MOLECULAR CHARACTERISATION OF *GYRA, PARC AND QEPA* GENES IN QUINOLONE RESISTANCE EXTENDED SPECTRUM BETA-LACTAMASE PRODUCING *ESCHERICHIA COLI* ISOLATES IN HTAA, KUANTAN

BY

RAHMATULLAH

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Kulliyyah of Medicine
International Islamic University Malaysia

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ABSTRACT

Quinolone resistance (QR) and extended-spectrum β-lactamase (ESBL) production have increased in *Escherichia coli* and considered a serious problem worldwide. It is worth to monitor the resistance mechanism in *E. coli* to provide guidance for optimising antimicrobial treatments and control the spread of resistance. The objective of this study was to molecularly characterize *gyrA, parC* and plasmid-mediated *qepA* efflux pump genes, in QR-ESBL *E. coli* isolates obtained from patients in HTAA, Kuantan. The antibiotic susceptibility profile was also studied. 32 QR-ESBL and six quinolone-susceptible *E. coli* isolates from (September to December 2018) were included in the study. The isolates were reconfirmed with known phenotypic tests. The antibiotic susceptibility test was performed according to CLSI, 2019 guidelines. PCR and DNA sequencing were performed for the identification of mutations in quinolone resistance determining region (QRDR) of *gyrA* and *parC* genes. Resistance to ampicillin, tetracycline, nalidixic acid was (100%) followed by cefotaxime (96.9%), ciprofloxacin (78.1%) trimethoprim-sulfamethoxazole (75%), ceftazidime (56.3%), cefepime (43.8%) and gentamycin (25%). None of the isolates was resistant to piperacillin-tazobactam, amikacin, imipenem, meropenem, ertapenem, and colistin. PCR successfully amplified the *gyrA* and *parC* genes. However, *qepA* gene was not detected by PCR in the isolates. The majority of the isolates had a point mutation in QRDR of *gyrA* at codons 83 and 87 and in *parC* at codons 80 and 84. Two isolates had mutations outside of QRDR at codons 144 and 167 in *parC*. A strong positive correlation was found between MIC levels of ciprofloxacin and the number of resistance mutations. The sequencing of 6QS-ESBL *E. coli* revealed the absence of resistance mutations. Quinolone resistance in the isolates was mainly due to mutations in *gyrA, parC* genes. Acquisition of multidrug resistance (MDR) genes through innate gene mutations and mobile genetic elements contributed to the emergence of MDR. This study reinforces the importance of being vigilant in utilising molecular techniques to monitor the emergence of resistance genes in different locations.

خلاصة البحث

ان مقاومة الكينولونات وتصنيع انزيمات البيتالاكتام ذات الطيف الممتد قد ازدادت انتشارا في بكتريا اشريشيا القولون مما جعلها معضله هامه على مستوى العالم. ان مراقبة آلية المقاومه في اشريشيا القولون مهمه لإيجاد الطرق المثلى لعلاج الأخماج البكتيري، السيطره ومنع انتشار المقاومه. الغايه من هذه الدراسه هوالتوصيف الجزيئي لجينات (*gyrA,parC*) وجينات مضخات التدفق (*qepA*) في البلازميد المتواجده في اشريشيا القولون المقاومه للكوينولون والمصنعه لانزيمات البيتالاكتام ذات الطيف الممتد (QR-ESBL) التي تم الحصول عليها من نماذج المرضى الراقدين في مستشفى (HTAA) في كوانتان. كما تمت دراسة حساسية العزلات للمضادات الحيوية. أجريت الدراسه على 32 عزله (QR-ESBL) وست عزلات من جرثومة الأشرشية كولى الحساسه لمجموعة الكوينولون. جمعت هذه العينات من شهر أيلول الى شهر كانون الاول من سنة ٢٠١٨ . تم اعادة التأكد من هوية العزلات بطرق النمط الظاهري. درست الحساسيه للمضادات والتركيز المثبط الأدنى حسب توصيات (CLSI). تم إجراء فحوصات تفاعل البوليميراز المتسلسل التقليدية ومن ثم تسلسل قواعد الحمض النووي للاستدلال على وجود طفرات وراثيه في المنطقه المسؤوله عن مقاومة الكوينولون. كانت نسبة المقاومه للامبيسيلين، تتراسيكلين، ونااليديكسك اسيد ١٠٠٪ يأتي بعدها سيفوتاكسيم (٩٦,٩٪), سيبروفلوكساسين (٧٨,١٪) ترايمثوبريم-سلفامثاكسازول (٧٥٪), سفتازيديم (٥٦,٣٪)، سفيبيم (٤٣,٨٪) وجنتامايسين (٢٥٪). ولا واحد من العزلات كانت مقاومه للبيبراسيلين-تازوباكتام، اميكاسين، اميبنيم، مروبنم، ارتابنيم وكوليستين. تفاعل البوليميراز المتسلسل ضخم بنجاح الجينات (*gyrA,parC*)، ولكن لم يتم الاستدلال على الجين (*qepA*) بواسطة تفاعل البوليميراز المتسلسل في أي من العزلات. بين تحليل تسلسل قواعد الد أن إية (٩٦,٩٪) من العزلات احتوت على طفره نقطيه في (QRDR) من (GyrA) عند كودون ٨٣, و(٧٥٪) عند كودون ٨٧. بالاضافه لذالك، احتوت العزلات على طفرات من (ParC) عند كودون ٨٠ (٧٨,١٪) وكودون ٨٤ (٥٦,٢ ٪). أثنين من العزلات احتوت على طفرات خارج عن هذه الطفرة (QRDR) في (*parC*) عند كودون ١٤٤ و١٦٧. كذالك وجدت علاقته مرتبطة بالتراكيز المثبطه للسيبروفلوكساسين و الدأن أية وكذالك عدد الطفرات المسببه للمقاومه. لقد بين تحليل تسلسل قواعد الد أن إية لستة عزلات من بكتريا اشريشيا الكولون حساسه للكوينولات وكذالك أظهرت حساسية بالنسبة لانزيمات البيتالاكتام ذات الطيف الواسع، عدم وجود أي طفرات مقاومه. ان خاصية مقاومة الكوينولات في العزلات البكتيريه نتج بصورة رئيسية من وجود طفرات في الجينات (*gyrA,parC*) . ان الاستحواذ على الجينات المسببه للمقاومه المتعدده من خلال الطفرات الجينيه الفطريه والعناصر الجينيه المتحركه، كانتا السبب الرئيس لظهور البكتريا ذات المقاومه المتعدده للمضادات الحيويه. تعزز هذه الدراسه أهمية اليقضه والتقصي عند اللجوء الى التقنيات الجزيئيه لمراقبة ظهور الجينات التي تضفي صفة المقاومه للبكتريا في الأماكن المختلفة.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Medical Sciences

……..
Hairul Aini Hamzah
Supervisor

……..
Mohammed Imad Al-Deen Mustafa Mahmud
Co-Supervisor

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Medical Sciences

……..
Engku Nuraishah Huda E-Zainudin
Internal Examiner

……..
Zulkefley Othman
External Examiner

This thesis was submitted to the Department of Basic Medical Sciences and is accepted as a fulfilment of the requirement for the degree of Master of Medical Sciences

……..
Mohammed Imad Al-Deen Mustafa Mahmud
Head, Department of Basic Medical Sciences

This thesis was submitted to the Kulliyyah of Medicine and is accepted as a fulfilment of the requirement for the degree of Master of Medical Sciences

……..
Azmi Md Nor
Dean, Kulliyyah of Medicine

DECLARATION

I hereby declare that this thesis is the result of my own investigation, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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DEDICATION

TO MY FATHER

ATTA GUL

THE SOURCE OF KINDNESS AND STRENGTH

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LIST OF ABBREVIATIONS

T	Temperature
≥	Greater or equal to
≤	Lesser or equal to
Bp	base pair
%	Percentage
Mg	Milligram
µg	Microgram
mm	Millimeter
µl	Microlitre
Ml	Millilitre
°C	Centigrade
FQs	Fluoroquinolones
QS	Quinolone sensitive
QR	Quinolone resistant
QRDR	Quinolone resistance determining region
ESBL	Extended Spectrum β-lactamases
PMQR	Plasmid-mediated quinolone resistance
Ser	Serine
Leu	Leucine
Asp	Asparagine
Glu	Glutamic acid
Val	Valine
Tyr	Tyrosine
ILe	Isoleucine
β	Beta
PCR	Polymerase chain reaction
UV	Ultraviolet

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND AND JUSTIFICATION OF THE STUDY

Antibiotic resistance has become one of the global health priorities of the World Health Organization (WHO). Enterobacteriaceae family members of gram-negative bacteria have shown antibacterial resistance worldwide. *Escherichia coli* is a member of the Enterobacteriaceae family, and one of the most common pathogens causing severe community-acquired and hospital-acquired infections, such as blood infections, intra-abdominal infections, and urinary tract infections. It is associated with drug resistance and can exhibit resistance to multiple classes of antibiotics. Resistance to the last resort antibiotics carbapenems and colistin has been reported among *E. coli* (Blair, Webber, Baylay, Ogbolu, & Piddock, 2015; Röderova et al., 2017).

β-lactam antibiotics have been used widely and considered as the first line of antibacterial drugs against many pathogenic bacteria including the members of the Enterobacteriaceae family. Despite, their great efficacy against many bacteria, the number of resistant bacteria against β-lactams is increasing worldwide. The emergence of β-lactam resistance in many bacteria which are causing community and hospital-acquired infections, has resulted in massive usage of other antibiotics such as quinolones and fluoroquinolones worldwide (Silva-Sánchez et al., 2013).

The major mechanism responsible for resistance to beta-lactam antibiotics is the production of beta-lactamases. These enzymes, which have the ability to inactivate extended-spectrum beta-lactam antibiotics, like cefotaxime and ceftazidime are called extended-spectrum β-lactamases (ESBL). *E. coli* is notoriously known as an ESBL

producer. Production of ESBLs limiting the treatment options in infections caused by *E. coli*. In such cases, β-lactamases inhibitors and carbapenems would be great alternative treatment options of these multidrug-resistant bacteria. Unfortunately, resistance to carbapenems has also been reported among Enterobacteriaceae, particularly in *E. coli*. Resistance to carbapenems further decreases the treatment options and requires second-line and last resort antibiotics such as polymyxin, tigecycline, aminoglycosides and quinolones (Rodriguez-Bano, Gutiérrez-Gutiérrez, Machuca, & Pascual, 2018).

Quinolones and fluoroquinolones have shown greater efficacy against many bacteria which cause community-acquired and hospital-acquired infections. These are the drugs of choice in the treatment of many human infections such as gastrointestinal infections, urinary tract infections, and respiratory tract infections (Zurfluh, Abgottspon, Hächler, Nüesch-Inderbinen, & Stephan, 2014).

Quinolone resistance among Enterobacteriaceae including *E. coli* had remained low for many decades. However, the increase in quinolone resistance had started from the early 1990s when its rate in the United States of America was less than 1% and rose only to 3% in 2008. In the United States, the quinolone resistance rate among community-associated Enterobacteriaceae has jumped from 10% to 30%. Furthermore, much higher quinolone resistance rates greater than 50% have been reported worldwide due to the spread of a specific clone of *E. coli* ST131-*H*30 (Colpan et al., 2013; Johnson, Johnston, Clabots, Kuskowski, & Castanheira, 2010; Spellberg & Doi, 2015). Antibiotic resistance is similarly a problematic and serious health-related concern in Malaysia. According to National surveillance of antibiotic resistance (NSAR) reports published by the Ministry of Health Malaysia, between 2010 and 2017, *E. coli* showed

a high resistance rate to quinolones and fluoroquinolones 24.4% (MKK, Rashid, MHM, Baharudin, & Ramli, 2019).

Bacteria are considered as multi-drug resistance (MDR) when showing resistance to at least one antimicrobials from three different groups (Magiorakos et al., 2012).

Furthermore, the production of different ESBLs in Enterobacteriaceae particularly in *E. coli* is limiting treatment options and causing the emergence of co-existing resistance determinants such as aminoglycosides and fluoroquinolones (Livermore, 2012; Parajuli et al., 2017).

The presence of MDR bacteria in Malaysia is a great health threat and showing an increasing trend of antibiotic resistance observed over the years according to the NSAR 2017 report (MKK et al., 2019).

Gram-negative bacteria-harboring ESBLs limit the effectiveness of available antibiotics because they can demonstrate a high level of quinolone resistance as well. In a study conducted on ESBL producing *Klebsiella pneumoniae* isolates in Malaysia found 37% resistance rate to ciprofloxacin, a commonly used fluoroquinolone antibiotic worldwide (Al-Marzooq, Yusof, Yasim, & Tay, 2014).

Therefore, surveillance on ESBL-producing bacteria, especially among Enterobacteriaceae such as *E. coli*, is a must to prevent further spread of MDR. Even though the development of antimicrobial resistance (AMR) control guidelines by the Ministry of Health contains important general measures to ensure patient care and to implement antimicrobial stewardship strategies, these should be customised according to the background of each geographic region. Therefore, it is mandatory to understand local epidemiology through clinical and microbiological monitoring. The epidemiology

of quinolone resistance has been described extensively in many different countries (Ahmad, 2019; Akova, 2016; Turnidge, 1995).

However, few investigators conducted further research into detecting resistance genes. Likewise, many studies tackling the antibiotic resistance rely on the problem of screening resistant isolates solely, which may substantially affect susceptibility reports, representing a strong bias towards resistance.

Quinolones and fluoroquinolones resistance mechanism in bacteria is mainly due to alteration of the target DNA gyrase and topoisomerase IV enzymes which can be caused by a mutation in the quinolone resistance determining region (QRDR) of the encoding genes *gyrA, gyrB, parC, and parE.* However, plasmid-mediated quinolone resistance (PMQR) determinants such as *qnr*, aminoglycoside acetyltransferase, *AAC(6')-Ib-cr* and efflux pumps *QepA* and *OqxAB* genes may also confer low-level quinolone resistance (Hooper & Jacoby, 2015).

Studies on quinolone resistance have been done in Malaysia. In one study, 30.7% of 283 of *Salmonella* strains isolated from food, humans, and animals were resistant to ciprofloxacin and the majority (69.3%) of them possessed missense mutation in QRDR of *gyrA* gene. Furthermore, the plasmid-mediated *qnrS1* variant was found in 36.6% of the isolates (Thong, Ngoi, Chai, & Teh, 2016).

In another study in Malaysia, a total of 21 ciprofloxacin-resistant, 8 intermediate, and 18 sensitive *K. pneumoniae* isolates were studied for the quinolone-resistant *gyrA* mutations and PMQR determinants. The findings of the study revealed a high prevalence (48.9%) of *qnr* determinants and *gyrA* mutation was detected in 18 (85.7%) ciprofloxacin-resistant isolates. Thus, the study concluded that ciprofloxacin resistance was significantly associated with *gyrA* gene alteration (Saiful, Mohd, & Tay, 2013).

In another study, a total of 93 (ESBL)-producing *K. pneumoniae* isolates were investigated for chromosomally-mediated and PMQR. Ciprofloxacin resistance was significantly associated with the presence of multiple mutations in *gyrA* and *parC* genes in several positions (Al-Marzooq et al., 2014).

Thus, early identification and continually monitoring the existence of resistant *E. coli* isolates in every region is highly recommended by several authorities to plan for the control and prevention of the further spread of these drug-resistant bacteria in the region (CDC, 2013).

To date, there is no extensive report on quinolone resistance mechanisms among *E. coli* isolates across Malaysia, particularly from Kuantan, Pahang, Malaysia (Ab Rahman, Teng, & Sivasampu, 2016; Ahmad, 2019; Al-Marzooq et al., 2014; Lim, Yasin, Yeo, Puthucheary, & Thong, 2009; Yun, Myo, Emran, & Lin, 2017).

Therefore, the objectives of this study were as follows:

a. To characterise the chromosomal genes *gyrA* and *parC* in quinolone resistance extended-spectrum β-lactamases producing *E. coli* (QR-ESBL *E.coli*) from a tertiary hospital in Pahang.
b. To determine the correlation between the genetic mutations with phenotypic antibiotic resistance profile.
c. To detect the presence of plasmid-mediated gene *qepA* in the isolates.
d. To determine the antibiotic susceptibility profile in the QR-ESBL producing *E. coli* isolates.

CHAPTER TWO

LITERATURE REVIEW

2.1 ENTEROBACTERIAECAE

The Enterobacteriaceae are gram-negative, non-spore-forming, facultative anaerobes that ferment glucose and other sugars, reduce nitrate to nitrite, and produce catalase but do not produce oxidase. Most are motile by virtue of peritrichous flagella. The majority of the family members share some features and characteristics in common. Members of the Enterobacteriaceae are often referred to as enteric bacteria because the principal habitat of many of these organisms is the lower gastrointestinal tract of various animals. They comprise the most common gram-negative isolates in microbiology laboratories, including the vast majority of urinary isolates and a large proportion of isolates from the blood, the peritoneal cavity, and the respiratory tract (Bennett, Dolin, & Blaser, 2014).

Enterobacteriaceae are a large family of gram-negative bacteria that encompass many clinically important bacteria such as *E. coli, Klebsiella* spp, *Pseudomonas aeruginosa, Salmonella species, Shigella, Enterobacter* spp which are commonly isolated from clinical cultures. Bacteria of this large family often associated with severe community-acquired and hospital-acquired infections (Van Duin & Doi, 2017).

2.2 *E. COLI*

E. coli is a common member of the large gram-negative family Enterobacteriaceae, which was initially discovered and introduced by the German scientist “Theodor Escherich” in 1805. It is the common inhabitant and residential in a large portion of the

faecal flora of the colon and large intestine of mammals. The *E. coli* are considered as the abundant facultative anaerobes in these particular sites and their presence in the environment is due to the fecal contamination by animals and humans which carry these organisms as commensals in their body rather than the ability of the bacteria propagation in the environment. *E. coli* are non-spore-forming round shape bacteria with a diameter of 0.5 micrometres and 1.0 to 3.0 micrometres in length (Divya & Mohamed Hatha, 2014; Ghosh, 2017).

2.2.1 Classification of *E. coli*

As most *E. coli* are commensal members of the no2rmal intestinal flora, some strains are virulent which can cause severe gastrointestinal infections along with other serious extraintestinal health complications. Pathogenic *E. coli* is classified into intestinal pathogenic *E. coli* (InPEC) and extraintestinal pathogenic *E. coli* (ExPEC). Intestinal pathogenic *E. coli* strains can cause several forms of gastroenteritis and are classified into six groups. These are enterohemorrhagic (EHEC), enteropathogenic (EPEC), enteroaggregative (EAEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), and diffusely adherent (DAEC) *E. coli*, while ExPEC uropathogenic *E. coli* are causative agents of infections in anatomical sites outside of the gastrointestinal tract, and are associated with urinary tract infections (UTIs), neonatal meningitis, and septicemia (Adefisoye & Okoh, 2016).

2.2.2 Pathotypes of *E. coli*

The pathotypes of the *E. coli* are the intestinal and extraintestinal pathogenic strains which are classified based on certain characteristics such as the different virulence factors and the capabilities of causing the infection. The extraintestinal pathogenic

strains represent a large variety of diseases outside of the intestinal tract and therefore are called the Uropathogenic *E. coli*, septicemia-associated *E. coli*, meningitis-associated *E. coli* and other pathogen strains (Kaper, Nataro, & Mobley, 2004; Poolman & Wacker, 2015; Wang, 2017).

E. coli is an extremely common and complex human pathogen. It is the most frequently isolated species in clinical microbiology laboratories and is the leading cause of UTIs, with millions of cases and billions of dollars in associated health care costs annually in the USA (Russo and Johnson 2003). Although *E. coli* is most closely linked to UTI, it can infect any extraintestinal site, causing meningitis, skin infections, myositis, osteomyelitis and epididymo-orchitis and severe *E. coli* infections, which often involve bloodstream infection (Vila et al., 2016).

2.2.3 Pathogenesis of *E. coli*

The pathogenic *E. coli* can be classified into two groups which are the extraintestinal and the intestinal pathogens. The extraintestinal pathogenic *E. coli* encompass some strains that have the ability to produce infections outside the intestinal tract such as urinary infections, bacteremia, sepsis, pneumonia, and neonatal meningitis. On the other hand, infections by the intestinal pathogenic *E. coli* strains present with abdominal pain, watery and bloody diarrhoea often similar to dysentery in which the causative agent is rather *Shigella* spp. or *Campylobacter jejuni*. The pathogenesis to produce infection in the intestinal tract has been associated with their spreading and expression of virulence determinants such as adhesion, invasins, toxins and the survival abilities against host defense mechanisms as well as the acquisition of resistance to antibiotics (Wang, 2017).

The gain and loss of genes throughout the genome at a number of some hot spots have resulted in the gaining of virulence and fitness behavior among pathogenic *E. coli*