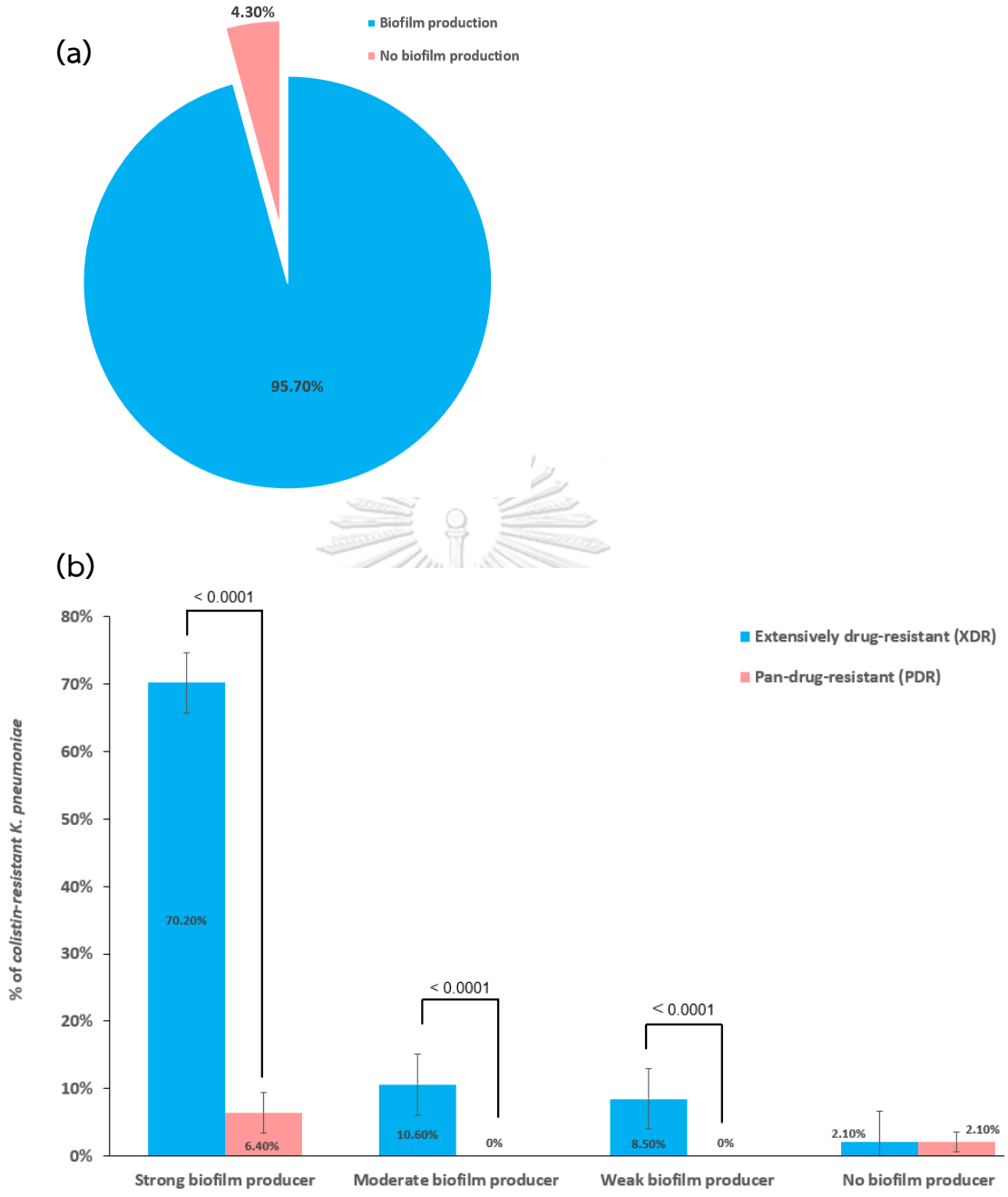


Figure 22. Determination of biofilm production, and classification of biofilm producers between XDR and PDR ColRkp clinical isolates in this study.

6. Coexistence and altered expressions of bacterial virulence factors were associated with ColRkp clinical isolates.

The existence of all tested bacterial virulence genes as *mrkD, kfu, ybtS, ompK35, ompK36, uge*, *wabG, luxS* combination was detected in 12.8%of XDR ColRkp isolates (Figure 23 a). Meanwhile, the most commonly observed virulence gene combination was *mrkD, ybtS, ompK35*, *ompK36, uge, wabG*, *luxS* combination, which was discovered in 70.2% of XDR ColRkp strains and 8.5% of PDR ColRkp strains, respectively (Figure 23 a). The combination of *mrkD, kfu, ompK35, ompK36, uge, wabG*, and *luxS* were identified in 4.3% of XDR ColRkp strains (Figure 23 a). The least common virulence gene combinations were *mrkD, ompK35, ompK36, uge, wabG, luxS* combination and *mrkD*, *ybtS, ompK35, ompK36, wabG, luxS* combination which were observed as 2.1% each of XDR ColRkp clinical isolates (Figure 23 a). In comparison to control ColSkp clinical isolates, ColRkp isolates displayed altered virulence factors expressions, showing significantly higher expressions of *ompK35, ompK36, kfu*, *uge*, and *luxS* virulence genes, as well as significantly lower expression of *wabG* virulence gene (p<0.0001) (Figure 23 b).

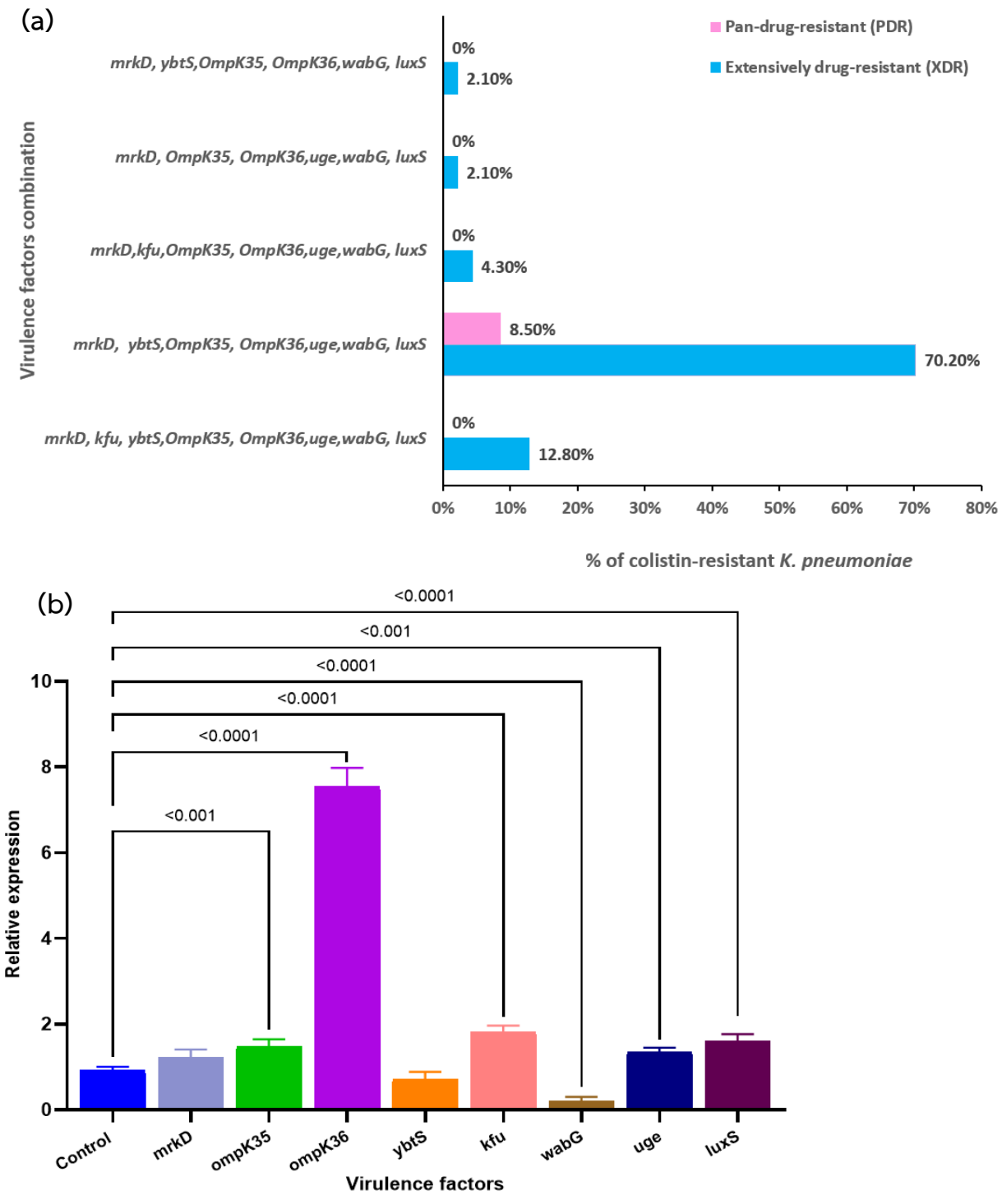


Figure 23. Coexistence of virulence genes combinations in XDR and PDR ColRkp isolates, and expression levels of virulence genes associated with XDR ColRkp clinical isolates.

7. Colistin-EDTA combination showed remarkable synergistic effects in both planktonic and mature biofilms of ColRkp clinical isolates *in vitro*.

When the effects of the proposed adjuvants including EDTA, sodium citrate, tramadol, curcumin, resveratrol, DNAase and antibiofilm peptide were evaluated, only EDTA was discovered to exhibit potent inhibitory effects in planktonic XDR and PDR ColRkp isolates by producing no visible signs of growth, at concentrations ranging from 3 to 24mg/mL (Table 12).

When the synergistic activity of colistin-EDTA combination was evaluated against planktonic ColRkp isolates using checkerboard synergy testing assays, colistin MIC was lowered to 0.25mg/L when it was given in conjunction with EDTA (12mg/mL) (Table 13), (Appendix D, Table 18). This colistin-EDTA combination displayed substantial synergistic effects (FICI≤ 0.5) on all tested planktonic XDR and PDR ColRkp clinical isolates.

To further validate the synergistic activities of the colistin-EDTA combination in planktonic ColRkp isolates, time-kill synergy confirmation assays were performed in 11 representative XDR and PDR ColRkp isolates encoded with chromosomal-mediated and plasmid-mediated colistin resistance mechanisms, by utilizing 1XMIC and 0.5XMIC of both drugs as monotherapy and colistin-EDTA combination therapy (Figure 24 a-k). Despite bacterial regrowth after 6 hours of colistin and EDTA monotherapy, colistin-EDTA combination exhibited remarkable synergistic activities in reducing >3$\log_{10}$ of bacteria starting 2 hours after treatment and produced prolonged bactericidal effects with no regrowth until 24 hour, in all tested XDR and PDR ColRkp isolates regardless of their underlying colistin resistance mechanisms (Figure 24 a-k).

Minimal biofilm eradication concentrations (MBEC) values for XDR and PDR ColRkp mature biofilms were observed to be 2-256-fold higher for colistin alone and 2-4-fold higher for EDTA alone (Table 12-13), (Appendix D, Table 17-18). Interestingly, colistin (0.5-1mg/L) in combination with EDTA (12 mg/mL) was able to completely eradicate the mature biofilms of both XDR and PDR ColRkp clinical isolates (p<0.05) (Table 13), (Appendix D, Table 18).

Table 12. Susceptibilities of planktonic and mature biofilms of ColRkp clinical isolates to different adjuvants.

Adjuvants	Planktonic		Biofilms	
	MIC (mg/mL)		MBEC (mg/mL)	
	Extensively drug-resistant ColRkp (XDR ColRkp) (n=43)	Pandrug-resistant ColRkp (PDR ColRkp) (n=4)	Extensively drug-resistant ColRkp (XDR ColRkp) (n=42)	Pandrug-resistant ColRkp (PDR ColRkp) (n=3)
EDTA	3-24	12-24	12-48	12-48
Sodium citrate	>48	>48	>48	>48
Tramadol	>48	>48	>48	>48
Curcumin	>48	>48	>48	>48
Resveratrol	>48	>48	>48	>48
DNAase	>48	>48	>48	>48
Antibiofilm peptide	>48	>48	>48	>48

MIC - Minimal inhibitory concentrations (mg/mL) for planktonic cells.

MBEC- Minimal biofilm eradication concentrations (mg/mL) for mature biofilms.

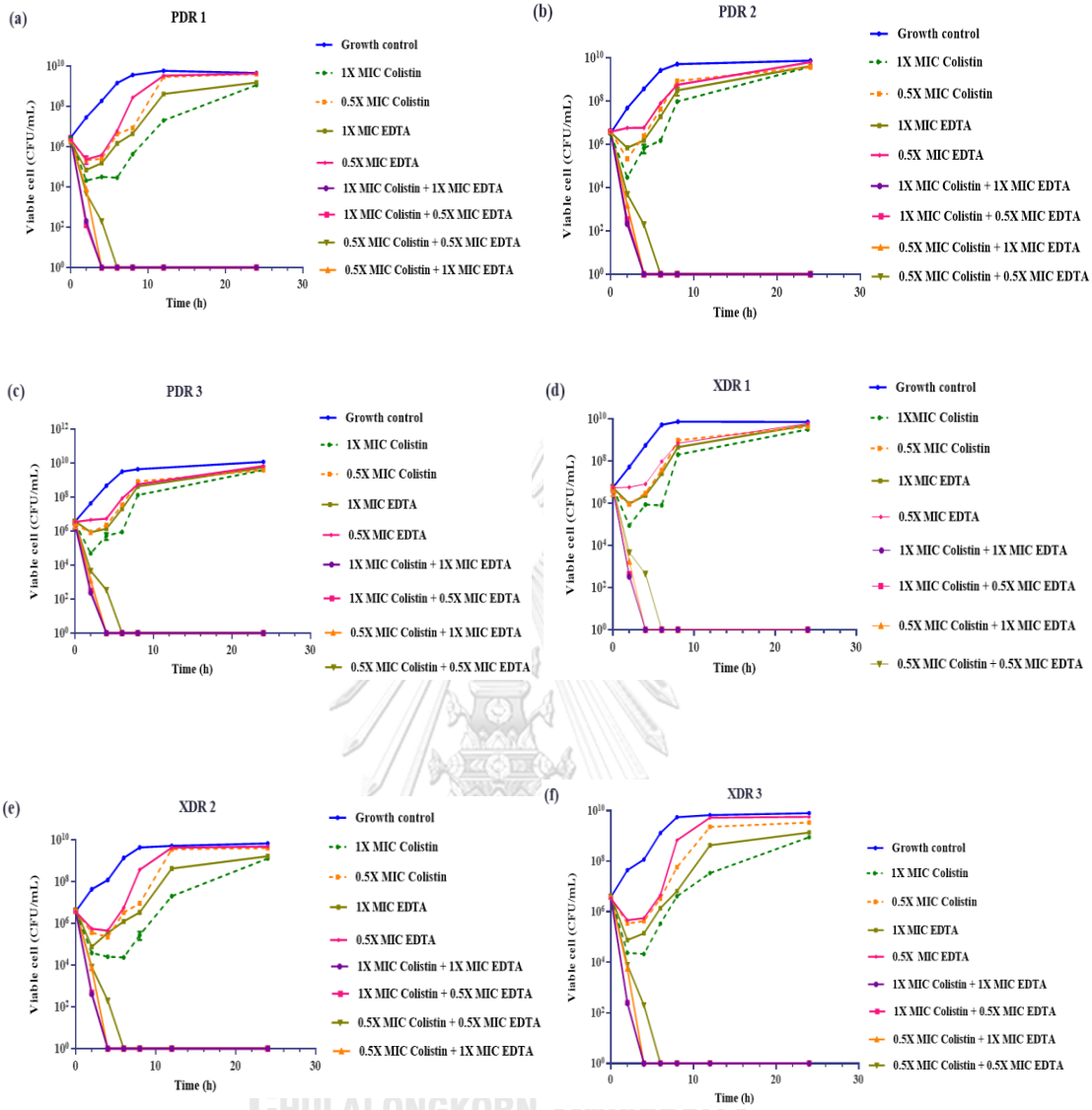
Table 13. Susceptibilities of planktonic and mature biofilms of ColRkp clinical isolates to colistin, EDTA and their combination *in vitro*.

Specimens	Planktonic				Biofilms		
	Colistin (mg/L)	EDTA (mg/mL)	Colistin (mg/L) + EDTA (mg/mL)		Colistin (mg/L)	EDTA (mg/mL)	Colistin (mg/L) + EDTA (mg/mL)
	MIC[#]	MIC[#]	MIC[#]	FICI[†]	MBEC[*]	MBEC[*]	MBEC[*]
Extensively drug-resistant ColRkp (XDR ColRkp) (n=42)	8->512	3 - 24	0.25 + 12	0.09375-0.375	16-2048	12 - 48	0.5 + 12
Pandrug-resistant ColRkp (PDR ColRkp) (n=3)	8->512	12 - 24	0.25 + 12	0.3125-0.5	64-2048	12 - 48	1 + 12

[#] MIC - Minimal inhibitory concentrations (mg/L and mg/mL) for planktonic cells.

[†]FIC index-Fractional inhibition concentration index calculated from checkerboard synergy assay.

[*]MBEC - Minimal biofilm eradication concentrations (mg/L and mg/mL) for mature biofilms.



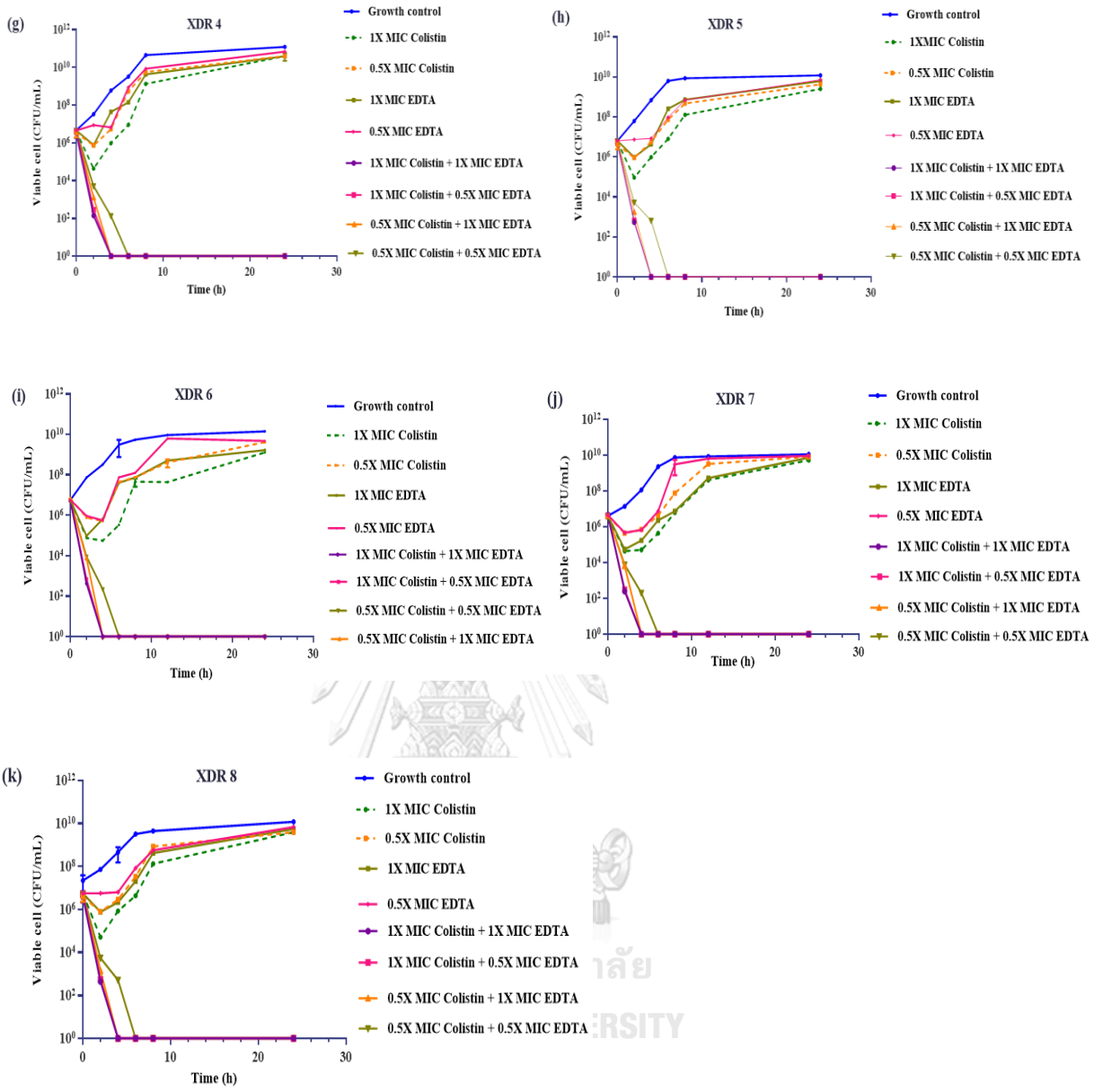


Figure 24. Time-kill effects of single and combination of colistin and EDTA on 11 representative PDR and XDR ColRkp clinical isolates with underlying chromosomal-mediated and plasmid-mediated colistin resistance mechanisms.

8. Colistin-EDTA combination showed potent efficacy in eradicating ColRkp biofilms within a 24-hour treatment exposure *in vitro*.

The single exposure to colistin (1mg/L) and EDTA (12mg/mL) in combination displayed progressive reductions in biofilm biovolume in a time-dependent manner with the most pronounced eradication effects within 24hour (p<0.01) (Figure 25 a). Within a short exposure time (6 hour), bacteria cell viability within the biofilm was significantly reduced (p<0.001) with colistin-EDTA combination treatment when compared to EDTA or colistin alone (Figure 25 b). When compared to the PBS control (untreated) group, colistin alone resulted in a lower cell viability but a higher rise in biofilm biovolume. Interestingly, as compared to colistin-treated biofilms, EDTA alone displayed significant reduction in bacteria cell viability within the biofilm on 6-hour exposure time but sharp increase in biofilm biovolume with increased viable bacteria on 12 and 24 hours exposure time (p<0.001) (Figure 25 b).

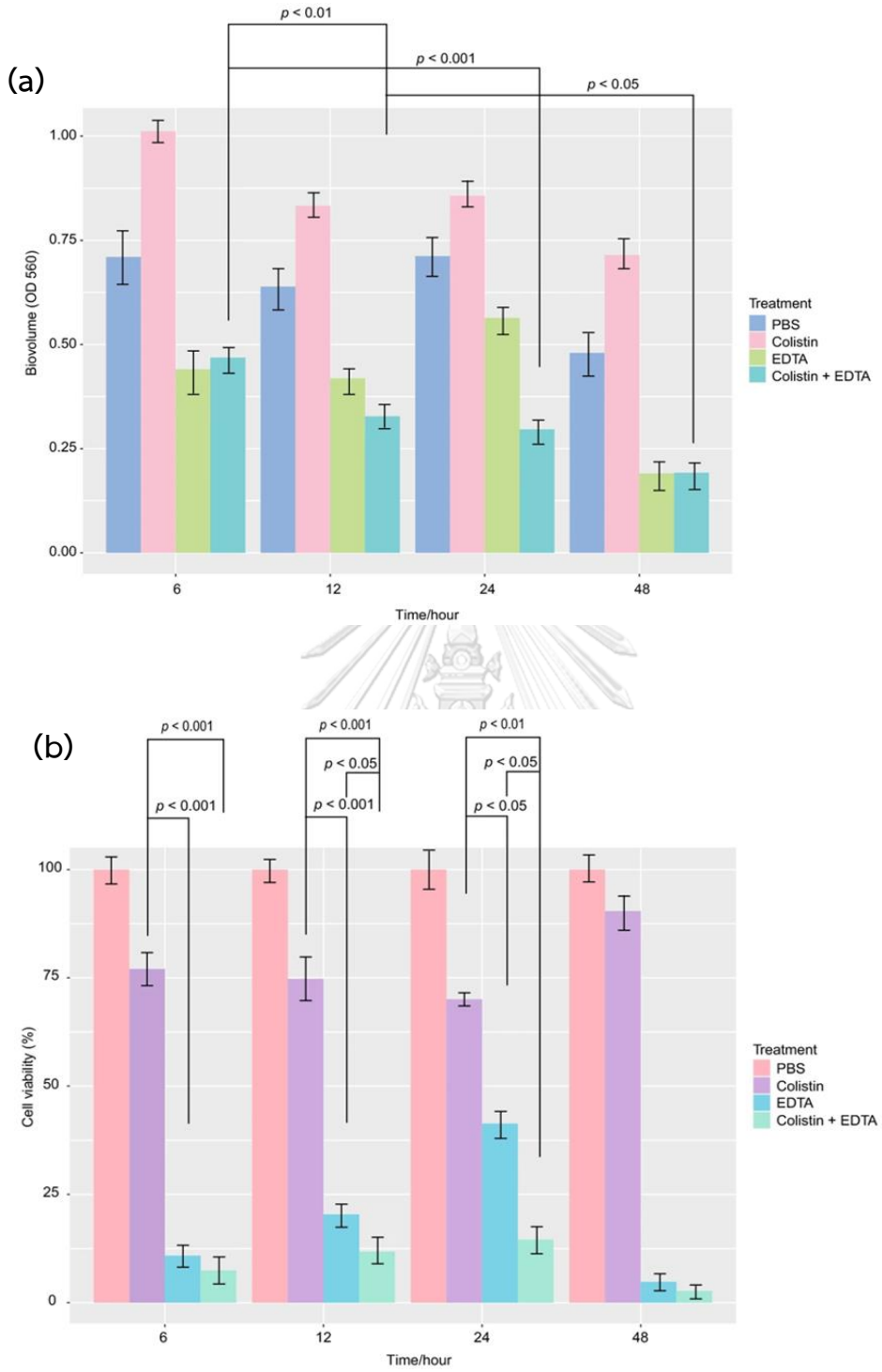


Figure 25. Effects of colistin, EDTA and colistin-EDTA combination on biofilm biovolume, and biofilm cell viability of ColRkp for different exposure time *in vitro*.

9. Colistin-EDTA combination significantly eradicated ColRkp catheter-related biofilm infections both *in vitro* and *in vivo*.

As compared to colistin (1mg/L) or EDTA (12mg/mL) alone treatments, there was significant reduction in biofilm biomasses ($p<0.001$) with significantly decreased bacterial viabilities showing significantly lower live/dead ratios of bacteria within biofilms ($p<0.01$) when treated with colistin-EDTA combination (1mg/ L+ 12mg/ mL), both *in vitro* (Figure 26 a-b) and *in vivo* (Figure 26 d-f). Furthermore, when compared to colistin and EDTA alone, colistin-EDTA combination displayed significantly increased inhibitory effects on biofilm biovolume of ColRkp ($p<0.001$), both *in vitro* (Figure 26 c) and *in vivo* (Figure 26 e).

Under confocal imaging analysis, *in vivo* catheter-related biofilm infection with well-defined biofilm structure showing green signal of viable bacteria inside and along the lumen of catheter was observed in PBS control (untreated) group (Figure 27 A-C), whereas colistin (Figure 27 D-F) and EDTA-treated groups (Figure 27 G-I), showed a few red signal of non-viable cells mixed with large numbers of viable cells and unchanged biofilm biovolume as compared to PBS group. Meanwhile, confocal imaging analysis revealed a significant reduction in biofilm biovolume and viable bacteria when *in vivo* ColRkp catheter-related biofilm infection was treated with a colistin-EDTA combination compared to EDTA or colistin alone (Figure 27 J-L).

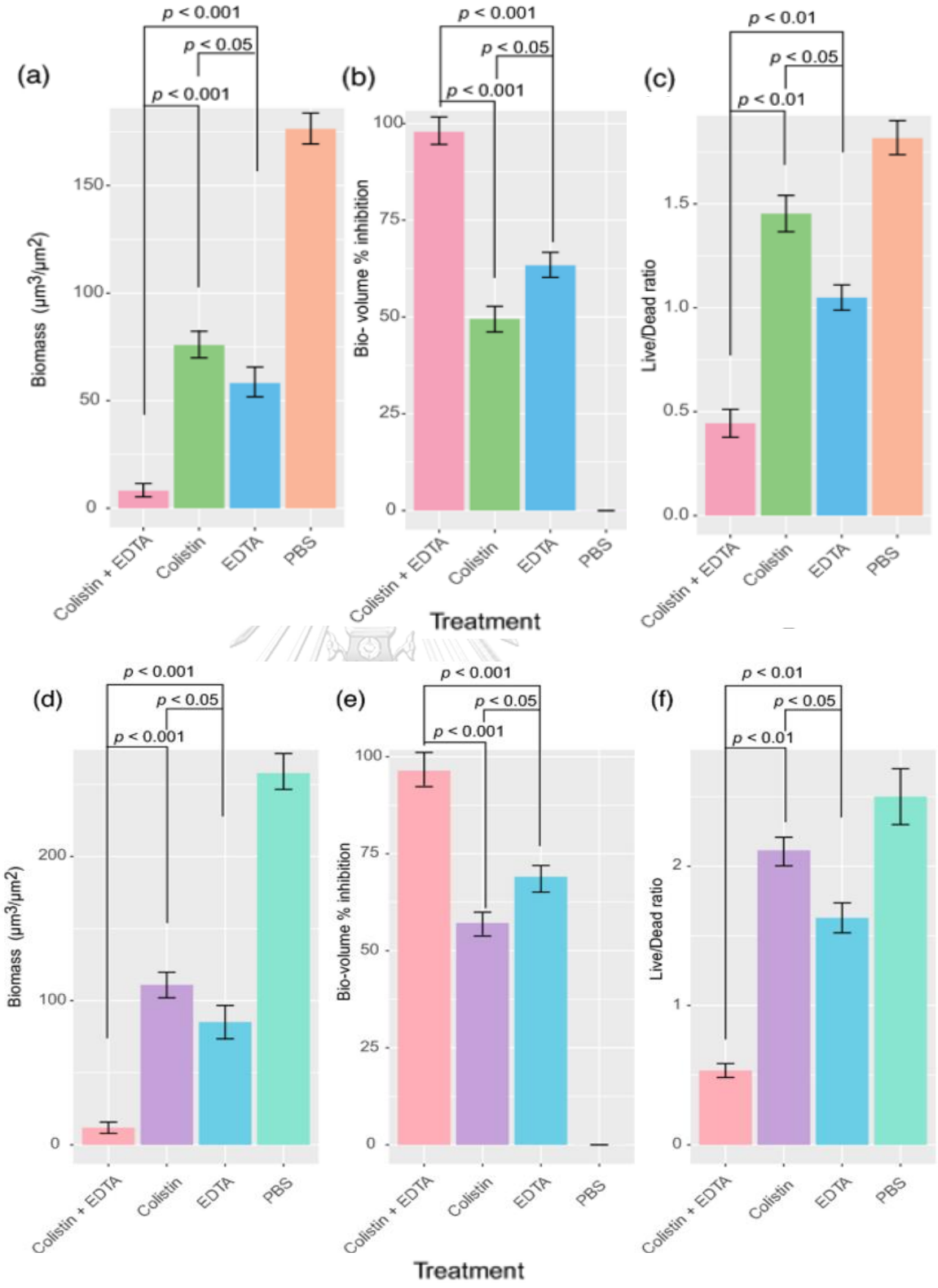


Figure 26. Effects of colistin, EDTA and colistin-EDTA combination on ColRkp catheter-related biofilm infections both *in vitro* and *in vivo*.

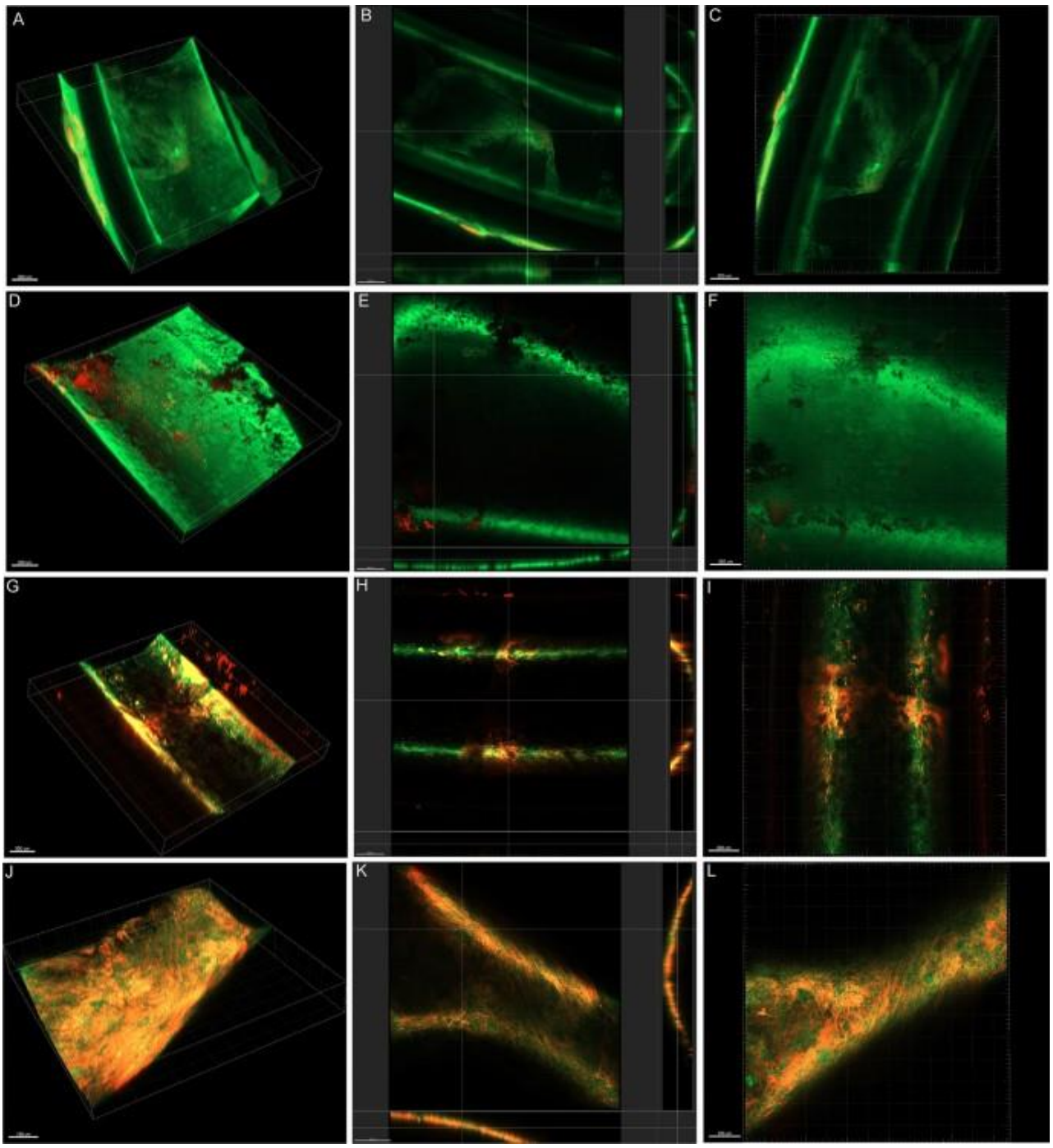


Figure 27. Confocal imaging analysis (3D and cross-sectional); (A-C) PBS-treated, (D-F) Colistin-treated, (G-I) EDTA-treated, (J-L) Colistin-EDTA combination-treated catheter-related biofilm infection of ColRkp *in vivo*.

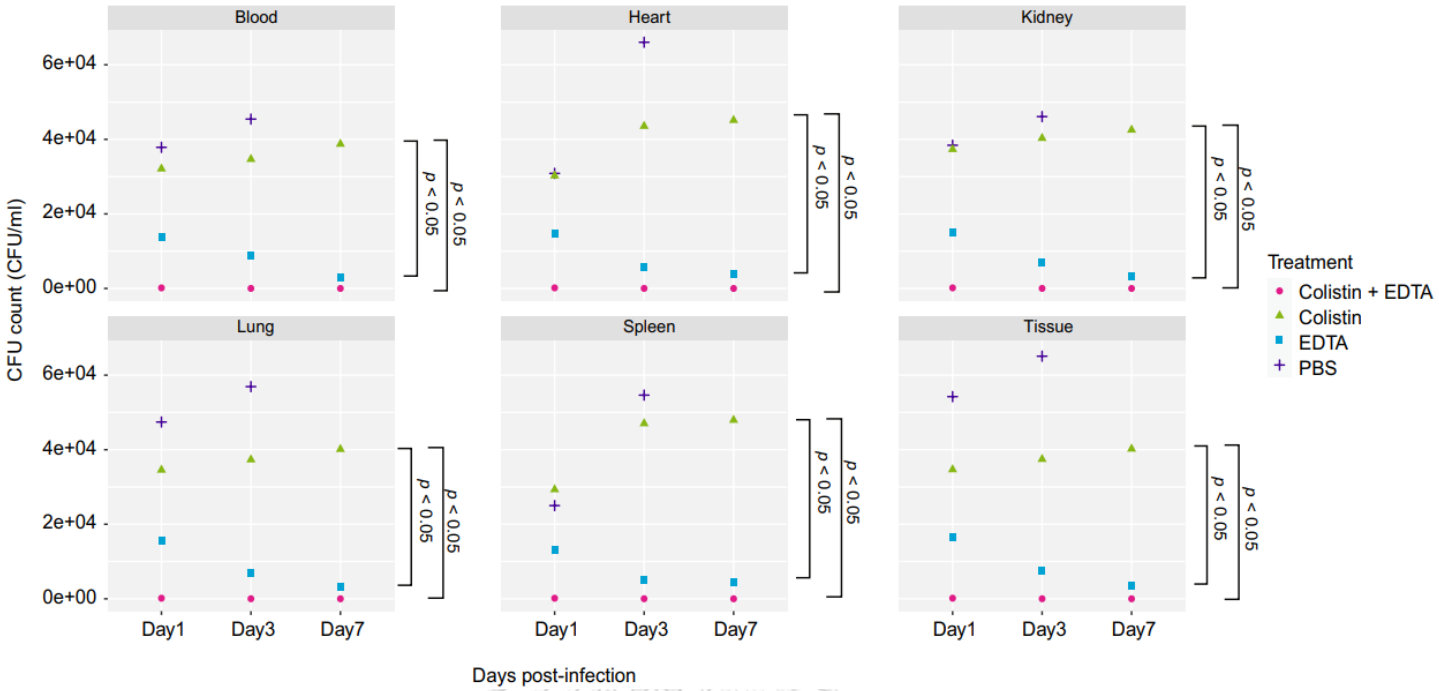
10. Colistin-EDTA combination significantly decreased bacterial load in internal organs and serum creatinine.

Throughout all tested days, mice treated with colistin-EDTA combination (1mg/L+ 12mg/mL) exhibited a significant reduction in ColRkp bacterial load in internal organs including blood, heart, kidneys, lungs, spleen, and tissues surrounding catheter, when compared to mice treated with colistin or EDTA alone ($p<0.05$) (Figure 28 a). Significantly, EDTA-treated mice had lower bacterial loads in various internal organs on all tested days than colistin-treated mice ($p<0.05$) (Figure 28 a). Furthermore, infected mice treated with colistin-EDTA combination showed significantly lower serum creatinine levels than mice treated with colistin or EDTA alone ($p<0.05$) (Figure 28 b). In comparison to colistin-treated mice, administration of EDTA alone significantly reduced serum creatinine levels in treated mice ($p<0.05$) (Figure 28 b).

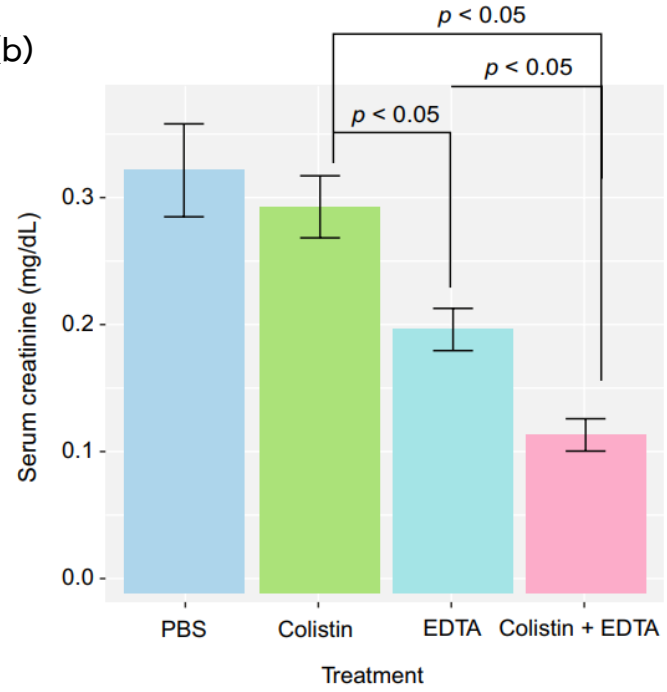
11. Colistin-EDTA combination significantly improved animals survival.

In PBS control (untreated) group, none of the infected mice survived to day 7 (Figure 28 c). Interestingly, EDTA-treated mice had significantly higher survival rates than the colistin-treated mice ($p<0.0001$) (Figure 28 c). Mice treated with the colistin-EDTA combination displayed significantly higher survival rates of 100% until day 7 when compared to mice given colistin alone ($p<0.0001$) or EDTA alone ($p<0.001$) (Figure 28 c).

(a)



(b)



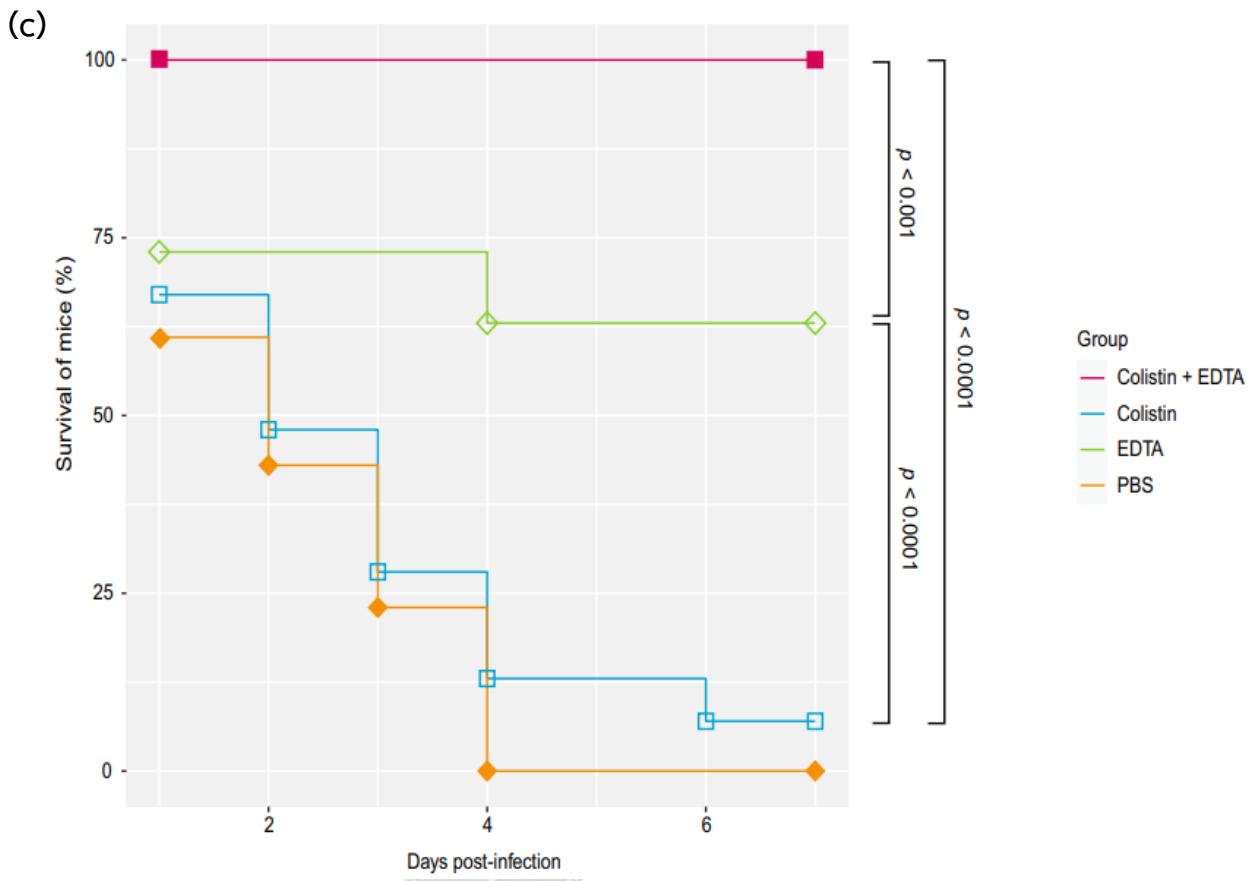


Figure 28. Effects of colistin, EDTA and colistin-EDTA combination on bacterial load in internal organs, serum creatinine and survival of mice.

12. Exposure to colistin-EDTA combination resulted in significantly altered expressions of bacterial virulence genes *in vivo*.

Expression of *kfu* was significantly increased after exposure to colistin-EDTA combination as compared to colistin and EDTA-treated groups ($p<0.05$) (Figure 29). Genes - *ybtS* and *luxS* expression were similar in both colistin and colistin-EDTA combination-treated groups, however their expression become significantly increased after EDTA treatment ($p<0.05$) (Figure 29). The expressions of *mrkD* were not significantly affected by all treatments (Figure 29). The virulence gene - *ompK 35* expression was increased significantly with EDTA as compared to colistin treatment but its expression level was decreased significantly with colistin-EDTA combination treatment ($p<0.05$) (Figure 29). The expression of *ompK36* was found to be comparable in colistin and colistin-EDTA combination treatments, despite EDTA decreased expression of this gene ($p<0.05$) (Figure 29). For *uge* and *wabG*, their expression levels were significantly increased after exposure to EDTA as compared to colistin and colistin-EDTA combination treatments ($p<0.05$) (Figure 29).

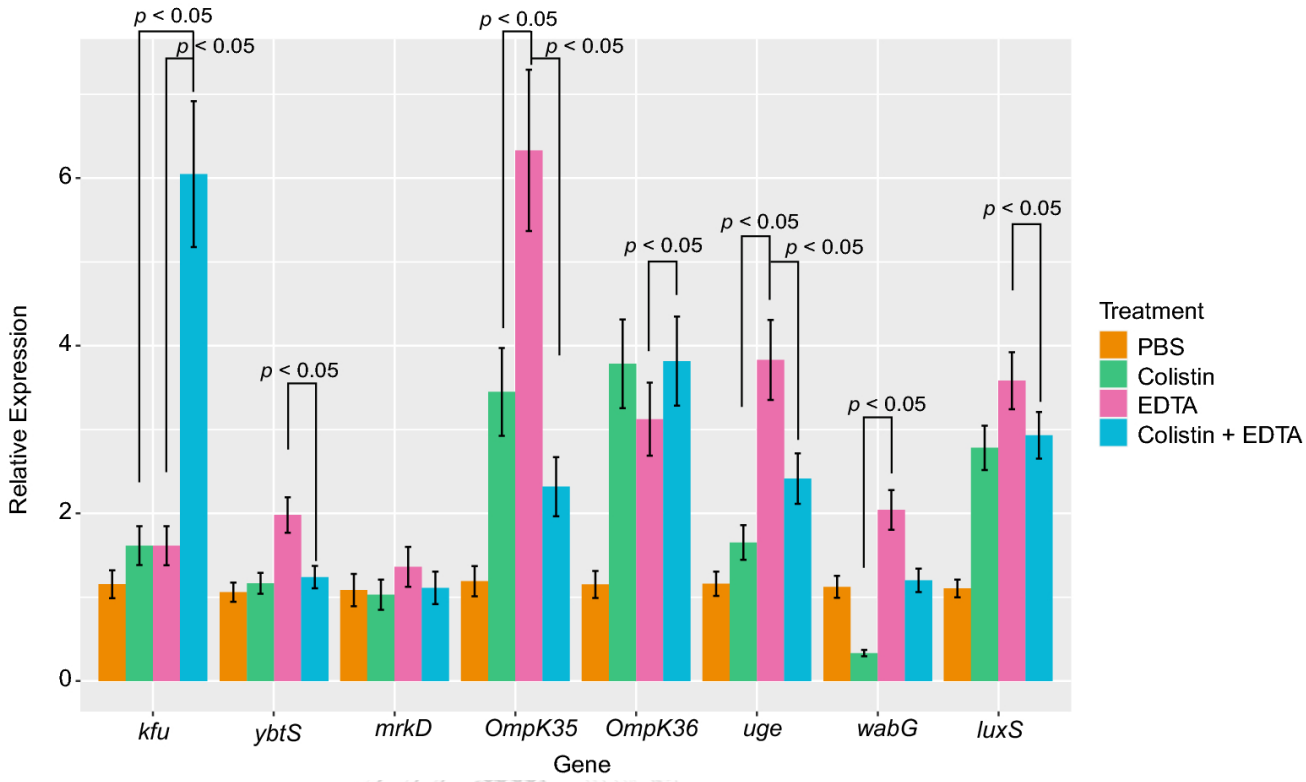


Figure 29. Effects of single and combination of colistin and EDTA on relative expressions of bacterial virulence genes *in vivo*.

CHAPTER VI

DISCUSSION

K. pneumoniae is a Gram-negative pathogen accountable for a variety of severe hospital-acquired infections, including catheter-related biofilm infections, pneumonia, urinary tract infection, wound or surgical site infection, and septicemia, notably in immunocompromised patients (1). What makes these pathogens more problematic is the increasing prevalence of carbapenem resistance in *K. pneumoniae* (CRkp) which have been reported as serious threats to human health around the world (8). Colistin has thus emerged as a feasible therapeutic option for treating these CRkp infections due to the shortage of effective therapeutic alternatives and restrictions in novel antibiotics developments (7, 12). Consequently, as a result of expanded usage of colistin, the global prevalence of colistin resistance among *K. pneumoniae* (ColRkp) have steadily increased which reveals significant threats for the emergence and spread of XDR and PDR *K. pneumoniae* strains around the world (12).

In this study, a total of 165 ESBL and carbapenemase-producing CRkp clinical isolates collected from Chulalongkorn Memorial Hospital were observed to exhibit different antibiotic susceptibilities profiles, showing highest resistance to ceftazidime and ciprofloxacin (100%), followed by imipenem (94.5%), meropenem (90.3%), fosfomycin (31.5%) and amikacin (23.6%), respectively in their planktonic environments. Among these CRkp isolates, we discovered a 28.5% prevalence of ColRkp (n=47) that displayed both XDR and PDR characteristics. Additionally, there was a rising trend of colistin resistance which increased over time from 14.9% in 2016 to 36.2% in 2021. The prevalence of colistin resistance in our clinical setting is comparable to India (30%) (145) and Italy (22.4%) (146), but higher than in other clinical settings in Thailand (6.6%) (147), Nigeria (9.1%) (80) and other regions of the world - Europe (1.8%), Latin America (1.5%), Middle East-Africa (1.4%), North America (1.3%), and the Asia-Pacific (1.3%), respectively (148). According to NARST data, the prevalence of colistin resistance among *K. pneumoniae* was reported to be 2.5% in 2018, 3.7% in 2019 and 2.9% in 2020, respectively (149, 150). Despite the lack of clinical data on colistin use in this study, the rising trend of ColRkp in our study could be due to selective pressure from increased colistin use in clinical settings to combat

an increasing CRkp burdens, and in agribusinesses settings especially in pig production as therapeutic and short-term preventive strategies for Gram-negative *Enterobacteriaceae* infections (68, 69, 151-153). These could lead to the emergence and colonization of ColRkp among patients, healthy adults, food animals, and the environment, causing further circulation with a higher regional prevalence of colistin resistance in Thailand (69, 151-154). Future research is needed to determine the most prevalent sequence types and clonal groups of ColRkp clinical isolates circulating in this region, along with their clonal relatedness, in order to rule out the occurrence of any local outbreak driven by clonal expansion and dissemination of a single dominant strain in our clinical setting (155-157). Epidemiological surveillance studies will also be further needed to investigate the clonal relatedness of local dominant clones with high-risk international clonal lineages, and explore the possible source of ColRkp clinical isolates in this region, along with their risks of transmission (155-157). Importantly, the development of PDR strains among ColRkp in our study also suggests that managing these infections will be incredibly challenging and this allows these PDR ColRkp isolates continue to evolve alarmingly in the upcoming years in this healthcare setting. Establishing a comprehensive and effective infection control guideline, as well as regular monitoring and surveillance of underlying colistin resistance mechanisms to investigate how they acquire colistin resistance, are the critical steps to limit the spread of resistant bacteria in both clinical and agribusiness settings (155-157).

Because colistin resistance triggered by chromosomal or plasmid-mediated Ara4N-related or PEtN-related LPS alterations is continuously increasing and varying between countries and across time, addressing the underlying colistin resistance mechanisms is becoming increasingly essential (12, 13, 69). Chromosomal *mgrB* alterations, such as insertional alterations by IS*1*-like, IS*kpn14*-like, IS*3*-like, IS*5*-like and IS*1380*-like elements (61. 7%), point mutations (12. 75%) and deletion (10.62%), were found to play a significant role in facilitating colistin resistance with 8->512mg/L of colistin MIC among ESBL and carbapenemase-producing ColRkp clinical isolates in this study. These findings are consistent with those of several other studies around the world that have highlighted the potential role of chromosomal *mgrB*

gene in establishing colistin resistance in *K. pneumoniae* of clinical origin (82-86, 124, 158, 159). There have been no reports of ColRkp clinical isolates from human patients in Thailand having altered chromosomal *mgrB* due to point mutations, deletions, or insertions by IS*kpn14*-like, IS*5*-like and IS1*380*-like elements. According to prior studies, transferable plasmids containing various IS act as donors for the integration of IS elements into chromosomal *mgrB* for facilitating the emergence of *mgrB*-related colistin resistance under colistin-induced selective pressure (82, 160, 161). Future study is therefore required to explore the roles of plasmids in carrying and transferring different IS elements into bacterial chromosomal genes for the development of colistin resistance in ColRKp clinical isolates. Our findings further emphasize the possibility of several independent insertional, deletional, or mutational alterations in *mgrB*, revealing that genetic alterations in *mgrB* can occur at a remarkably high frequency with unaffected bacterial fitness and enhanced virulence, as demonstrated in earlier studies (33, 34, 159). Additionally, previous studies have revealed that *mgrB*-related colistin resistance can be selected in the presence of colistin and can persist even in the absence of colistin selection pressure (34). These could explain the rapid expansion and rising prevalence of ColRkp clinical isolates exhibiting this resistance mechanism in clinical settings.

When the chromosomal *mgrB* is deactivated, PhoPQ signaling pathway becomes activated, which has previously been demonstrated to stimulate PmrAB two-component system indirectly through connector PmrD (12). In this study, ColRkp isolates with IS-mediated or point mutation-mediated *mgrB* alterations displayed significantly higher expressions of Ara4N-related *phoPQ* and *pmrK* transcripts when compared to ColSkp clinical isolates with wild type *mgrB*. Interestingly, the expressions of both Ara4N-related *phoPQ*, connector *pmrD* and PEtN-related *pmrCAB* transcripts were significantly upregulated in ColRkp with *mgrB* deletion. These findings demonstrate the activation of PhoPQ-PmrD-PmrAB signal transduction pathway via *mgrB* inactivation, as evidenced in earlier studies (82, 86, 124). According to the expression levels of LPS modifying genes, increased expressions of Ara4N-related *phoPQ* and *pmrK* transcripts with several genetic alterations in *mgrB* were

observed to be crucial in establishing colistin resistance in our isolates, as previously demonstrated (82, 84, 124).

Notably, 4.25% (n=2) of ESBL and carbapenemase-producing ColRkp isolates in this study exhibited deleterious T157P PmrB with overexpression of PEtN-related *pmrCAB* transcripts, which have previously been proved to be associated with colistin resistance in *K. pneumoniae* (81). Noticeably, T157P PmrB substitution has never been reported in ColRkp clinical isolates in Thailand. Additional amino acid substitution - R256G PmrB with significant *pmrCAB* transcriptions, was revealed in 4.25% of our isolates (n= 2) harboring either *mcr*-1.1 or *mcr*-8.2 genes. Although PROVEAN bioinformatic tool anticipated that R256G in PmrB would have a deleterious effect on protein function, this substitution was discovered as lineage-specific mutations in both polymyxin-susceptible as well as polymyxin-resistant *K. pneumoniae* isolates (162-164). Moreover, R256G PmrB substitution has been confirmed to be unrelated to colistin resistance in *K. pneumoniae* in a prior study (165).

On the other hand, PhoP is a dimeric protein comprising a N-terminal response regulatory domain which connected to a C-terminal DNA-binding effector domain (166). The abilities of PhoP effector domain to bind to DNA and further regulate the transcriptions of other genes are influenced by its activated response regulatory domain (166). In our study, E82K amino acid substitution in response regulator domain of PhoP with significantly upregulated expressions of Ara4N-related *phoPQ, pmrK* and *pmrD* transcripts, was identified in 2.12% of ESBL and carbapenemase-producing ColRkp isolate (n=1), which is consistent with a previous research (167). The presence of an E82K PhoP substitution in ColRkp clinical isolates has never been documented in Thailand. No study has also determined the role of E82K PhoP in the development of colistin resistance in *K. pneumoniae*. Although PROVEAN prediction score demonstrated a neutral effect on protein function, we hypothesize that replacing an acidic positive amino acid (glutamic acid) with a basic negative amino acid (lysine) in response regulatory domain of PhoP could induce transcriptional activation of *phoP* for developing LPS-induced colistin resistance. Additional research will be required in the future to validate the significance of E82K

PhoP on colistin resistance using molecular approaches including site-directed mutagenesis to target position 82 and other different amino acid positions of PhoP. Because efflux pumps overexpression, capsule polysaccharide overproduction, porins mutations, and genetic alterations in *crrAB* TCS are also involved in conferring colistin resistance in *K. pneumoniae* (15), future research will provide valuable information relating to their contributing roles as associated colistin resistance mechanisms among ColRkp clinical isolates with the same genetic alterations but different colistin MIC values observed in this study.

The plasmid-mediated *mcr* gene encodes for a phosphoethanolamine transferase that adds a phosphoethanolamine to bacterial LPS in order to generate colistin resistance through PEtN-related LPS alterations (12). Following the discovery of *mcr*-1 among *Enterobacteriaceae* in China since 2015, *mcr*-1 and several *mcr* alleles (*mcr*-1 to *mcr*-10) have been identified not only in patients and healthy individuals, but also in food chains such as raw meat, retail fruits, and food animals, as well as in the environment, including hospital environments, canal water, and wastewater treatment plants, all over the world (87, 88, 168-170). This demonstrates the increasing complexity and adaptability of *mcr* genes for the dissemination of colistin resistance among the bacteria (87, 88, 168-170). While *mcr*-1 was predominantly discovered in *E. coli* from human clinical samples, its prevalence is growing in other bacterial species including *K. pneumoniae* (88). Until recently, previous studies discovered *mcr*-1 in clinical isolates of *K. pneumoniae* and *mcr*-8 in *K. pneumoniae* complex from slaughtered pigs in different regions of Thailand (152, 171).

Our study is the first to show the presence of plasmid-mediated *mcr*-8.1, or *mcr*-8.2 in XDR ColRkp clinical isolates from human patients in Thailand. This study showed the plasmid-mediated colistin resistance with a prevalence of 8.5% , which is higher than the reports described in earlier studies in Thailand (<1% - 3.2%) (172-174). Additionally, different carbapenemase and ESBL genes were identified in both XDR and PDR ColRkp isolates with underlying plasmid-mediated and chromosomal-mediated colistin resistance mechanisms in this study. The findings of this study highlight the greater propensity for horizontal plasmid-mediated drug

resistance genes to transfer to other bacteria of the same or different genera residing in the patient's body, other patients, or the healthcare environment, which could potentially accelerate the regional emergence and further dissemination of colistin-resistant bacteria in the near future (88). Because the existence of plasmid-mediated *mcr-10* was not determined in this study, we cannot rule out the involvement of *mcr-10* as underlying plasmid-mediated colistin resistance mechanism in our ColRkp isolates. Future studies of *mcr-10* involvement, as well as plasmids analysis and conjugation experiments, will be needed to identify the most prevalent types of plasmids associated with their successful horizontal transferability of these *mcr* and other drug resistance genes in our ColRkp clinical isolates.

Nowadays, several studies observed the establishment of hypervirulent ColRkp expressing diverse bacterial virulence characteristics (16-19). Reportedly, not only PmrAB and PhoPQ supported bacterial virulence by regulating virulence gene transcripts, but also *mgrB*-related LPS alterations augmented bacterial virulence by suppressing both antimicrobial peptides expression and early host defense activation without compromising bacterial fitness (31-34). These emphasize the importance of exploring the association between colistin resistance and other virulence factors that influence bacterial pathogenicity. Within the host, *K. pneumoniae* displays biofilm formation (2, 28) and a variety of virulence factors for enhancing bacterial colonization, invasion, and pathogenicity. Theses bacterial virulence factors involve genes related to lipopolysaccharide (*wabG* and *uge*) for protecting bacteria from host immune defenses (23), outer membrane porins (*ompK35, ompK36*) for improved bacterial survival by maintaining membrane integrity and nutrients transport (25), iron acquisition siderophores (*kfu, ybtS*) for systemic survival and dissemination (26), type 3 adhesin (*mrkD*) for bacterial binding to develop biofilm on abiotic catheters surfaces (22) and type 2 QS regulatory system gene (*luxS)* for biofilm development by facilitating cell to cell communication (27), respectively.

In this study, we observed a significant association between XDR ColRkp clinical isolates and increased biofilm production *in vitro*. This findings indicate the alarming potential of enhanced bacterial adaptive biofilm-mediated

tolerance to both host defenses and antibiotics that could enable these XDR isolates to evolve into PDR isolates in the future (29, 175). Additionally, all of the evaluated virulence genes combination was encoded in 12.8% of XDR ColRkp stains, and other virulence gene combinations were encoded in varying frequencies in both XDR and PDR ColRkp clinical isolates. Interestingly, there were significantly altered virulence factors expressions associated with XDR ColRkp clinical isolates showing significant upregulations of *ompK35, ompK36, kfu, uge*, and *luxS* as well as remarkably decreased expression of *wabG* virulence gene. Due to the convergence of colistin resistance and altered bacterial virulence genes expressions observed in this study, these ColRkp could evade both immunological and antimicrobial effects, that enables them to evolve and disseminate as untreatable superbugs producing persistent and recurring infections in clinical settings (16, 18, 35).

Adding to the urgency of the problem, the occurrences of ColRkp infections are increasing in critically ill patients of intensive care units, where they have been linked to recurrent and often untreatable catheter-related biofilm infections, which emphasizes the urgent need to discover an effective innovative therapy to overcome these ColRkp catheter-related biofilm infections (4). An antibiotic lock strategy, which involves instilling a lock solution containing high concentrations of single or combined antimicrobial agents, is currently being attempted to target intraluminal biofilms of infected catheters (36). Colistin combination therapy, when used to treat drug-resistant bacteria, resulted in significantly lower treatment failure rates and enhanced patients survival (38). EDTA is an FDA-approved metal ions chelator with a favorable pharmacokinetic safety profile for intravenous treatment of lead poisoning (42). It can disrupt the permeability-associated resistance mechanisms and restore antibiotic potency even against resistant bacteria through its metal ions chelation activities (41). Furthermore, EDTA performs as an anti-virulence agent by disrupting the biofilm matrix via its strong metal ion chelation and produces preferable bactericidal activities against inner biofilm bacteria with lower metabolic activities (42). These suggest that EDTA could be utilized as a potent adjuvant in colistin combination therapy to combat colistin resistance in catheter-related biofilm infections of ColRkp clinical isolates.

Among the proposed adjuvants in this study, only EDTA (3-24mg/mL) was discovered to exhibit potent inhibitory effects in planktonic XDR and PDR ColRkp clinical isolates. Interestingly, when colistin (0.25-1mg/L) was given in combination with EDTA (12mg/mL), this combination produced potent synergistic antimicrobial activity in planktonic XDR and PDR ColRkp clinical isolates, regardless of the underlying chromosomal-mediated and plasmid-mediated colistin resistance mechanisms. However, when this colistin-EDTA combination was tested against XDR and PDR ColRkp isolates (n=2) with exceptionally high colistin MIC (>512mg/L), the checkerboard assay revealed indifference in terms of synergy testing. These findings agree with a prior study showing the synergistic antibacterial effects of antimicrobial peptide AA230 and EDTA on planktonic Gram-negative bacteria (176). The significantly increased antimicrobial spectrum of colistin-EDTA against planktonic XDR and PDR ColRkp isolates in this study could be attributed to EDTA ions sequestration activities for increased bacterial outer membrane permeabilities which then sensitize as well as synergize with colistin to regain colistin efficacy of increased bacterial membrane permeabilizations, resulting in enhanced intracellular content release and bacterial death (12, 42). Furthermore, chelation activities of EDTA could augment the entry of colistin into bacteria to exert its bactericidal effects by blocking essential respiratory enzymes and other important intracellular targets of colistin, thereby overcoming colistin resistance regardless of the underlying colistin resistance mechanisms encoded in our XDR and PDR ColRkp clinical isolates (177).

In this study, mature ColRkp biofilms showed significant antimicrobial tolerance with 2-256-fold higher colistin MBEC than their corresponding planktonic MIC values. These findings are in accordance with those of previous studies that indicated the higher antimicrobial tolerance of mature biofilms due to the barrier effects of biofilm matrix, which limit the penetration of antibiotics into the biofilms (178). Interestingly, increased biofilm biovolume of ColRkp following exposure to subinhibitory concentrations of colistin (1mg/L) observed in this study could be explained as bacterial adaptive survival responses to antibiotic stress (179). These findings are consistent with previous research which indicated an increase in development of MDR *A. baumannii* biofilms by sub-inhibitory doses of colistin and

polymyxin (0.5X and 0.25XMIC) through regulating efflux pumps and biofilm-related genes expressions (180). Because antibiotics at sub-inhibitory doses can facilitate the biofilm formation in clinically important pathogens through different strategies, it is valuable to further investigate the mechanisms involved in increasing ColRkp biofilms in the presence of antibiotic stress.

In our study, even though EDTA produced potent antimicrobial activities in reducing biofilm biovolume and biofilm-embedded viable bacteria, an incomplete biofilm eradication and a sharp increase in biofilm viable cells after 24-hour EDTA (12mg/ mL) treatment *in vitro* suggests that antibiotics should be administered in conjunction with EDTA for treating ColRkp biofilms. According to prior study, colistin displayed antibiofilm effects on metabolically inactive cells in the inner layers of *E. coli* and MRSA biofilms with intrinsic colistin resistance, although there was regrowth of colistin-resistant phenotypes at 24 hour following treatment with 16mg/L of colistin monotherapy (39, 99). In this study, colistin-EDTA combination displayed improved antibiofilm activities in completely eradicating mature biofilms and decreasing biofilm bacteria viabilities of ColRkp clinical isolates within 24 hour of treatment *in vitro*. It may have occurred due to the biofilm matrix disruption by EDTA's metal ions chelation, which may not only facilitate colistin penetration into biofilms inner layers, but also increase bacterial release, where they act synergistically to produce lethal effects on released biofilm bacteria (42, 43, 99, 181). Previous studies also demonstrated that EDTA increased bactericidal effects of gentamicin on *Enterobacteriaceae* biofilms by matrix disruption and synergistic bactericidal effects (44).

To further confirm the therapeutic efficacy of novel colistin-EDTA combination, catheter-related biofilm infections were developed both *in vitro* and in immunocompromised mice to obtain a clinically relevant *in vivo* animal model that relates to patients' conditions commonly seen in clinical settings. Administration of colistin-EDTA combination not only successfully eradicated catheter-related biofilm infections, but also decreased biofilm-embedded bacteria viabilities within 24-hour exposure time, which demonstrates the potent therapeutic efficacy of colistin-EDTA combination in eradicating ColRkp catheter-related biofilm infections and eliminating

the risk of recurrence both *in vitro* and *in vivo*. These findings are in agreement with a prior study that demonstrated the effectiveness of gentamicin-EDTA combination for the eradication of catheter-related Gram-negative pathogens in Totally Implantable Venous-Access Ports (TIVAP) catheters in both *in vitro* and *in vivo* (44, 45).

Furthermore, colistin-EDTA combination was observed to reduce bacterial loads in internal organs, decrease serum creatinine, and provide significant protection against mortalities in treated mice, which further indicate the significant therapeutic efficacy and safety of colistin-EDTA combination therapy *in vivo*. These results are in accordance with a previous study in which EDTA enhanced imipenem susceptibilities to treat bla_{NDM-1}-producing *E. coli* and reduced systemic bacterial burden in murine sepsis model (142). Additionally, in *P. aeruginosa*-induced pneumonic mice model, reduced pulmonary bacterial burden and striking improvement with 100% survival of treated animals were observed after subcutaneous and intranasal administration of imipenem-EDTA combination therapy (182). According to the clinical reports, colistin exposure has been linked to increased serum creatinine in treated patients due to their induced oxidative stress in proximal renal tubules (183). However, the lower dose of colistin in the colistin-EDTA combination therapy, as well as the combined vasodilatation and antioxidant effects of EDTA (184), may help to mitigate the potential nephrotoxic side effects of colistin in treated mice of our study. Because a subinhibitory dose of colistin (1mg/L) in this colistin-EDTA combination therapy has been shown to have significant *in vivo* therapeutic efficacy for treating ColRkp catheter-related biofilm infections without causing nephrotoxicity or bacterial regrowth, this colistin-EDTA combination therapy could overcome not only the development of renal complications, but also the resurgence of resistant populations, both of which are the major limiting factors for colistin use in clinical settings (185). Although EDTA flushing or diffusion into the systemic circulation when used as a lock solution inside vascular catheters can result in pseudo-thrombocytopenia (186), the dose of EDTA used in this study was 12mg/mL, which is lower than FDA-approved doses for lead poisoning (1000mg/m^2 per day intravenously for 5 days) (187, 188), hemodialysis catheters (30mg/mL in

catheter lock solution) (189), and reduction of cardiovascular events in diabetic patients with peripheral vascular disease (3g in 500mL for intravenous infusion) (190).

This study also discovered a significantly altered *in vivo* expression of various bacterial virulence genes following exposure to different antimicrobial treatments in murine ColRkp catheter-related biofilm infections. Exposure to antimicrobial agents create stressful environment for bacteria and it could result in altered expressions of bacterial genes which reflect how the bacteria deal with the stress inside the host (191). Altered *in vivo* expressions of bacterial virulence genes found in this study point to the bacterial adaptive responses to survive in hostile environment that occurred as the impacts of different treatments tested in mice (192, 193). Given the scarcity of knowledge on the involvement of these virulence genes in response to various stresses induced by colistin and EDTA treatments in *K. pneumoniae*, further study is needed to investigate the consequences of altered virulence genes expressions following these treatments.

This is the first *in vitro* and *in vivo* study to demonstrate that a novel colistin-EDTA combination therapy produced potent synergistic activity for eradicating ColRkp catheter-related biofilm infections while also demonstrating a favorable safety profile with low resistance and toxicity risks. Although the subcutaneous catheter-related biofilm infection model used in this study is relevant to the conditions seen in clinical settings and linked to device-associated infections or catheter-related bloodstream infections, additional randomized control trials will be required to validate the clinical efficacy, tolerance and safety of this colistin-EDTA combination therapy in the treatment of catheter-related biofilm infection. The effects of altered virulence gene expressions associated with ColRkp will need to be investigated further to learn more about how colistin-resistant bacteria modulate their pathogenicity inside the host, which will support the implementation of more effective targeted strategies to overcome and mitigate their infectivity.

CHAPTER VII

CONCLUSION

Our study demonstrated a 28.5% prevalence of ColRkp exhibiting XDR and PDR characteristics among 165 ESBL and carbapenemase-producing CRkp clinical isolates collected from Chulalongkorn Memorial Hospital in Thailand between 2016 and 2021. Additionally, this study found a rising trend of colistin resistance that increased over time from 14.9% in 2016 to 36.2% in 2021. Both chromosomal-mediated colistin resistance mechanisms (91.5%), including *mgrB* alterations (85.1%), *pmrB* mutation (4.25%), or *phoQ* mutation (2.12%), and plasmid-mediated colistin resistance mechanisms with the presence of *mcr*-1.1, or *mcr*-8.1, or *mcr*-8.2 genes (8.5%) were found to be associated with colistin resistance in our ColRkp isolates. Several independent insertions, deletions, or substitutions in *mgrB* (85.1%) associated with increased expressions of Ara4N-related *phoPQ* and *pmrK* transcripts were observed to be crucial in establishing colistin resistance in our ColRKp isolates. Additionally, we observed a significant association between XDR ColRkp and increased biofilm production. Moreover, significantly altered bacterial virulence factors expressions were found to be associated with XDR ColRkp clinical isolates. In this study, a combination of colistin (0.25-1mg/L) and EDTA (12mg/mL) produced potent synergistic effects not only in planktonic but also in mature biofilms of both XDR and PDR ColRkp clinical isolates *in vitro*, regardless of the underlying colistin resistance mechanisms. This novel colistin-EDTA combination in our study has also been demonstrated to exhibit potent therapeutic efficacy in eradicating ColRkp catheter-related biofilm infections and eliminating the possibility of recurrence both *in vitro* and *in vivo*. Furthermore, colistin-EDTA combination demonstrated its significant therapeutic efficacy and safety in reducing bacterial load in internal organs, lowering serum creatinine, and protecting treated mice from mortality in this study. This is the first *in vitro* and *in vivo* study to highlight that a novel colistin-EDTA combination therapy is a promising alternative therapeutic strategy that can successfully eradicate ColRkp catheter-related biofilm infections while also demonstrating a favorable safety profile with low resistance and toxicity risks.

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APPENDIX A

REAGENTS AND INSTRUTMENTS

Reagent	Manufacturer
Agarose	Amresco, USA
Amikacin sulphate	Hi-media, India
Ammonium hydroxide	Sigma-Aldrich, USA
Boric acid	Sigma-Aldrich, USA
Bovine pancreatic DNase I	Sigma-Aldrich, USA
Ciprofloxacin	Sigma-Aldrich, USA
Ceftazidime	Sigma-Aldrich, USA
Colistin sulphate	Sigma-Aldrich, USA
Curcumin	Sigma-Aldrich, USA
DNA gel loading dye	Thermo fisher scientific, USA
dNTP	Thermo fisher scientific, USA
Ethylenediaminetetraacetic acid	Sigma-Aldrich, USA
Fosfomycin sodium	Meiji, Japan
Gene ruler 100 bp plus DNA ladder	Thermo fisher scientific, USA
Glucose-6-phosphate	Sigma-Aldrich, USA
Glycerol	Merck, Germany
Hiyield® Gel/PCR DNA mini kit	RBCBioscience, Taiwan
Hydrochloric acid	Merck, Germany
Imipenem	Wako, Japan
LB broth	BBL, USA
Meropenem	Wako, Japan
Methanol	Sigma-Aldrich, USA
Mueller-Hinton II agar	BBL, USA
Mueller-Hinton II broth (cation-adjusted)	BBL, USA
Phosphate buffer saline	Sigma-Aldrich, USA
Resveratrol	Sigma-Aldrich, USA

Reagent	Manufacturer
Sodium chloride	Amresco, USA
Sodium citrate	Thermo fisher scientific, USA
Sodium Hydroxide	Merck, Germany
Tramadol	Sigma-Aldrich, USA
Taq DNA polymerase	Thermo fisher scientific, USA
Tris	Amresco, USA
Tryptic soy broth (TSB)	BBL, USA

Instrument	Manufacturer
Incubator	Thermo fisher scientific, USA
Incubator shaker	Thermo fisher scientific, USA
Microcentrifuge	Eppendorf, Germany
UV/Visible spectrometer	Bio-Rad, USA
Thermal cycler	Applied Bioscience, USA
Nanodrop 1000 spectrometer	Thermo fisher scientific, USA
UV transilluminator	Montreal Biotech, Canada
QuantStudio 6 Flex Real-Time PCR System	Applied Biosystems, USA
Varioskan Flash Multimode spectrophotometer	Thermo Fisher Scientific, USA
Confocal laser scanning microscope	Zeiss, Oberkochen, Germany

APPENDIX B

MEDIA AND ANTIMICROBIAL AGENTS SOLUTION PREPARATION

1. Media preparation

1.1. Luria-Bertani broth (BBL, USA)

Suspend 25g of the dehydrated Luria-Bertani broth in 1000mL of distilled water and mixed homogenously then sterilized by autoclaving at 121°C for 15 minutes. The Luria-Bertani broth was stored at 4°C.

1.2. McConkey agar (Oxoid, USA)

Suspend 51.5g of the dehydrated MacConkey agar in 1000mL of distilled water and mixed homogeneously and sterilized by autoclaving at 121°C for 15 minutes. The MacConkey agar plates were stored at 4°C.

1.3. Mueller-Hinton II agar (BBL, USA)

Suspend 38g of the dehydrated Mueller-Hinton II agar in 1000mL of distilled water and mixed homogenously then sterilized by autoclaving at 121°C for 15 minutes. The Mueller-Hinton II agar was stored at 4°C.

1.4. Cation- adjusted Mueller-Hinton II broth (BBL, USA)

Suspend 22g of the dehydrated cation- adjusted Mueller-Hinton II broth in 1000mL of distilled water and mixed homogenously then sterilized by autoclaving at 121°C for 15 minutes. The cation- adjusted Mueller-Hinton II broth was stored at 4°C.

1.5. Tryptic soy broth (TSB) (BBL, USA)

Suspend 30g of the dehydrated TSB in 1000mL of distilled water and mixed homogenously then sterilized by autoclaving at 121°C for 15 minutes. The TSB was stored at 4°C.

1.6. Sterile 0.85% normal saline

Suspend 8.5g of the dehydrated sodium chloride in 1000mL of distilled water and mixed homogenously then sterilized by autoclaving at 121°C for 15 minutes. The sterile normal saline was stored at 4°C.

2. Antibiotic stock solution

1.1. Imipenem, stock concentration 1,024 mg/L

For preparation of stock, 0.0102g of imipenem was weighed then dissolved by 10mL of 5mL of sterile distilled water.

1.2. Meropenem, stock concentration 1,024 mg/L

For preparation of stock, 0.0102g of meropenem was weighed then dissolved by 10mL of 5mL of sterile distilled water.

1.3. Amikacin, stock concentration 1,024 mg/L

For preparation of stock, 0.0102g of amikacin was weighed then dissolved by 10mL of 5mL of sterile distilled water.

1.4. Ciprofloxacin, stock concentration 1,024 mg/L

For preparation of stock, 0.0102g of ciprofloxacin was weighed then dissolved by 10mL of 5mL of sterile distilled water.

1.5. Ceftazidime, stock concentration 1,024 mg/L

For preparation of stock, 0.0102g of ceftazidime was weighed then dissolved by 10mL of 5mL of sterile distilled water.

1.6. Colistin, stock concentration 1,024 mg/L

For preparation of stock, 0.0102g of colistin was weighed then dissolved by 10mL of 5mL of sterile distilled water.

1.7. Fosfomycin, stock concentration 1,024 mg/L

For preparation of stock, 0.0102g of fosfomycin was weighed then dissolved by 10mL of 5mL of sterile distilled water.

1.8. Ethylenediaminetetraacetic acid (EDTA), stock concentration 48 mg/ mL

For preparation of stock, 0.48g of the dehydrated EDTA was weighed then dissolved by 5mL of distilled water. Mixed homogenously, adjust the pH 8.0 and adjust the volume to 10mL. Then sterilized by filtration. The sterile EDTA was stored at 4°C.

1.9. Sodium citrate, stock concentration 48 mg/ mL

For preparation of stock, 0.48g of the dehydrated sodium citrate was weighed then dissolved by 10mL of distilled water. Mixed homogenously. Then sterilized by filtration. The sterile sodium citrate was stored at 4°C.

1.10. Tramadol, stock concentration 48 mg/mL

For preparation of stock, 0.48g of the dehydrated tramadol was weighed then dissolved by 10mL of distilled water. Mixed homogenously. Then sterilized by filtration. The sterile tramadol was stored at 4°C.

1.11. Curcumin, stock concentration 48 mg/mL

For preparation of stock, 0.48g of the dehydrated curcumin was weighed then dissolved by 10mL of distilled water. Mixed homogenously. Then sterilized by filtration. The sterile curcumin was stored at 4°C.

1.12. Resveratrol, stock concentration 48 mg/mL

For preparation of stock, 0.48g of the dehydrated resveratrol was weighed then dissolved by 10mL of distilled water. Mixed homogenously. Then sterilized by filtration. The sterile resveratrol was stored at 4°C.

1.13. DNAase, stock concentration 48 mg/mL

For preparation of stock, 0.48 g of DNAase was weighed then dissolved by 10mL of distilled water. Mixed homogenously. Then sterilized by filtration. The sterile DNAase was stored at 4°C.

1.14. Antibiofilm peptide, stock concentration 48 mg/mL

For preparation of stock, 0.48g of the dehydrated antibiofilm peptide was weighed then dissolved by 10mL of distilled water. Mixed homogenously. Then sterilized by filtration. The sterile antibiofilm peptide was stored at 4°C.

1.15. Glucose-6-phosphate

For preparation of stock, 0.0102g of glucose-6-phosphate was weighed then dissolved by 10mL of 5mL of sterile distilled water.

APPENDIX C

REAGENT PREPARATION

1. 0.5 M EDTA (pH 8.0)

   For preparation of 0.5M EDTA, 186.1g of EDTA was dissolved in 800mL of distilled water. Adjust the pH 8.0 and the volume to 1000mL. This reagent was stores at room temperature.

2. 10X Tris-Borate buffer (TBE)

   For preparation of 10X TBE, 108g of Tris base, 55g of boric acid, and 40ml of 0.5M EDTA (pH 8.0) were mixed and dissolved in 1000mL of distilled water. The TBE buffer was sterilized by autoclaving at 121°C for 15 minutes. This reagent was stores at room temperature.

3. 1.5% agarose gel

   For preparation of 1.5% agarose gel, 1.5g of agarose was suspended and dissolved by heating in 100mL of 0.5X TBE buffer.

4. Phosphate buffer saline (pH 7.4)

   For preparation of phosphate buffer saline (pH 7.4), 1 pouch of phosphate buffer saline powder was dissolved in 1000mL of distilled water. The phosphate buffer saline solution was sterilized by autoclaving at 121°C for 15 minutes. This reagent was stored at room temperature.

5. 0.1% Crystal violet solution

   For preparation of crystal violet 0.1% solution, 100mL of 10% crystal violet was suspended in 900mL of distilled water. The crystal violet 0.1% solution was sterilized by filtration. This reagent was stored at room temperature.

6. 30% Acetic acid solution

   For preparation of acetic acid 30% solution, 300mL of 100% acetic acid was suspended in 700mL of distilled water. This reagent was stored at room temperature.

APPENDIX D

ADDITIONAL RESULTS

Table 14. Distributions of antibiotics MICs tested against 165 CRkp clinical isolates.

Strains	Type of specimens	Age (years)	Sex	MIC (mg/L)						
				COL	IPM	MEM	CAZ	CIP	AMK	FOS
kp 921	Bronchoalveolar lavage	54	M	64	128	256	>512	512	16	>512
kp 924	Pus	93	F	64	128	128	>512	256	16	>512
kp 926	Sputum	84	F	64	128	128	>512	256	16	>512
kp 944	Endotracheal aspirate	6/12	M	16	8	64	>512	64	>512	>512
kp 946	Body fluid	39	M	64	64	256	>512	512	16	>512
kp 947	Sputum	38	F	64	256	512	>512	512	32	512
kp1078	Urine	44	F	8	1	0.25	128	>512	>512	8
kp1189	Sputum	56	M	32	64	64	>512	512	16	>512
kp1194	Sputum	61	M	16	256	512	>512	>512	>512	256
kp1225	Pus (Wound)	77	F	64	256	256	>512	>512	32	>512
kp 80	Sputum	85	F	8	>512	>512	>512	128	32	256
kp 104	Blood	51	F	64	128	256	>512	256	16	128
kp 114	Urine	87	M	64	128	256	>512	256	16	128
kp 119	Endotracheal aspirate	71	F	32	1	0.25	>512	256	64	32
kp 151	Sputum	71	M	64	128	256	>512	>512	>512	64
kp 122	Urine	62	F	>512	128	256	>512	256	16	>512
kp 202	Blood	19	F	16	64	128	>512	256	32	>512
kp 243	Pus	1/12	F	64	256	256	>512	32	>512	>512
kp 248	Endotracheal aspirate	57	M	64	16	128	>512	256	512	128

Strains	Type of specimens	Age (years)	Sex	MIC (mg/L)						
				COL	IPM	MEM	CAZ	CIP	AMK	FOS
kp 264A	Sputum	81	M	16	128	128	>512	128	>512	>512
kp 270A	Pus (Wound)	54	M	16	8	16	>512	64	32	>512
kp 259	Wound swab	55	F	0.25	32	1	>512	64	2	32
kp 260	Body fluid	56	F	0.125	64	128	>512	256	16	256
kp 261	Endotracheal aspirate	89	F	16	64	128	>512	128	>512	>512
kp 262	Urine	97	F	0.25	64	0.25	>512	>512	>512	>512
kp 263	Sputum	77	M	2	64	8	>512	256	16	64
kp 264	Sputum	56	M	0.25	128	256	>512	256	32	64
kp 265	Blood	29/365	F	0.25	64	32	>512	8	16	64
kp 266	Urine	56	F	0.25	128	32	>512	256	16	256
kp 267	Sputum	54	M	0.25	128	32	>512	256	16	>512
kp 268	Sputum	92	M	0.25	16	2	512	512	8	16
kp 269	Sputum	56	M	0.25	128	128	>512	256	32	64
kp 270	Sputum	56	F	0.25	64	16	>512	64	1	8
kp 271	Sputum	59	M	0.25	128	128	>512	>512	>512	>512
kp 272	Blood	56	M	0.25	128	256	>512	256	32	64
kp 273	Sputum	56	M	0.25	128	256	>512	256	32	128
kp 274	Sputum	70	M	0.25	32	1	512	512	16	64
kp 275	Sputum	84	F	0.25	32	0.25	32	2	16	32
kp 276	Body fluid	66	M	0.25	128	256	>512	256	16	32
kp 277	Bile	64	M	0.25	64	32	>512	512	4	>512
kp 278	Body fluid	39	M	0.25	128	128	>512	512	16	256
kp 280	Urine	79	M	0.25	128	128	>512	256	32	512
kp 281	Endotracheal aspirate	8/12	M	0.25	16	16	>512	256	>512	128
kp 282	Sputum	49	F	0.25	16	16	>512	512	4	>512
kp 283	Urine	62	F	0.25	64	128	>512	512	16	32

Strains	Type of specimens	Age (years)	Sex	MIC (mg/L)						
				COL	IPM	MEM	CAZ	CIP	AMK	FOS
kp 284	Urine	84	M	0.25	8	64	>512	256	8	256
kp 285	Sputum	49	F	0.25	16	32	>512	16	16	16
kp 286	Endotracheal aspirate	84	F	0.25	64	32	>512	16	4	256
kp 287	Tip catheter	66	M	0.25	8	32	>512	512	16	8
kp 288	Sputum	92	M	0.25	128	128	>512	256	32	16
kp 289	Urine	62	M	16	128	64	>512	512	8	128
kp 290	Urine	77	M	0.5	8	64	>512	>512	>512	128
kp 291	Urine	38	F	8	>512	256	>512	>512	64	256
kp 292	Urine	84	M	0.5	0.5	2	>512	256	8	32
kp 293	Urine	79	M	0.5	128	128	>512	>512	8	64
kp 294	Urine	72	M	0.5	64	128	>512	512	32	32
kp 295	Urine	79	M	0.5	0.5	16	>512	512	8	64
kp 296	Pus	79	F	0.5	8	32	>512	256	16	32
kp 297	Urine	60	F	0.5	64	128	>512	512	32	64
kp 298	Bile	57	F	0.5	64	128	>512	256	16	64
kp 299	Urine	51	M	0.5	0.5	32	>512	>512	64	128
kp 300	Body fluid	92	M	2	512	256	>512	512	1	128
kp 301	Urine	73	M	64	128	128	>512	>512	16	>512
kp 302	Blood	37	M	0.5	128	128	>512	512	16	64
kp 303	Bile	57	F	0.5	0.5	32	>512	256	8	16
kp 304	Blood	79	M	0.5	128	256	>512	256	16	64
kp 305	Endotracheal aspirate	78	F	0.5	128	128	>512	256	32	16
kp 306	Urine	83	M	0.5	128	128	>512	>512	>512	>512
kp 308	Urine	88	F	0.5	128	128	>512	>512	32	>512
kp 309	Sputum	37	M	64	128	128	>512	>512	32	>512
kp 311	Urine	4	M	0.5	32	8	>512	4	1	32

Strains	Type of specimens	Age (years)	Sex	MIC (mg/L)						
				COL	IPM	MEM	CAZ	CIP	AMK	FOS
kp 312	Urine	56	M	0.5	4	4	>512	512	4	>512
kp 313	Endotracheal aspirate	82	M	0.5	128	128	>512	512	32	128
kp 314	Pus	41	M	0.5	32	128	>512	>512	32	128
kp 315	Urine	75	M	0.5	0.25	1	>512	512	32	256
kp 316	Intraabdominal fluid	16	M	0.5	128	128	>512	512	16	32
kp 317	Urine	61	F	0.5	128	128	>512	>512	>512	>512
kp 318	Urine	79	F	0.5	0.25	4	>512	512	>512	128
kp 319	Sputum	36	F	64	128	128	>512	>512	2	64
kp 320	Urine	65	M	0.5	2	2	>512	>512	2	8
kp 321	Urine	54	M	0.5	128	128	>512	256	32	64
kp 322	Pus	1	M	0.5	4	8	>512	256	8	>512
kp 323	Urine	77	M	0.5	16	32	>512	256	2	512
kp 324	Sputum	59	F	0.5	64	128	>512	>512	16	128
kp 325	Sputum	65	F	0.5	32	32	>512	128	1	32
kp 326	Urine	60	F	0.5	2	4	>512	256	2	32
kp 327	Urine	65	M	0.5	128	128	>512	>512	8	>512
kp 328	Blood	54	M	32	128	128	>512	>512	32	512
kp 329	Urine	78	M	0.5	16	64	>512	512	1	64
kp 330	Blood	65	M	0.5	2	0.125	>512	512	1	8
kp 331	Sputum	62	M	0.5	128	128	>512	>512	16	>512
kp 332	Urine	71	F	64	128	128	>512	>512	16	64
kp 333	Pus	1	M	0.5	128	128	>512	>512	>512	>512
kp 334	Urine	83	M	0.5	128	128	>512	>512	>512	>512
kp 335	Blood	82	M	0.5	32	64	>512	256	2	>512
kp 338	Pleural fluid	56	M	0.5	128	128	>512	512	16	64
kp 339	Sputum	82	F	0.5	32	128	>512	512	16	16

Strains	Type of specimens	Age (years)	Sex	MIC (mg/L)						
				COL	IPM	MEM	CAZ	CIP	AMK	FOS
kp 340	Sputum	65	M	16	256	256	>512	512	8	512
kp 341	Urine	86	F	0.5	8	16	>512	512	4	8
kp 342	Urine	65	M	0.5	4	0.25	>512	>512	4	8
kp 344	Urine	6	M	0.5	8	64	>512	128	16	64
kp 345	Urine	29	F	0.5	1	1	>512	>512	1	64
kp 346	Urine	89	F	0.5	128	128	>512	>512	16	16
kp 347	Urine	80	M	0.5	32	64	>512	>512	16	>512
kp 348	Urine	41	F	0.5	256	64	>512	64	16	32
kp 349	Sputum	74	M	0.5	128	64	>512	256	16	128
kp 350	Urine	75	M	0.5	16	16	>512	128	4	8
kp 351	Urine	65	M	0.5	32	16	>512	128	2	4
kp 352	Urine	75	M	0.5	32	8	>512	>512	4	32
kp 353	Urine	60	M	64	128	128	>512	>512	32	32
kp 415	Urine	70	F	0.25	128	256	>512	512	16	16
kp 355	Sputum	76	F	0.5	32	0.5	>512	>512	2	>512
kp 356	Sputum	60	M	0.5	64	64	>512	512	16	16
kp 357	Endotracheal aspirate	1/12	M	16	64	128	>512	512	>512	64
kp 358	Endotracheal aspirate	84	M	0.5	32	2	>512	32	16	8
kp 359	Endotracheal aspirate	54	M	0.5	32	64	>512	>512	16	8
kp 361	Blood	23	F	0.5	128	128	>512	>512	16	64
kp 362	Sputum	61	M	0.5	64	32	>512	>512	16	4
kp 364	Sputum	80	M	0.5	256	256	>512	256	16	32
kp 365	Urethral swab	84	F	0.5	32	64	>512	512	16	8
kp 366	Blood	2 /12	M	16	256	128	>512	512	>512	64
kp 367	Bile	86	F	0.5	8	4	>512	128	2	8

Strains	Type of specimens	Age (years)	Sex	MIC (mg/L)						
				COL	IPM	MEM	CAZ	CIP	AMK	FOS
kp 368	Urine	52	M	0.5	0.5	1	>512	512	>512	64
kp 369	Urine	60	M	0.5	64	64	>512	>512	>512	64
kp 371	Urine	82	F	0.5	128	64	>512	32	1	16
kp 372	Urine	46	M	>512	128	128	>512	512	>512	>512
kp 373	Sputum	54	M	0.5	32	4	>512	>512	4	32
kp 374	Bile	86	F	0.5	8	4	>512	128	>512	16
kp 375	Pus	79	F	32	64	128	>512	512	16	16
kp 376	Blood	87	F	0.5	128	256	>512	512	32	16
kp 377	Sputum	74	M	0.5	64	32	>512	512	>512	128
kp 378	Bile	86	F	0.5	64	64	>512	64	8	32
kp 379	Urine	74	M	0.5	512	256	>512	>512	>512	>512
kp 380	Urine	77	M	32	256	128	>512	>512	32	16
kp 381	Urine	84	F	0.5	64	64	>512	>512	16	64
kp 382	Urine	68	F	0.5	512	256	>512	>512	32	128
kp 383	Urine	84	F	32	64	32	>512	512	32	16
kp 384	Body fluid	61	M	0.5	64	8	>512	32	1	2
kp 385	Sputum	60	M	0.5	256	256	>512	>512	>512	8
kp 386	Urine	69	M	0.5	128	64	>512	32	8	32
kp 387	Endotracheal aspirate	74	M	0.5	64	8	>512	>512	>512	64
kp 388	Blood	60	M	0.5	16	128	>512	>512	32	16
kp 389	Urine	57	M	32	128	128	>512	>512	32	64
kp 390	Endotracheal aspirate	73	M	32	128	64	>512	>512	32	>512
kp 391	Blood	74	M	0.5	64	64	>512	128	>512	64
kp 392	Urine	67	F	0.5	16	64	>512	>512	8	64
kp 393	Urine	71	M	0.5	4	4	>512	256	>512	16
kp 394	Urine	83	F	0.5	128	128	>512	512	32	32

Strains	Type of specimens	Age (years)	Sex	MIC (mg/L)						
				COL	IPM	MEM	CAZ	CIP	AMK	FOS
kp 395	Sputum	74	M	16	64	64	>512	>512	16	16
kp 396	Sputum	50	F	16	256	128	>512	>512	>512	32
kp 397	Urine	86	F	0.5	64	64	>512	>512	>512	128
kp 398	Bile	61	F	0.5	128	128	>512	>512	>512	512
kp 399	Endotracheal aspirate	65	M	0.5	32	32	>512	512	16	>512
kp 400	Urine	83	M	32	>512	256	>512	>512	4	128
kp 402	Urine	70	M	64	128	128	>512	>512	64	32
kp 406	Urine	95	M	0.5	64	128	>512	>512	64	16
kp 407	Blood	95	M	0.5	128	64	>512	>512	16	4
kp 408	Blood	43	M	0.5	64	32	>512	>512	64	64
kp 409	Pus (wound)	45	M	0.5	256	256	>512	>512	64	16
kp 410	Urine	77	F	0.25	128	128	>512	>512	16	32
kp 411	Urine	75	F	32	16	32	>512	>512	32	4
kp 412	Blood	45	M	0.25	128	64	>512	>512	4	16
kp 413	Sputum	75	M	32	64	128	>512	>512	32	32
kp 414	Endotracheal aspirate	25	F	8	32	64	>512	>512	16	8
kp 354	Tip catheter	72	F	32	128	256	>512	512	16	>512

Col - Colistin	MEM - Meropenem	CIP- Ciprofloxacin	FOS - Fosfomycin
IPM - Imipenem	CAZ - Ceftazidime	AMK - Amikacin	

Table 15. Interpretation of drug-resistance characteristics of 47 ColRkp clinical isolates by testing against 10 different antibiotic classes.

ColRkp isolates	Antibiotics tested against ColRkp clinical isolates										Interpretation (XDR/PDR)
	Cationic peptide - COL	Penicillin-AMP	β lactam-β lactamase inhibitor-AMC	Cephalo-sporin-CAZ	Carbapenem-IPM,MEM, DOR, ETP	Quinolone - CIP	Amino-glycoside-AMK	Fosfomycin - FOS	Sulfonamide - SXT	Tetra-cycline -TET	
kp 921	R	R	R	R	R	R	S	R	R	R	XDR
kp 924	R	R	R	R	R	R	S	R	R	R	XDR
kp 926	R	R	R	R	R	R	S	R	R	R	XDR
kp 944	R	R	R	R	R	R	R	R	R	I	XDR
kp 946	R	R	R	R	R	R	S	S	R	R	XDR
kp 947	R	R	R	R	R	R	S	R	S	R	XDR
kp 1078	R	R	R	R	R	R	R	S	R	R	XDR
kp 1189	R	R	R	R	R	R	S	R	R	R	XDR
kp 1194	R	R	R	R	R	R	R	R	R	I	XDR
kp 1225	R	R	R	R	R	R	I	R	R	R	XDR
kp 80	R	R	R	R	R	R	I	R	S	S	XDR
kp 104	R	R	R	R	R	R	S	R	R	R	XDR
kp 114	R	R	R	R	R	R	S	R	R	R	XDR

ColRkp isolates	Antibiotics tested against ColRkp clinical isolates										Interpretation (XDR/PDR)
	Cationic peptide - COL	Penicillin-AMP	β lactam-β lactamase inhibitor-AMC	Cephalo-sporin-CAZ	Carbapenem-IPM,MEM, DOR, ETP	Quinolone - CIP	Amino-glycoside-AMK	Fosfomycin - FOS	Sulfonamide - SXT	Tetra-cycline -TET	
kp 119	R	R	R	R	R	R	R	S	R	R	XDR
kp 151	R	R	R	R	R	R	R	S	R	R	XDR
kp 122	R	R	R	R	R	R	S	R	R	R	XDR
kp 202	R	R	R	R	R	R	I	R	R	R	XDR
kp 243	R	R	R	R	R	R	R	R	R	S	XDR
kp 248	R	R	R	R	R	R	R	R	R	R	PDR
kp 264A	R	R	R	R	R	R	R	R	R	R	PDR
kp 270A	R	R	R	R	R	R	I	R	R	R	XDR
kp 261	R	R	R	R	R	R	R	R	S	I	XDR
kp 289	R	R	R	R	R	R	S	R	S	R	XDR
kp 291	R	R	R	R	R	R	R	R	R	R	PDR
kp 301	R	R	R	R	R	R	S	R	R	R	XDR
kp 309	R	R	R	R	R	R	I	R	R	R	XDR
kp 340	R	R	R	R	R	R	S	R	R	I	XDR

ColRkp isolates	Antibiotics tested against ColRkp clinical isolates										Interpretation (XDR/PDR)
	Cationic peptide - COL	Penicillin-AMP	β lactam-β lactamase inhibitor-AMC	Cephalo-sporin-CAZ	Carbapenem-IPM,MEM, DOR, ETP	Quinolone - CIP	Amino-glycoside-AMK	Fosfomycin - FOS	Sulfonamide - SXT	Tetra-cycline -TET	
kp 357	R	R	R	R	R	R	R	S	S	R	XDR
kp 366	R	R	R	R	R	R	R	S	R	I	XDR
kp 375	R	R	R	R	R	R	S	S	R	R	XDR
kp 380	R	R	R	R	R	R	I	S	R	R	XDR
kp 395	R	R	R	R	R	R	S	S	S	I	XDR
kp 400	R	R	R	R	R	R	S	I	R	S	XDR
kp 402	R	R	R	R	R	R	R	S	R	R	XDR
kp 414	R	R	R	R	R	R	S	R	R	R	XDR
kp 354	R	R	R	R	R	R	S	S	R	R	XDR
kp 319	R	R	R	R	R	R	S	S	R	R	XDR
kp 328	R	R	R	R	R	R	I	R	R	R	XDR
kp 332	R	R	R	R	R	R	S	S	R	R	XDR
kp 353	R	R	R	R	R	R	I	S	R	R	XDR
kp 413	R	R	R	R	R	R	S	S	R	R	XDR

ColRkp isolates	Antibiotics tested against ColRkp clinical isolates										Interpretation (XDR/PDR)
	Cationic peptide - COL	Penicillin-AMP	β lactam-β lactamase inhibitor-AMC	Cephalo-sporin-CAZ	Carbapenem-IPM,MEM, DOR, ETP	Quinolone - CIP	Amino-glycoside-AMK	Fosfomycin - FOS	Sulfonamide - SXT	Tetra-cycline -TET	
kp 372	R	R	R	R	R	R	R	R	R	R	PDR
kp 383	R	R	R	R	R	R	I	S	R	R	XDR
kp 390	R	R	R	R	R	R	I	S	R	R	XDR
kp 396	R	R	R	R	R	R	R	S	R	R	XDR
kp 411	R	R	R	R	R	R	I	S	R	R	XDR
kp 389	R	R	R	R	R	R	I	S	R	R	XDR

COL - Colistin	AMC - Amoxicillin-clavulanic acid	IPM - Imipenem	DOR - Doripenem	CIP - Ciprofloxacin	SXT- Trimethoprim-sulfamethoxazole
AMP - Ampicillin	CAZ - Ceftazidime	MEM - Meropenem	ETP - Ertapenem	AMK - Amikacin	TET - Tetracycline

Table 16. Mechanisms of colistin resistance with their respective virulence genes and drug resistance genes profiles observed among 47 ColRkp clinical isolates.

ColRkp isolates	Mechanisms of colistin resistance	Virulence gene profile	ESBL profile	Carbapenemase profile
kp 921	Altered *mgrB* due to insertion of IS *1-like* between Nucleotide +55 and +56	*mrkD, ybtS,OmpK35, OmpK36,uge,wabG, luxS*	TEM,CTX-M	NDM,OXA-48
kp 924	Altered *mgrB* due to insertion of IS *1-like* between Nucleotide +55 and +56	*mrkD, ybtS,OmpK35, OmpK36,uge,wabG, luxS*	TEM,CTX-M	NDM,OXA-48
kp 926	Altered *mgrB* due to insertion of IS *1-like* between Nucleotide +71 and +72	*mrkD, ybtS,OmpK35, OmpK36,uge,wabG, luxS*	TEM,CTX-M	NDM,OXA-48
kp 944	Altered *mgrB* due to insertion of IS *3-like* between Nucleotide +121 and +122	*mrkD, kfu, ybtS,OmpK35, OmpK36,uge,wabG, luxS*	SHV,TEM, CTX-M	OXA-48
kp 946	Altered *mgrB* due to insertion of IS *1-like* between Nucleotide +55 and +56	*mrkD, ybtS,OmpK35, OmpK36,uge,wabG, luxS*	TEM,CTX-M	OXA-48
kp 947	Altered *mgrB* due to insertion of IS *1-like* between Nucleotide +71 and +72	*mrkD, ybtS,OmpK35, OmpK36,uge,wabG, luxS*	TEM,CTX-M	OXA-48

ColRkp isolates	Mechanisms of colistin resistance	Virulence gene profile	ESBL profile	Carbapenemase profile
kp 1078	*mcr-8.2* with Wild type *mgrB*, *pmrAB* and *phoPQ*	*mrkD, kfu, OmpK35, OmpK36,uge,wabG, luxS*	SHV,TEM, CTX-M	-
kp 1189	Genetic alteration in *mgrB* due to point mutation (A7T, AAA>TAA) causing premature internal stop codon in *mgrB*	*mrkD, ybtS,OmpK35, OmpK36,uge,wabG, luxS*	TEM,CTX-M	OXA-48
kp 1194	Deleterious T157P PmrB due to point mutation in *pmrB* (A469C, ACC>CCC)	*mrkD, kfu, ybtS,OmpK35, OmpK36,uge,wabG, luxS*	OXA,CTX-M	OXA-48
kp 1225	Altered *mgrB* due to insertion of IS *Kpn14-like* (IS *1*) between Nucleotide +115 and +116	*mrkD, ybtS, OmpK35, OmpK36,uge,wabG, luxS*	TEM,CTX-M	NDM
kp 80	Altered *mgrB* due to insertion of IS *Kpn14-like* (IS *1*) between Nucleotide +117 and +118	*mrkD, kfu, ybtS,OmpK35, OmpK36,uge,wabG, luxS*	SHV	NDM

ColRkp isolates	Mechanisms of colistin resistance	Virulence gene profile	ESBL profile	Carbapenemase profile
kp 104	Altered *mgrB* due to insertion of IS *1-like* between Nucleotide -7 and -8 (in promoter region, upstream of *mgrB* start codon)	*mrkD, ybtS,OmpK35, OmpK36,uge,wabG, luxS*	TEM,CTX-M	NDM,OXA-48
kp 114	Altered *mgrB* due to insertion of IS *1-like* between Nucleotide -7 and -8 (in promoter region, upstream of *mgrB* start codon)	*mrkD, ybtS,OmpK35, OmpK36,uge,wabG, luxS*	TEM,CTX-M	NDM,OXA-48
kp 119	Altered *mgrB* due to insertion of IS *1-like* between Nucleotide +105 and +106	*mrkD, kfu,OmpK35, OmpK36,uge,wabG, luxS*	OXA-1, CTX-M	NDM
kp 151	Deleterious T157P PmrB due to point mutation in *pmrB* (A469C, ACC>CCC)	*mrkD, kfu, ybtS,OmpK35, OmpK36,uge,wabG, luxS*	SHV,TEM, OXA-1, CTX-M	NDM,OXA-48
kp 122	Altered *mgrB* due to insertion of IS *1-like* between Nucleotide +104 and +105	*mrkD,ybtS,OmpK35, OmpK36,uge,wabG, luxS*	TEM,CTX-M	NDM,OXA-48
kp 202	Loss of *mgrB*	*mrkD, ybtS,OmpK35, OmpK36,uge,wabG, luxS*	TEM,CTX-M	NDM,OXA-48

ColRkp isolates	Mechanisms of colistin resistance	Virulence gene profile	ESBL profile	Carbapenemase profile
kp 243	Genetic alteration in *mgrB* due to point mutation in initial codon of *mgrB* (G3A, GTG>GTA)	*mrkD, kfu, ybtS,OmpK35, OmpK36,uge,wabG, luxS*	TEM,CTX-M	NDM,OXA-48
kp 248	E82K on the response regulator domain of PhoP due to point mutation in *phoP* (G244A, GAA>AAA)	*mrkD, ybtS,OmpK35, OmpK36,uge,wabG, luxS*	CTX-M	OXA-48
kp 264A	Loss of *mgrB*	*mrkD, ybtS,OmpK35, OmpK36,uge,wabG, luxS*	SHV,TEM, CTX-M	OXA-48
kp 270A	Loss of *mgrB*	*mrkD, ybtS,OmpK35, OmpK36,uge,wabG, luxS*	SHV,TEM, CTX-M	NDM, VIM
kp 261	Loss of *mgrB*	*mrkD, ybtS,OmpK35, OmpK36,uge,wabG, luxS*	SHV,TEM, CTX-M	OXA-48
kp 289	Altered *mgrB* due to insertion of IS *Ecp 1-like* (IS *1380-like*) between Nucleotide +124 and +125	*mrkD, kfu, ybtS,OmpK35, OmpK36,uge,wabG, luxS*	SHV,OXA-1, TEM	NDM,OXA-48
kp 291	*mcr-1.1* with Wild type *mgrB, pmrAB* and *phoPQ*	*mrkD, ybtS,OmpK35, OmpK36,uge,wabG, luxS*	SHV,TEM, CTX-M	NDM,OXA-48, VIM

ColRkp isolates	Mechanisms of colistin resistance	Virulence gene profile	ESBL profile	Carbapenemase profile
kp 301	Altered *mgrB* due to insertion of IS *1-like* between Nucleotide -7 and -8 (in promoter region, upstream of *mgrB* start codon)	*mrkD, OmpK35, OmpK36,uge,wabG, luxS*	SHV,TEM, CTX-M	NDM,OXA-48, VIM
kp 309	Loss of *mgrB*	*mrkD, ybtS,OmpK35, OmpK36,uge,wabG, luxS*	SHV,TEM, CTX-M	NDM,OXA-48, VIM
kp 340	Altered *mgrB* due to insertion of IS *1-like* between Nucleotide +55 and +56	*mrkD, ybtS,OmpK35, OmpK36,uge,wabG, luxS*	SHV,TEM, CTX-M	NDM, VIM
kp 357	Altered *mgrB* due to insertion of IS *1-like* between Nucleotide +71 and +72	*mrkD, ybtS,OmpK35, OmpK36,uge,wabG, luxS*	SHV,TEM, OXA-1, CTX-M	NDM
kp 366	Altered *mgrB* due to insertion of IS *1-like* between Nucleotide +71 and +72	*mrkD, ybtS,OmpK35, OmpK36,uge,wabG, luxS*	SHV,TEM, CTX-M	NDM
kp 375	Genetic alteration in *mgrB* due to point mutation (G60A, TGG>TGA) causing premature internal stop codon in *mgrB*	*mrkD, ybtS,OmpK35, OmpK36,uge,wabG, luxS*	SHV,TEM, CTX-M	NDM,OXA-48, VIM
kp 380	Genetic alteration in *mgrB* due to point mutation (G60A, TGG>TGA) causing premature internal stop codon in *mgrB*	*mrkD, ybtS,OmpK35, OmpK36,uge,wabG, luxS*	SHV,TEM, CTX-M	NDM,OXA-48, VIM

ColRkp isolates	Mechanisms of colistin resistance	Virulence gene profile	ESBL profile	Carbapenemase profile
kp 395	*mcr-8.1* with R256G PmrB due to point mutation in *pmr*B (C766G, CGC>GGC), Wild type *mgrB, pmrA* and *phoPQ*	*mrkD, ybtS,OmpK35, OmpK36,uge,wabG, luxS*	SHV,TEM, CTX-M	NDM,OXA-48
kp 400	*mcr-1.1* with R256G PmrB due to point mutation in *pmr*B (C766G, CGC>GGC), Wild type *mgrB, pmrA* and *phoPQ*	*mrkD, ybtS,OmpK35, OmpK36,uge,wabG, luxS*	SHV,TEM, OXA-1, CTX-M	NDM
kp 402	Altered *mgrB* due to insertion of IS *903-like* (IS *5-like*) between Nucleotide +74 and +75	*mrkD, ybtS,OmpK35, OmpK36,wabG, luxS*	SHV,TEM, CTX-M	OXA-48
kp 414	Altered *mgrB* due to insertion of IS *1-like* between Nucleotide +55 and +56	*mrkD, ybtS,OmpK35, OmpK36,uge,wabG, luxS*	SHV,TEM, CTX-M	OXA-48
kp 354	Genetic alteration in *mgrB* due to point mutation in initial codon of *mgrB* (G3A, GTG>GTA)	*mrkD, ybtS,OmpK35, OmpK36,uge,wabG, luxS*	TEM,CTX-M	NDM,OXA-48
kp 319	Altered *mgrB* due to insertion of IS *1-like* between Nucleotide +55 and +56	*mrkD, ybtS,OmpK35, OmpK36,uge,wabG, luxS*	TEM,CTX-M	OXA-48

ColRkp isolates	Mechanisms of colistin resistance	Virulence gene profile	ESBL profile	Carbapenemase profile
kp 328	Genetic alteration in *mgrB* due to point mutation (A7T, AAA>TAA) causing premature internal stop codon in *mgrB*	*mrkD, ybtS,OmpK35, OmpK36,uge,wabG, luxS*	TEM,CTX-M	OXA-48
kp 332	Altered *mgrB* due to insertion of IS *1-like* between Nucleotide -7 and -8 (in promoter region, upstream of *mgrB* start codon)	*mrkD,ybtS,OmpK35, OmpK36,uge,wabG, luxS*	SHV,TEM, CTX-M	NDM,OXA-48, VIM
kp 353	Altered *mgrB* due to insertion of IS *3-like* between Nucleotide +121 and +122	*mrkD, ybtS,OmpK35, OmpK36,uge,wabG, luxS*	TEM,CTX-M	OXA-48
kp 413	Altered *mgrB* due to insertion of IS *1-like* between Nucleotide +71 and +72	*mrkD, ybtS,OmpK35, OmpK36,uge,wabG, luxS*	TEM,CTX-M	OXA-48
kp 372	Altered *mgrB* due to insertion of IS *1-like* between Nucleotide +104 and +105	*mrkD,ybtS,OmpK35, OmpK36,uge,wabG, luxS*	TEM,CTX-M	NDM,OXA-48

ColRkp isolates	Mechanisms of colistin resistance	Virulence gene profile	ESBL profile	Carbapenemase profile
kp 383	Altered *mgrB* due to insertion of IS *Kpn14-like* (IS *1*) between Nucleotide +117 and +118	*mrkD, ybtS,OmpK35, OmpK36,uge,wabG, luxS*	SHV	NDM
kp 390	Altered *mgrB* due to insertion of IS *1-like* between Nucleotide -7 and -8 (in promoter region, upstream of *mgrB* start codon)	*mrkD, ybtS,OmpK35, OmpK36,uge,wabG, luxS*	TEM,CTX-M	NDM,OXA-48
kp 396	Altered *mgrB* due to insertion of IS *Kpn14-like* (IS *1*) between Nucleotide +115 and +116	*mrkD, ybtS,OmpK35, OmpK36,uge,wabG, luxS*	TEM,CTX-M	NDM
kp 411	Altered *mgrB* due to insertion of IS *Ecp 1-like* (IS *1380-like*) between Nucleotide +124 and +125	*mrkD, ybtS,OmpK35, OmpK36,uge,wabG, luxS*	SHV,OXA-1, TEM	NDM,OXA-48
kp 389	Altered *mgrB* due to insertion of IS *903-like* (IS *5-like*) between Nucleotide +74 and +75	*mrkD, ybtS,OmpK35, OmpK36,uge,wabG, luxS*	SHV,TEM, CTX-M	OXA-48

Table 17. Susceptibilities of planktonic and mature biofilms of ColRkp clinical isolates to different antibiotics.

Antibiotics	Planktonic		Biofilms	
	MIC (mg/mL)		MBEC (mg/mL)	
	Extensively drug-resistant ColRkp (XDR ColRkp) (n=43)	Pandrug-resistant ColRkp (PDR ColRkp) (n=4)	Extensively drug-resistant ColRkp (XDR ColRkp) (n=42)	Pandrug-resistant ColRkp (PDR ColRkp) (n=3)
Colistin	8->512	8->512	16-2048	64-2048
Imipenem	1->512	16->512	>2048	>2048
Meropenem	0.25->512	128-256	>2048	>2048
Ceftazidime	>512	>512	>2048	>2048
Ciprofloxacin	64->512	128->512	>2048	>2048
Amikacin	2->512	64->512	>2048	>2048
Fosfomycin	8->512	128->512	>2048	>2048

Table 18. Respective MICs, FICIs and MBECs of colistin and EDTA tested against ColRkp clinical isolates.

ColRkp isolates	Planktonic					Biofilms		
	Colistin (mg/L)	EDTA (mg/ml)	Colistin (mg/L) + EDTA (mg/ml)	FICI	Interpretation	Colistin (mg/L)	EDTA (mg/ml)	MBEC [Colistin (mg/L) + EDTA (mg/ml)]
kp 921	64	24	0.25 + 12	0.28125	Synergy	2048	48	0.5 + 12
kp 924	64	24	0.25 + 12	0.15625	Synergy	2048	48	0.5 + 12
kp 926	64	24	0.25 + 12	0.15625	Synergy	2048	48	0.5 + 12
kp 944	16	24	0.25 + 12	0.3125	Synergy	2048	48	0.5 + 12
kp 946	64	24	0.25 + 12	0.3125	Synergy	2048	48	0.5 + 12
kp 947	64	6	0.25 + 12	0.375	Synergy	2048	48	0.5 + 12
kp 1078	8	3	0.25 + 12	0.1875	Synergy	512	24	0.5 + 12
kp 1189	32	24	0.25 + 12	0.3125	Synergy	2048	48	0.5 + 12
kp 1194	16	12	0.25 + 12	0.1875	Synergy	2048	48	0.5 + 12
kp 1225	64	24	0.25 + 12	0.375	Synergy	2048	48	0.5 + 12
kp 80	8	6	0.25 + 12	0.1875	Synergy	2048	24	0.5 + 12
kp 104	64	24	0.25 + 12	0.265625	Synergy	2048	48	0.5 + 12
kp 114	64	24	0.25 + 12	0.28125	Synergy	2048	48	0.5 + 12
kp 119	32	12	0.25 + 12	0.125	Synergy	2048	48	0.5 + 12
kp 151	64	24	0.25 + 12	0.1875	Synergy	2048	48	0.5 + 12
kp 122	>512	24	2048+24	1.25	Indifference	2048	48	0.5 + 12
kp 202	16	12	0.25 + 12	0.28125	Synergy	2048	48	0.5 + 12
kp 243	64	24	0.25 + 12	0.1875	Synergy	-	-	-
kp 248	64	6	0.25 + 12	0.5	Synergy	2048	12	1 + 12
kp264A	16	24	0.25 + 12	0.3125	Synergy	-	-	-
kp270A	16	24	0.25 + 12	0.28125	Synergy	2048	48	0.5 + 12

ColRkp isolates	Planktonic					Biofilms		
	Colistin (mg/L)	EDTA (mg/ml)	Colistin (mg/L) + EDTA (mg/ml)	FICI	Interpretation	Colistin (mg/L)	EDTA (mg/ml)	MBEC [Colistin (mg/L) + EDTA (mg/ml)]
kp 261	16	12	0.25 + 12	0.3125	Synergy	2048	48	0.5 + 12
kp 289	16	6	0.25 + 12	0.1875	Synergy	2048	12	0.5 + 12
kp 291	8	24	0.25 + 12	0.3125	Synergy	64	48	1 + 12
kp 301	64	12	0.25 + 12	0.3125	Synergy	2048	48	0.5 + 12
kp 309	64	3	0.25 + 12	0.265625	Synergy	2048	24	0.5 + 12
kp 340	16	6	0.25 + 12	0.3125	Synergy	2048	24	0.5 + 12
kp 357	16	24	0.25 + 12	0.265625	Synergy	2048	48	0.5 + 12
kp 366	16	24	0.25 + 12	0.125	Synergy	2048	48	0.5 + 12
kp 375	32	12	0.25 + 12	0.3125	Synergy	2048	48	0.5 + 12
kp 380	32	24	0.25 + 12	0.15625	Synergy	2048	48	0.5 + 12
kp 395	16	24	0.25 + 12	0.3125	Synergy	2048	48	0.5 + 12
kp 400	32	12	0.25 + 12	0.3125	Synergy	2048	48	0.5 + 12
kp 402	64	6	0.25 + 12	0.375	Synergy	2048	24	0.5 + 12
kp 414	8	3	0.25 + 12	0.09375	Synergy	16	12	0.5 + 12
kp 354	32	24	0.25 + 12	0.28125	Synergy	2048	48	0.5 + 12
kp 319	64	12	0.25 + 12	0.28125	Synergy	2048	48	0.5 + 12
kp 328	32	24	0.25 + 12	0.375	Synergy	2048	48	0.5 + 12
kp 332	64	6	0.25 + 12	0.3125	Synergy	2048	24	0.5 + 12
kp 353	64	3	0.25 + 12	0.265625	Synergy	2048	12	0.5 + 12
kp 413	32	12	0.25 + 12	0.28125	Synergy	2048	48	0.5 + 12
kp 372	>512	24	2048+24	1.25	Indifference	2048	48	1 + 12
kp 383	32	12	0.25 + 12	0.125	Synergy	2048	48	0.5 + 12
kp 390	32	3	0.25 + 12	0.3125	Synergy	2048	24	0.5 + 12
kp 396	16	6	0.25 + 12	0.1875	Synergy	2048	12	0.5 + 12

ColRkp isolates	Planktonic					Biofilms		
	Colistin (mg/L)	EDTA (mg/ml)	Colistin (mg/L) + EDTA (mg/ml)	FICI	Interpretation	Colistin (mg/L)	EDTA (mg/ml)	MBEC [Colistin (mg/L) + EDTA (mg/ml)]
kp 411	32	12	0.25 + 12	0.28125	Synergy	2048	48	0.5 + 12
kp 389	32	24	0.25 + 12	0.265625	Synergy	2048	48	0.5 + 12

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AWARD RECEIVED	Young Investigator Award, ISAAR 2021