# ASSESSMENT ON VARIOUS ASPECTS OF ANTIBIOTIC RESISTANCE IN ENTEROBACTERIACEAE

By

**LEE KAH LENG**

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## ABSTRACT

### ASSESSMENT OF VARIOUS ASPECTS OF ANTIBIOTIC RESISTANCE IN ENTEROBACTERIACEAE

**Lee Kah Leng**

Enterobacteriaceae that colonised in human intestine are highly exposed to the antibiotic pressures. These selective pressures may induce antibiotic resistance, as well as the transfer of resistance from the resistant bacteria to the enteric bacteria. However, the bacteria may also lose its resistance upon the withdrawal of antibiotic pressure. The aim of this study is to assess the losing, induction and the transfer of antibiotic resistance on *Escherichia coli* and *Klebsiella pneumoniae.* Firstly, the ability of losing antibiotic resistance was studied on clinical isolate *E. coli* 594370 which resistant to ciprofloxacin, moxifloxacin, gentamicin and trimethoprim. The bacteria was serially passaged in Mueller Hinton (MH) broth in the absence of antibiotics. Secondly, the induction of trimethoprim resistance was assessed on ATCC 25922 *E. coli* and ATCC 13883 *K. pneumoniae* that were susceptible to trimethoprim. These bacteria were serially passaged in MH broth in the presence of sub-lethal trimethoprim concentration (12 μg/mL). Thirdly, the transfer of antibiotic resistance was assessed in clinical isolate *E. coli* 594370 and clinical isolate *K. pneumoniae* 594394. Clinical isolate *E. coli* 594370 acted as donor bacteria

and clinical isolate *K. pneumoniae* 594394 acted as recipient bacteria that showed susceptibility to ciprofloxacin, moxifloxacin and gentamicin. These bacteria were grown and serially passaged together. There was no noticeable losing in resistance in clinical isolate *E. coli* 594370 after 85 passages. Next, the zone-of-inhibition diameter was noticeably reduced for more than 55% in ATCC 13883 *K. pneumoniae.* Gradual reduction in zone-of-inhibition diameter was observed in ATCC 25922 *E. coli*. However, there was no evidence in the transfer of antibiotic resistance from clinical isolate *E. coli* 594370 to clinical isolate *K. pneumoniae* 594394 over 30 passages. Clinical isolate *K. pneumoniae* 594394 remained susceptible to ciprofloxacin, moxifloxacin and trimethoprim. As a conclusion, the losing of antibiotic resistance did not occurred over 85 passages, and induction of trimethoprim resistance was occurred in both of the ATCC strains. The transfer of antibiotic resistance from donor bacteria to recipient bacteria did not happen over 30 passages.

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## DECLARATION

I hereby declare that the project is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at Universiti Tunku Abdul Rahman or other institutions.

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LEE KAH LENG

## APPROVAL SHEET

This project report entitled "**ASSESSMENT ON VARIOUS ASPECTS OF ANTIBIOTIC RESISTANCE IN ENTEROBACTERIACEAE**" was prepared by LEE KAH LENG and submitted as partial fulfillment requirements for the degree of Bachelor of Science (Hons) Biomedical Science at Universiti Tunku Abdul Rahman.

Approved by:

_____

(Mr. YUEN HAWK LEONG) Date: .....

Supervisor

Department of Biomedical Science

Faculty of Science

Universiti Tunku Abdul Rahman

**FACULTY OF SCIENCE**

**UNIVERSITI TUNKU ABDUL RAHMAN**

Date: _____

**PERMISSION SHEET**

It is hereby certified that **LEE KAH LENG** (ID No: 10ADB03790) has completed this final year project entitled "**ASSESSMENT ON VARIOUS ASPECTS OF ANTIBIOTIC RESISTANCE IN ENTEROBACTERIACEAE**" supervised by Mr. Yuen Hawk Leong (Supervisor) from the Department of Biomedical Science , Faculty of Science.

I hereby give permission to my supervisor to write and prepare manuscripts of these research finding for publishing in any form, if I did not prepare it within six (6) months from this date, provided that my name is included as one of the author for this article. The arrangement of the name depends on my supervisor.

Yours truly,

_____

(LEE KAH LENG)

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## LIST OF ABBREVIATONS

| | |
|---|---|
| CIP | Ciprofloxacin |
| CLSI | Clinical and Laboratory Standards Institute |
| CN | Gentamicin |
| DHFR | Dihydrofolate reductase |
| EMB | Eosin methylene blue |
| ESBL | Extended spectrum beta-lactamase |
| ENCAST | European Committee on Antimicrobial Susceptibility Testing |
| GI | Gastrointestinal |
| HGT | Horizontal gene transfer |
| IAIs | Intra-abdominal infection |
| ICU | Intensive care unit |
| KB | Kirby-Bauer |
| µg/ml | Microgramme per milliliter |
| MDR | Multidrug-resistant |
| MHA | Mueller Hinton agar |
| MHB | Mueller Hinton broth |
| MIC | Minimum inhibitory concentration |
| $MIC_{res}$ | minimal inhibitory concentration of resistant strain |
| $MIC_{susc}$ | Minimal inhibitory concentration of the susceptible strain |
| MXF | Moxifloxacin |
| OTC | Over-the-counter |
| QRDR | Quinolone resistance determining region |

| | |
|---|---|
| RND | Resistance-nodulation-cell division |
| RNA | Ribonucleic acid |
| ROAR | Reservoirs of antibiotic resistance |
| rpm | Revolutions per minute |
| UTI | Urinary tract infection |
| W | Trimethoprim |
| ZI | Zoneof-inhibition |

# CHAPTER 1

## INTRODUCTION

Emergence of antibiotic resistance in *ESKAPE* pathogens restricts the antibiotic choices available for treatment (Lisboa and Nagel 2011). Remarkably, Enterobacteriaceae that colonize human intestinal tract are highly exposed to the antibiotic pressures exerted by the oral antibiotic therapy and food with antibiotic residues (Jernberg et al., 2010). Moreover, reservoir hypothesis mentioned by Salyers et al. (2004) highlighted the role of the enteric bacteria as the reservoir of antibiotic resistant genes. Thus, the study on dissemination and transfer of antibiotic resistance may provide a better idea on the acquisition of antibiotic resistance among the enteric bacteria.

Aspects of antibiotic resistance, such as losing of antibiotic resistance, induction of antibiotic resistance and transfer of antibiotic resistance were assessed in this study. There are several community studies that evaluate the interventions of antibiotic prescribing policies. Some of these studies showed that the reduction in antibiotic prescription managed to bring down the resistance rate in bacteria (Seppala et al., 1997; Enne et al., 1999). In contrast, other studies yielded controversial findings in this aspect (Enne et al., 2001; Sundqvist et al., 2010). On the other hand, there are increasing evidence that

supported the fact that antibiotic resistance can be induced using sub-lethal antibiotic concentration (Drlisa and Zhao 2007; Andersson and Hughes 2011).

According to Schjørring and Krogfelt (2010), *in vivo* and *in vitro* model systems are used in the study of antibiotic resistance. Assessment of antibiotic resistance is frequently conducted in *in vivo* models and community settings, but the *in vitro* experimental studies are relatively less established. In this project, various aspects of antibiotic resistance were assessed using *in vitro* model system which in parallel with the objectives as stated:

- ✓ To assess the duration required for the losing of antibiotic resistance in resistant bacteria.
- ✓ To study the parameters (duration and concentration of antibiotics) required for the induction of antibiotic resistance.
- ✓ To analyze the duration required for the transfer of antibiotic resistance from the resistant, donor bacteria to susceptible, recipient bacteria.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Enterobacteriaceae

*Escherichia coli* and *Klebsiella pneumoniae* are gram-negative bacteria that are classified under Enterobacteriaceae. These bacteria are commensal flora in human gastrointestinal tract. Enterobacteriaceae are also opportunistic pathogens that account for more than 50% of the bacteremia cases (Livermore 2012).

*E. coli* and *K. pneumoniae* are categorized in the notorious *ESKAPE* pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* sp.) that are associated to the production of extended spectrum beta-lactamase (ESBL) in intensive care units (ICUs) (Fraimow and Tsigrelis, 2011; Lisboa and Nagel 2011). Common diseases that are caused by *E. coli* include urinary tract infection (UTI) (Vellinga et al., 2012), intra-abdominal infections (IAIs) (Paterson et al., 2005) and bacteremia (Wilson et al., 2011; Livermore 2012). As mentioned by Drago et al. (2010), similar diseases are caused by *K. pneumoniae,* in which nosocomial infections are more prominent than community-acquired infection. Longer length of hospital

stay is another risk factor for the nosocomial infections caused by *K. pneumoniae* (Lim and Webb 2005).

## 2.2 Antibiotics that are involved in this study

*Escherichia coli* that used in this study is classified as multidrug resistant (MDR) bacteria. Hereby, multidrug resistant (MDR) is defined as resistance of bacteria to at least 3 classes of antibiotics (Cantón and Ruiz-Garbajosa 2011; Barie 2012). The *E. coli* strain used in this study is resistant to 3 classes of antibiotics: gentamicin (aminoglycoside), ciprofloxacin and moxifloxacin (fluoroquinolone) and trimethoprim (folate pathway inhibitor).

In a nutshell, the mechanism action of the antibiotics and resistance mechanisms are summarized in Figure 2.1. Mechanisms confer to the resistance to aminoglycoside include: modification of aminoglycoside by enzymes like acetyltransferase, phosphotransferase and nucleotidyltransferase (Alekshun and Levy 2007); elimination of aminoglycosides using efflux pumps like AcrD, AcrA and TolC pump (Kumar and Schweizer 2005); and decreased membrane permeability to aminoglycoside due to absence of porin (Kumar and Schweizer 2005). In addition to efflux of fluoroquinolone by resistance-nodulation-cell division (RND) pump (Kumar and Schweizer 2005), mutation on the targets of fluoroquinolone, DNA gyrase and topoisomerase also confer to the resistance to fluoroquinolone (van Hoek et al., 2011). Next, the resistance to trimethoprim that serves as anti-folate is acquired by

alteration in chromosomal dihydrofolate reductase (DHFR) (van Hoek et al., 2011), which contributes to the excess production of DHFR (Huovinen 2001).

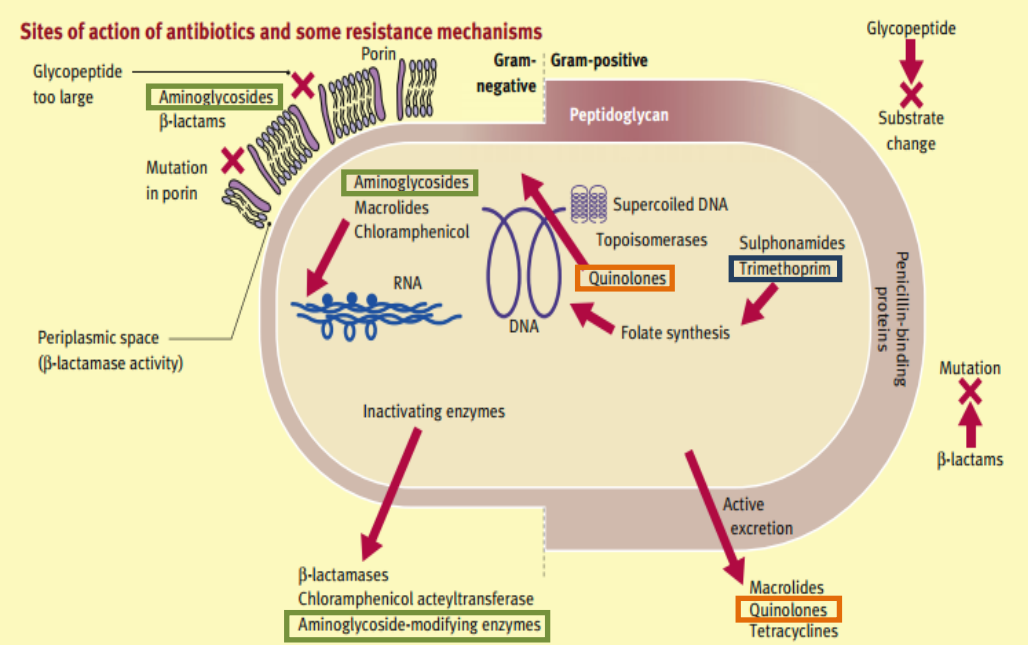



**Figure 2.1:** Mechanism action of antibiotics and resistance mechanisms on aminoglycosides, fluoroquinolone and folate pathway inhibitor (Scott 2009, p.553).

## 2.3 Impact of antibiotics on normal intestinal microbiota composition

The ecological balance between normal flora and pathogenic microorganisms will be affected by the administration of antibiotics. The impact of antibiotic administration is explained by Jernberg et al. (2010, Figure 2.2). In healthy individual, human gastrointestinal tract is colonized by normal flora and the number of resistant, pathogenic bacteria is below the detection threshold. Upon the initiation of antibiotic treatment, the number of antibiotic resistant strains (represented by purple rods) increases drastically as they are able to survive in the presence of antibiotic pressure. Most of the susceptible enterobacteria (represented by green rods) are eliminated during the antibiotics. As a result of the antibiotic treatment, the susceptible intestinal bacteria may acquire resistance from horizontal gene transfer or mutational events. This ecological imbalance among the enteric bacteria confers to both short-term and long-term impacts on the human host.

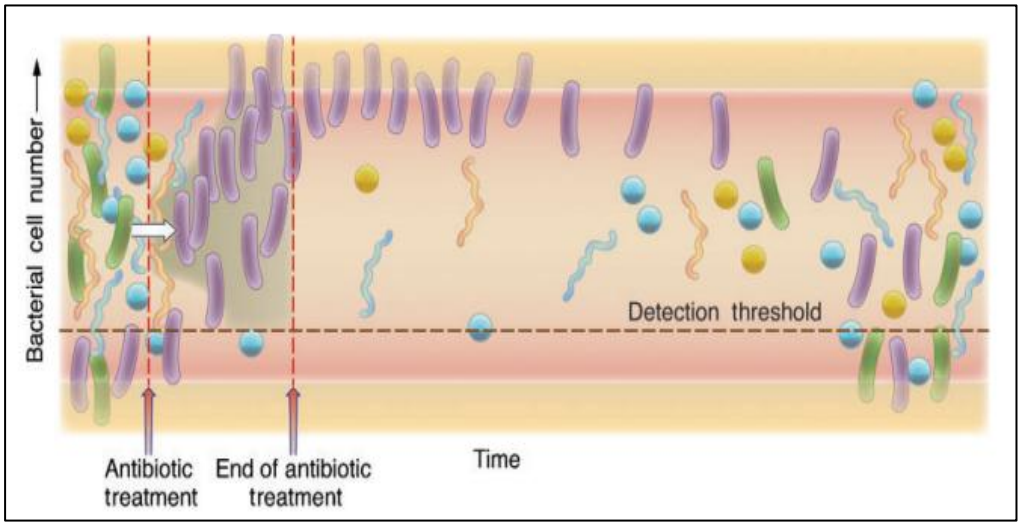



**Figure 2.2:** Representation of the impact of antibiotic administration on the bacteria community of the colon (Jernberg et al., 2010, p. 3217).

As explained by Sullivan et al. (2001), the short-term impacts of antibiotic administration were manifested as diarrhea and fungal infections. Administration of antibiotics may results in the emergence of antibiotic resistance in other intestinal bacteria, and gives rise to long-term impacts on the patient. As studied by Sjölund et al. (2003), antibacterial agents (clarithromycin, metronidazole and omeprazole) used for *Helicaobacter pylori* infection resulted in the emergence of clarithromycin-resistant enterococci. In addition, Nyberg et al. (2007) also reported an increase in clindamycin-resistant *E. coli* after the administration of the antibiotic. Similar study from Lindgren et al. (2009) also mentioned that erythromycin- and clindamycin-resistant *Enterococcus* sp. persisted for 9 months as a consequence of 7-day clindamycin course. Thus, the antibiotics course may results in the undesirable impacts on intestinal flora, eventually leading to the increase level of antibiotic resistance in other intestinal bacteria.

### 2.3.1 Losing Antibiotic Resistance

Several studies that were conducted in community setting showed promising findings, in which the reduction of antibiotic prescription managed to decrease the resistance rate to the antibiotic. The resistance rate of *Streptococcus pyogenes* abated from 19% to 8.6% between 1993 and 1996, as a result of 63% reduction in the administration of macrolide (Seppala et al., 1997). Study from Enne et al. (1999) revealed that reduction in overall antibiotics usage over 3-year time resulted in 25% reduction of penicillin resistance in *Streptococcus pneumoniae*.

Notwithstanding the successful findings, restriction in antibiotic prescription does not always cause a decrease in the resistance rate. 97% reduction in the prescription of sulphonamide-containing antimicrobials in UK conferred to zero change on the resistance rate in *E. coli* between 1991 and 1999 (Enne et al., 2001). A recent 24-month study conducted in Swedish concluded that 85% cutback in trimethoprim from 2004 to 2006 did not reverse the resistance in *E. coli* (Sundqvist et al., 2010).

Fitness cost and compensatory mutations are factors that influence the persistence of antibiotic resistance, even though the antibiotic consumption is discontinued (Levin, 2001; Andersson and Hughes 2011). In terms of community studies, co-selection is an important factor that leads to the persistence of resistant bacteria (Courvalin and Trieu-Cuot 2001; Martinez

2009; Andersson and Hughes 2010; Andersson and Hughes 2011). Resistance gene to a particular antibiotic is often linked to genes that confer resistance to other antibiotics and toxic metals. Thus, the use of any of the compounds will co-select other resistance mechanisms as well (Courvalin and Trieu-Cuot 2001; Martiniz 2009; Andersson and Hughes 2010). As shown in Figure 2.3, the antibiotic resistant genes (*r*) is genetically linked to heavy metal determinants (*b*) and ecologically rewarding elements (*e*). In pathway B, plasmid-encoded antitioxin (A) is produced to prevent the bacterial killing by toxin (T). Thus, co-selection may favor the expression of antibiotic resistance even in the absence of antibiotics (Martinez 2009).

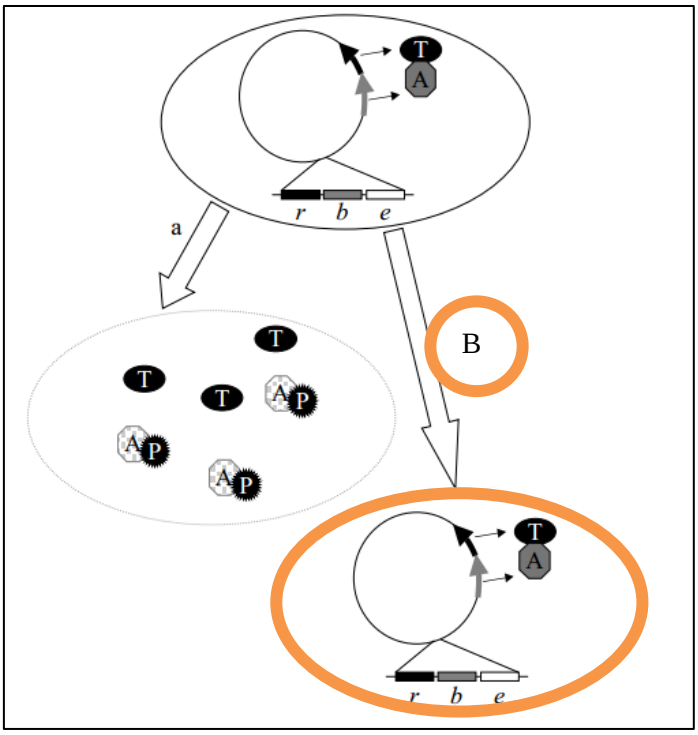



**Figure 2.3:** Cluster of antibiotic resistance genes (b) with other genetic elements (Martinez 2009, p. 2526).

### 2.3.2 Induction of antibiotic resistance

The association of antibiotic usage and emergence of antibiotic resistance was postulated by Gallini et al. (2010). This study relates the ciprofloxacin-resistant *E. coli* in nosocomial setting to the administration of fluoroquinolone in hospital and community. Indeed, their study was in line with the finding from Vellinga et al. (2010). As shown in Figure 2.4, greater prescription of ciprofloxacin and trimethoprim in general practice increases the risk of infection caused by resistant strains of *E. coli* (Vellinga et al., 2010).

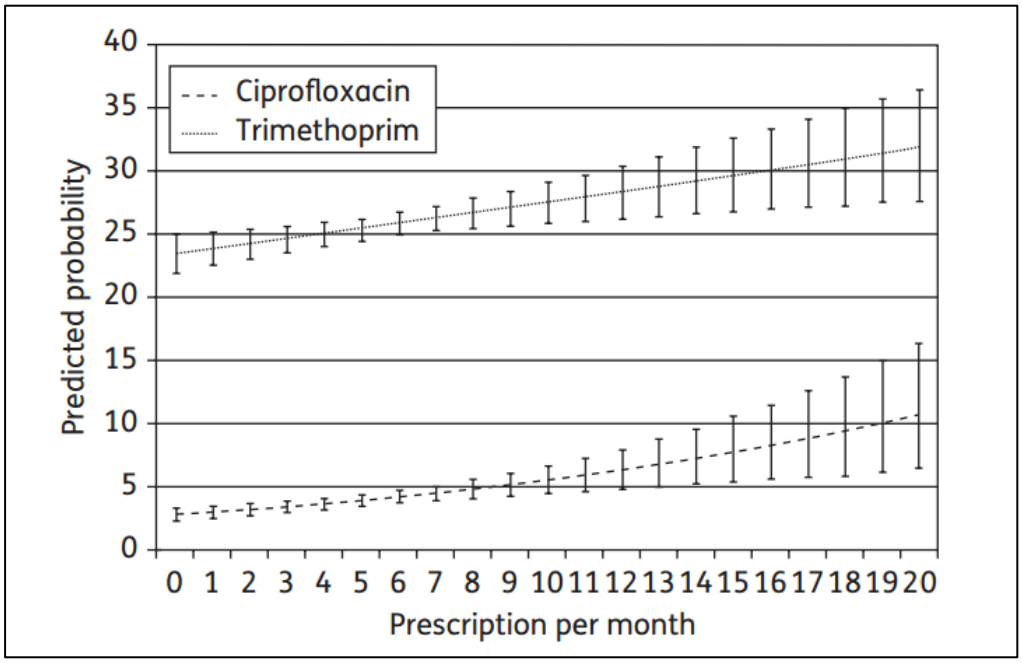



**Figure 2.4**: Predicted probability of resistance due to increase in prescription of ciprofloxacin and trimethoprim (Vellinga et al., 2010, p. 1518).

Another study carried out by Vellinga et al. (2012) suggested that the consumption of ciprofloxacin increases the prevalence of infections by extended spectrum beta-lactamase (ESBL) producing *E. coli*. In addition,

bacteremia caused by fluoroquinolone-resistant *E. coli* is steered by the usage of moxifloxacin and levofloxacin in community settings (Cuevas et al., 2011). Overall, the administration of antibiotics in nosocomial and community settings may induce the antibiotic resistance in pathogenic microorganism.

### 2.3.3 Transfer of antibiotic resistance

Antibiotic resistant genes can be transferred from the resistant, donor bacteria to susceptible, recipient bacteria via horizontal gene transfer (HGT) (Kelly, Vespermann and Bolton 2009a). The antibiotic resistance genes may involve in efflux pump, alteration of target molecules, degradation of antibiotic molecules, etc. (Andersson and Hughes 2010). As shown in Figure 2.5, the mechanisms for horizontal gene transfer include: conjugation, transformation and transduction. Conjugative gene transfer involves the cell-to-cell communication between the donor bacteria and recipient bacteria, whereby the genetic materials are transferred via direct contact and sex pili (Thomas and Nielsen 2005; Scott 2009). Next, transformation refers to the uptake of extracellular genetic materials when the bacteria are in normal physiological state (Thomas and Nielsen 2005). Then, transduction is mediated by bacteriophage which transfers the virulence gene between bacteria (Kelly, Vespermann and Bolton 2009b). Mutations that occurred either in the chromosome or plasmid may result in the acquisition of antibiotic resistance (Andersson and Hughes 2010). Quinolone resistance that caused by point mutation on the genes encode for DNA gyrase and topoisomerase is one of the examples for chromosomal mutation (Guan et al., 2013).

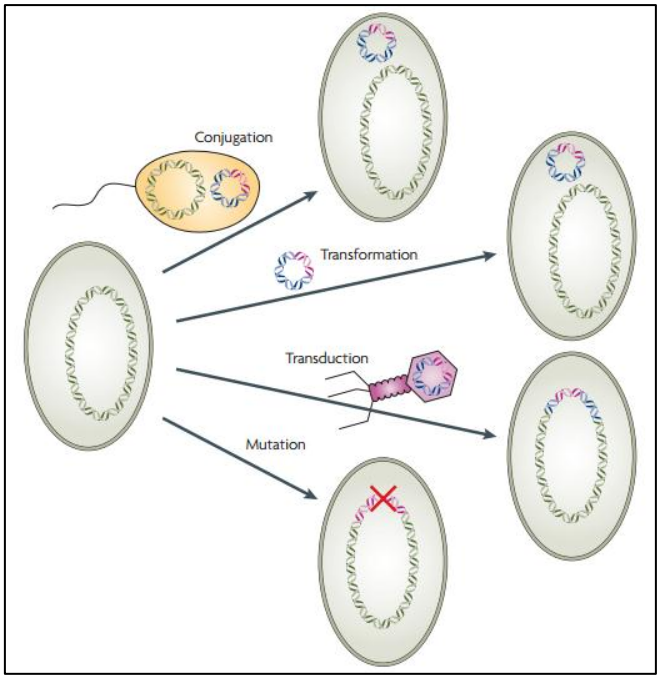



**Figure 2.5:** Mechanisms of horizontal gene transfer (HGT) (Andersson and Hughes 2010, p. 201).

Plasmids and conjugative transposons are examples of mobile genetic elements that can be transferred from one bacterial to another (Hooper, 2001; Bennett 2008; Scott 2009; van Hoek et al., 2011). Plasmid-carried genes play important role in bacteria survival, and some may confer to the resistance to cephalosporins, fluroquinolones and aminoglycosides (Bennett 2008). These plasmids are subjected to conjugative transfer among broad range of hosts, in which transmission among different species of bacteria is possible (Bennett 2008; van Hoek et al., 2011). Transposon, as defined by Scott (2008), refers to the large genetic elements that carry multiple resistant genes, and encodes for pheromones which promote the conjugative gene transfer among the bacteria. Examples of transposons include Tn*5* that encodes aminoglycoside resistance, Tn*10* that encodes for tetracycline resistance and Tn*3* that encodes for resistance to β-lactam antibiotics are prevalently found in Enterobacteriaceae (Bennett 2008).

In real, human gastrointestinal (GI) tract act as the habitat for the diverse community of enteric bacteria, which serve as reservoirs of antibiotic resistance (ROAR) (Schjørring and Krogfelt 2010; Vespermann and Bolton 2009). Reservoir hypothesis as shown in Figure 2.6 suggested that the bacteria in colon are capable of acquiring antibiotic resistance genes, and possibly transfer the resistant gene to other intestinal bacteria and transient bacteria in the gut (Salyers et al. 2004; Salyers et al. 2007; Kelly, Vespermann and Bolton 2009). The transfer of antibiotic resistance occurs mostly via conjugation between the intestinal bacteria, whereas gene transfer by bacteriophage transduction and transformation are most likely to occur between members of the same species.

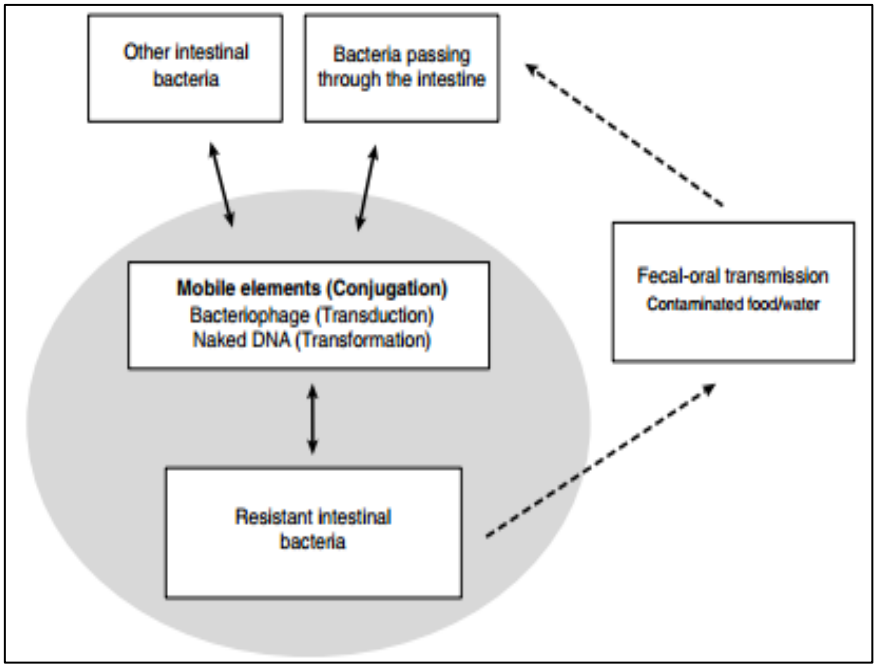



**Figure 2.6:** The reservoir hypothesis. (Salyers et al., 2007, p. 18)

## 2.4 Clinical Significance in Assessment of Antibiotics Resistance

Discovery of antimicrobial drugs provide solutions for life-threatening bacterial infections that are critical in intensive care unit (ICU) (Lim and Webb 2005; Fraimow and Tsigrelis 2011), surgical procedures (Carlet et al., 2012) and also the management for the cancer patients and patients that received organ transplantation (Livermore 2012). However, the emergence of drug resistant bacteria have cause the antibiotic pipeline to run dry as some of the antibiotics are no long active against these resistant bacteria (Gould 2009; Carlet et al., 2012). Moreover, over-the-counter (OTC) prescription of antibiotics in the third world countries even worsens the current scenario (Morgan, Okeke and Laxminarayan 2011). The antibiotic treatment without prescription will not give the optimal potency and thus create an optimal antibiotic pressure that may induce the antibiotic resistance in commensal flora (Bisht et al., 2009; Andersson and Hughes 2012).

Most of the susceptible flora are killed in the traditional antibiotic dosing approach, leading to the selection of antibiotic resistant bacteria (Zhao and Drlica 2008). Thus, the dosing regimen should be optimized and the selected drug used for treatment should be prescribed accordingly (Geoff, Bauer and Mangino 2012). Indeed, this preliminary project may provide an overview on the induction of antibiotic resistance, particularly in *E. coli* and *K. pneumoniae* using antibiotic with sub-lethal concentration.

In real, one of the major factors that lead to the emergence of antibiotic resistance is the patient attitude on the antibiotic prescription (Bisht et al., 2009). As mentioned by the survey conducted by McNulty et al. (2007), 11.3% of the respondent failed to complete the entire course of antibiotic therapy, and some even recycle the "left-over" antibiotics. The discontinued of antibiotics consumption and delay in between the antibiotics intake will reduce the antibiotic concentration in the body to less than the minimum inhibitory concentration (MIC), which is insufficient to eradicate the pathogenic bacteria in the body (Jackson et al., 2006). This amount of antibiotics will not only favor the selection of antibiotic resistant bacteria, but also promote the antibiotic resistance transfer from the pathogenic microorganisms to the susceptible intestinal flora (Andersson and Hughes 2012).

## 2.5 Methods used in the Study of Antibiotic Resistance

Approaches like dissemination and reversal of antibiotic resistance are mostly conducted in community settings. As elaborated in Section 2.3.1, this field of research was studied by Seppala et al. (1997), Enne et al. (1999), Enne et al. (2001), Sundqvist et al. (2010).

Schjørring and Krogfelt (2010) mentioned that both *in vivo* and *in vitro* methods are used to study the various aspects of antibiotic resistance on the gut flora. Some of the examples of the *in vivo* methods include the antibiotic-treated mouse model (Freter 1989) and human microbiota-associated rodent (HMA) model (Hirayama 1999). Indeed, *in vitro* studies which are conducted in liquid media and on agar plates stand an advantage. This is because parameters such as temperature, type and amount of media, incubation time and selective pressure. that are involved in the assessment of antibiotic resistance can be closely monitored in laboratory settings (Schjørring and Krogfelt 2010). Moreover, antibiotic susceptibility profiles and phenotypic characteristics of the bacteria can be evaluated using bacteria cultures (Jernberg et al., 2010). Nevertheless, current *in vitro* models are still less established in relative to *in vivo* models. Thus, this reflects the importance of this study in assessing the aspects of antibiotic resistance using *in vitro* methods.

# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 Materials

### 3.1.1 Bacteria strains

In this study, the clinical strains of *Escherichia coli* (Lab Number: 594370) and *Klebsiella pneumoniae* (Lab Number: 594394) were collected from Gleneagles Medical Center, Penang. *E. coli* 594370 was resistant to ciprofloxacin, gentamicin, moxifloxacin and trimethoprim. Whereas, *K. pneumoniae* 594394 showed susceptibility to these antibiotics.

On the other hand, the ATCC bacteria strains were obtained from Department of Biomedical Science, University of Tunku Abdul Rahman. The ATCC strains were *Escherichia coli* (ATCC 25922) and *Klebsiella pneumoniae* (ATCC 13883). These bacteria were susceptible to trimethoprim.

### 3.1.2 Chemicals and media used

The preparation of agar and media that were used in this study are listed in Appendix A.

### 3.1.3 Equipment and lab wares used

The equipment and lab wares that were used in this study are listed in Appendix B.

## 3.2 Methodology

### 3.2.1 Preparation of bacteria stock and master plate

Each of the bacteria strain was plated on both MacConkey agar and EMB agar. After overnight incubation at 37 ˚C, these master cultures were kept at 4˚C. 80% glycerol stock for each bacteria sample were prepared and preserved at -80 ˚C. This storage condition allowed long-term preservation of bacteria.

### 3.2.2 Assessment of the losing of antibiotic resistance

Resistant strain of *E. coli* 594370 was inoculated into 20 ml of MH broth and cultured at 37 ˚C with agitation at the speed of 200 rpm. After 4.5 hour incubation, 200 µL of the bacteria culture was serially passaged into 20 ml of fresh MH broth and cultured continually with the previous growing condition. The antibiotics susceptibility profile of this bacteria was monitored constantly every 5 passages.

### 3.2.3 Induction of trimethoprim resistance in *Escherichia coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 13883

*E. coli* ATCC 25922 was serially passaged in the presence of trimethoprim at the concentration of 12 µg/mL. The preparation of this antibiotic solution was shown in Appendix C. The viability of the bacteria was closely monitored by culturing the overnight bacteria on both EMB agar and MacConkey agar. In addition, the antibiotic susceptibility of the bacteria was assessed in every 5 passages. Same procedure was carried on *K. pneumoniae* ATCC 13883 as well.

### 3.2.4 Assessment of the transfer of antibiotic resistance

Resistant strain of *E. coli* 594370 and sensitive strain of *K. pneumoniae* 594394 were inoculated into 20 ml MH broth. The bacteria mixture was co-incubated at 37 ˚C with agitation at the speed of 200 rpm. After 5 hours of incubation, 200 µL of the bacteria solution was serially passaged into 20 ml MH broth and cultured continuously with the previous growing condition. The antibiotics susceptibility profile of *K. pneumoniae* 594394 was monitored constantly every 5 passages.

## 3.3 Antibiotic susceptibility testing

The susceptibility of each batch of bacteria was carried out by Kirby-Bauer (KB) method. Antibiotics that were used in the testing were ciprofloxacin (CIP, 5 µg), gentamicin (CN, 10 µg), moxifloxacin (MXF, 5 µg) and trimethoprim (W, 5 µg). The preparation of trimethorprim disks are mentioned in Appendix C. 3 to 5 colonies with identical morphology were isolated and suspended into 0.87% saline. The turbidity of the inoculum was adjusted to 0.5 MacFarland. Within 15 minutes, a sterile cotton swab was then used to streak the bacteria suspension over the MH agar. Next, antibiotics disks were distributed evenly on the MH agar. Within 15 minutes after placing the antibiotic disks, the plates were incubated at 37 ˚C for 12 to 14 hours.

The reference breakpoints for the zone diameter of inhibition for ciprofloxacin, gentamicin and trimethoprim are available in Clinical and Laboratory

Standards Institute (CLSI, 2007). On the other hand, the reference range for inhibition diameter of moxifloxacin is available in the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2013). In this study, *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 13883 were used as reference strains and served as quality control for the susceptibility testing. The reference ranges for ciprofloxacin, gentamicin, moxifloxacin and trimethoprim were shown in Table 3.1.

**Table 3.1:** Reference ranges of ciprofloxacin, gentamicin, moxifloxacin and trimethoprim.

| **Antimicrobial Agent** | **Zone Diameter, Nearest Whole mm** | | |
|---|---|---|---|
| | **R** | **I** | **S** |
| Ciprofloxacin | ≤ 15 | 16 -20 | ≥ 21 |
| Gentamicin | ≤ 12 | 13 - 14 | ≥ 15 |
| Trimethoprim | ≤ 10 | 11 -15 | ≥ 16 |
| Moxifloxacin | < 17 | - | ≥ 20 |

# CHAPTER 4

# RESULTS

## 4.1 Losing of antibiotic resistance in *Escherichia coli* 594370

The antibiotic susceptibility of clinical isolate of *E. coli* 594370 to ciprofloxacin, gentamicin, moxifloxacin and trimethoprim was assessed in every 5 passages. The ZI diameter measured that recorded as "0" referred to the absence of zone-of-inhibition, as shown in Figure 4.11. The measurement for the diameter of ZI was duplicated, and both of the readings were tabulated accordingly in Appendix D. There was no noticeable increase in the diameter of ZI of ciprofloxacin, gentamicin, moxifloxacin and trimethoprim, as referred to Table 4.1, Table 4.2, Table 4.3 and Table 4.4 respectively. Based on Table 4.4, the resistant pattern to trimethoprim was relatively constant, as there was no inhibition zone observed around the antibiotic disk throughout the 85 passages. Overall, the bacteria were resistant to ciprofloxacin, gentamicin, moxifloxacin and trimethoprim after the 85 passages.

The trends of antibiotic susceptibility of ciprofloxacin, gentamicin, moxifloxacin and trimethoprim throughout the 85 passages were tabulated in line graph as shown in Figure 4.1, Figure 4.4, Figure 4.7 and Figure 4.10 respectively. Over the 85 passages, there were minor fluctuations in the resistance pattern of ciprofloxacin, gentamicin and moxifloxacin as observed

in Figure 4.1, Figure 4.4 and Figure 4.7 respectively. On the other hand, the resistance trend to trimethoprim as shown in Figure 4.10 remained constant throughout the 85 passages in which there was no increase in the diameter of ZI. The diameter of zone-of-inhibition (ZI) on ciprofloxacin was showed in Figure 4.2 and Figure 4.3. Next, Figure 4.5 and Figure 4.6 showed the diameter of zone-of-inhibition (ZI) on gentamicin. The diameter of zone-of-inhibition (ZI) on moxifloxacin was showed in Figure 4.8 and Figure 4.9.

**Table 4.1:** Diameter of zone-of-inhibition (ZI) of ciprofloxacin in *E. coli* 594370.

| Number of Passages | Average reading on the diameter of inhibition zone (mm) | Indication |
|---|---|---|
| 0 (Initial ASP) | 8.00 | Resistant |
| 5 | 8.50 | Resistant |
| 10 | 10.00 | Resistant |
| 15 | 8.50 | Resistant |
| 20 | 8.50 | Resistant |
| 25 | 8.50 | Resistant |
| 30 | 8.50 | Resistant |
| 35 | 7.75 | Resistant |
| 40 | 9.00 | Resistant |
| 45 | 0 | Resistant |
| 50 | 8.25 | Resistant |
| 55 | 8.50 | Resistant |
| 60 | 0 | Resistant |
| 65 | 0 | Resistant |
| 70 | 9.25 | Resistant |
| 75 | 0 | Resistant |
| 80 | 9.50 | Resistant |
| 85 | 8.50 | Resistant |

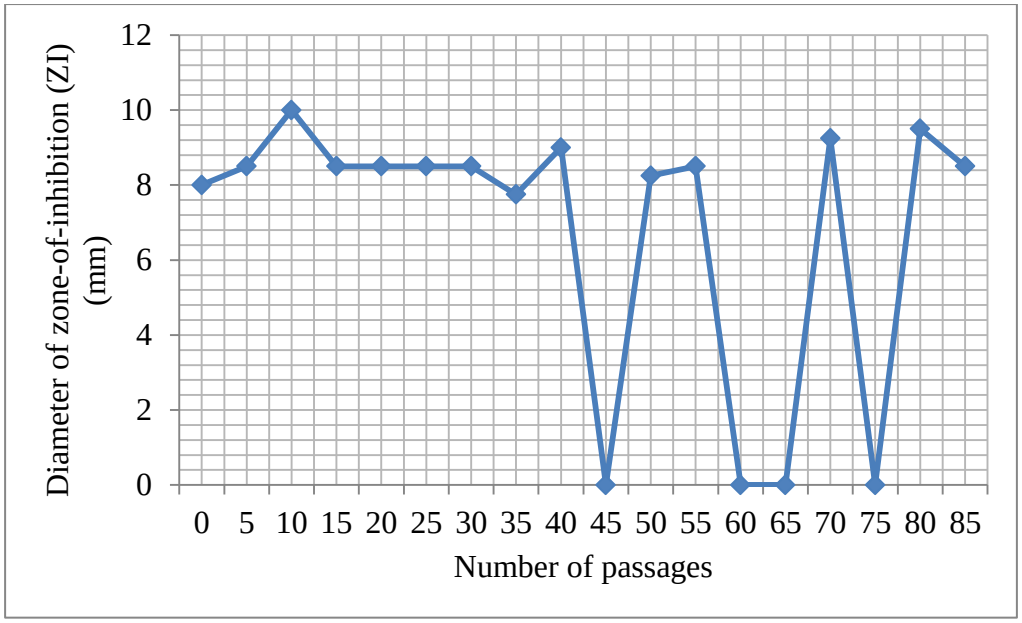



**Figure 4.1:** Trends of antibiotic susceptibility trends in *E. coli* 594370 to ciprofloxacin.

**Figure 4.2:** Diameter of zone-of-inihibition (ZI) on ciprofloxacin at Passage 0.

**Figure 4.3:** Diameter of zone-of-inihibition (ZI) on ciprofloxacin at Passage 85.

**Table 4.2:** Diameter of zone-of-inhibition (ZI) of gentamicin in *E. coli* 594370.

| Number of Passages | Average reading on the diameter of inhibition zone (mm) | Indication |
|---|---|---|
| 0 (Initial ASP) | 8.50 | Resistant |
| 5 | 9.00 | Resistant |
| 10 | 9.00 | Resistant |
| 15 | 8.00 | Resistant |
| 20 | 0 | Resistant |
| 25 | 7.75 | Resistant |
| 30 | 8.25 | Resistant |
| 35 | 7.75 | Resistant |
| 40 | 8.00 | Resistant |
| 45 | 7.25 | Resistant |
| 50 | 7.25 | Resistant |
| 55 | 0 | Resistant |
| 60 | 0 | Resistant |
| 65 | 0 | Resistant |
| 70 | 10.00 | Resistant |
| 75 | 7.50 | Resistant |
| 80 | 10.00 | Resistant |
| 85 | 7.00 | Resistant |

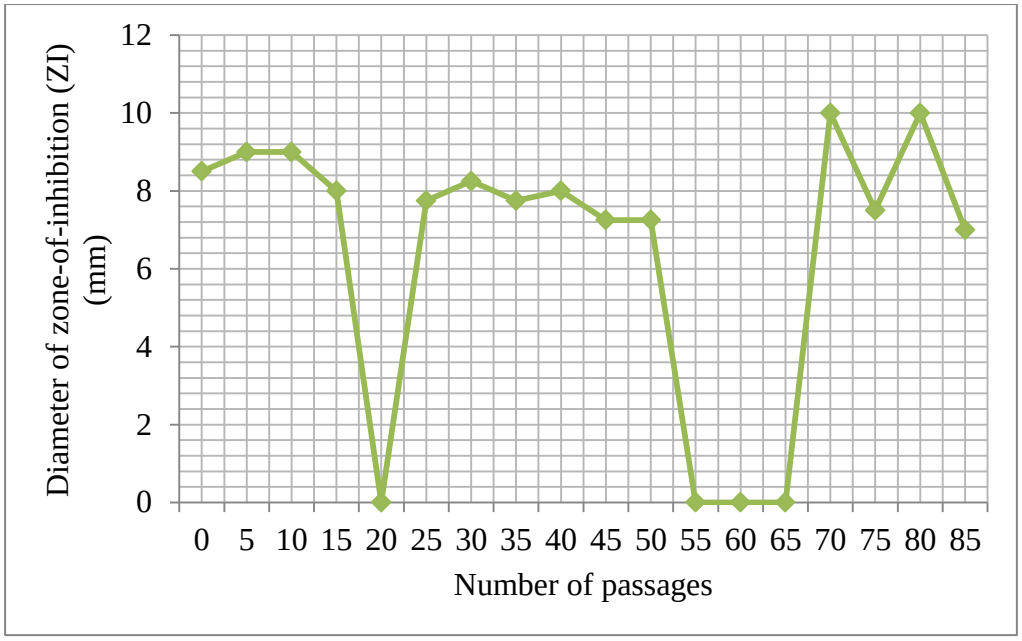



**Figure 4.4:** Trends of antibiotic susceptibility trends of *E. coli* 594370 to gentamicin.

**Figure 4.5**: Diameter of zone-of-inihibition (ZI) on gentamicin at Passage 0.


**Figure 4.6**: Diameter of zone-of-inihibition (ZI) on gentamicin at Passage 85.

**Table 4.3:** Diameter of zone-of-inhibition (ZI) of moxifloxacin in *E. coli* 594370.

| Number of passages | Average reading on the diameter of inhibition zone (mm) | Indication |
|---|---|---|
| 0 (Initial ASP) | 9.50 | Resistant |
| 5 | 10.75 | Resistant |
| 10 | 12.00 | Resistant |
| 15 | 9.75 | Resistant |
| 20 | 8.75 | Resistant |
| 25 | 9.50 | Resistant |
| 30 | 10.25 | Resistant |
| 35 | 9.75 | Resistant |
| 40 | 9.50 | Resistant |
| 45 | 8.75 | Resistant |
| 50 | 9.00 | Resistant |
| 55 | 7.75 | Resistant |
| 60 | 8.25 | Resistant |
| 65 | 8.25 | Resistant |
| 70 | 9.50 | Resistant |
| 75 | 8.50 | Resistant |
| 80 | 9.50 | Resistant |
| 85 | 9.25 | Resistant |

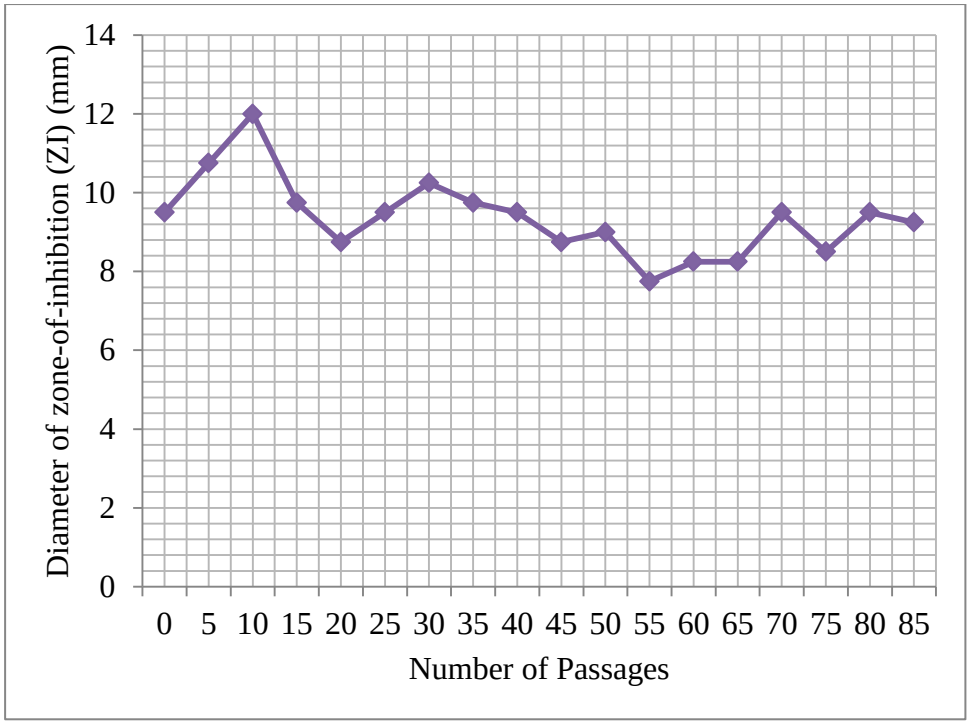



**Figure 4.7:** Trends of antibiotic susceptibility trends of *E. coli* 594370 to moxifloxacin.

**Figure 4.8:** Diameter of zone-of-inihibition (ZI) on moxifloxacin at Passage 0.

**Figure 4.9:** Diameter of zone-of-inihibition (ZI) on moxifloxacin at Passage 85.

**Table 4.4:** Diameter of zone-of-inhibition (ZI) of trimethoprim in *E. coli* 594370.

| Number of passages | Average reading on the diameter of inhibition zone (mm) | Indication |
|---|---|---|
| 0 (Initial ASP) | 0 | Resistant |
| 5 | 0 | Resistant |
| 10 | 0 | Resistant |
| 15 | 0 | Resistant |
| 20 | 0 | Resistant |
| 25 | 0 | Resistant |
| 30 | 0 | Resistant |
| 35 | 0 | Resistant |
| 40 | 0 | Resistant |
| 45 | 0 | Resistant |
| 50 | 0 | Resistant |
| 55 | 0 | Resistant |
| 60 | 0 | Resistant |
| 65 | 0 | Resistant |
| 70 | 0 | Resistant |
| 75 | 0 | Resistant |
| 80 | 0 | Resistant |
| 85 | 0 | Resistant |

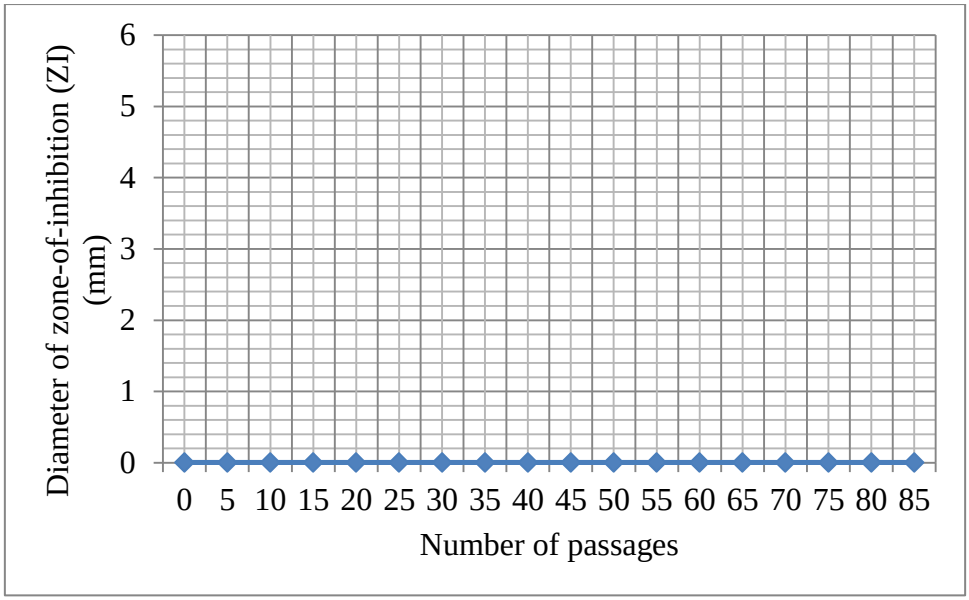



**Figure 4.10:** Trends of antibiotic susceptibility trends of *E. coli* 594370 to trimethoprim.

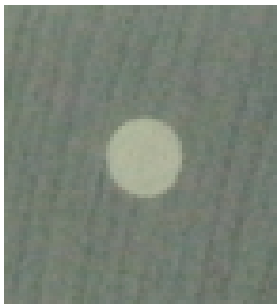


**Figure 4.11:** Absence of zone-of-inhibition.

## 4.2 Induction of Trimethoprim Resistance in *Escherichia coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 13883

Antibiotic susceptibility of *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 13883 to ciprofloxacin, gentamicin and moxifloxacin was assessed after incubation in the presence of 12 μg/ml trimethoprim. The diameter of ZI of trimethoprim on *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 13883 was tabulated in Table 4.5 and Table 4.6 respectively. Based on Table 4.5, the diameter of ZI on *E. coli* ATCC 25922 reduced after 15 passages. Based on Figure 4.12, gradual reduction in the diameter of ZI throughout 15 passages showed that the susceptibility of *E. coli* ATCC 25922 to trimethoprim reduced after serially passaged with 12 μg/ml trimethoprim.

On the other hand, the diameter of ZI on *K. pneumoniae* ATCC 13883 had decreased throughout the 15 passages (Table 4.6). The bacteria became resistant to trimethoprim at Passage 10 and there was no inhibition zone observed in Passage 15. Based on Figure 4.13, there was a drastic reduction in diameter of ZI between Passage 5 and Passage 10, followed by another substantial reduction in the diameter of ZI as observed in Passage 15. The diameter of ZI observed in Passage 0, Passage 5 and Passage 15 were clearly shown in Figure 4.14, Figure 4.15 and Figure 4.16 respectively.

**Table 4.5:** Diameter of zone-of-inhibition (ZI) of trimethorpim to *E. coli* ATCC 25922.

| Number of passages | Average reading on the diameter of inhibition zone (mm) | Indication |
|---|---|---|
| 0 | 35.00 | Susceptible |
| 5 | 29.75 | Susceptible |
| 10 | 26.50 | Susceptible |
| 15 | 25.75 | Susceptible |

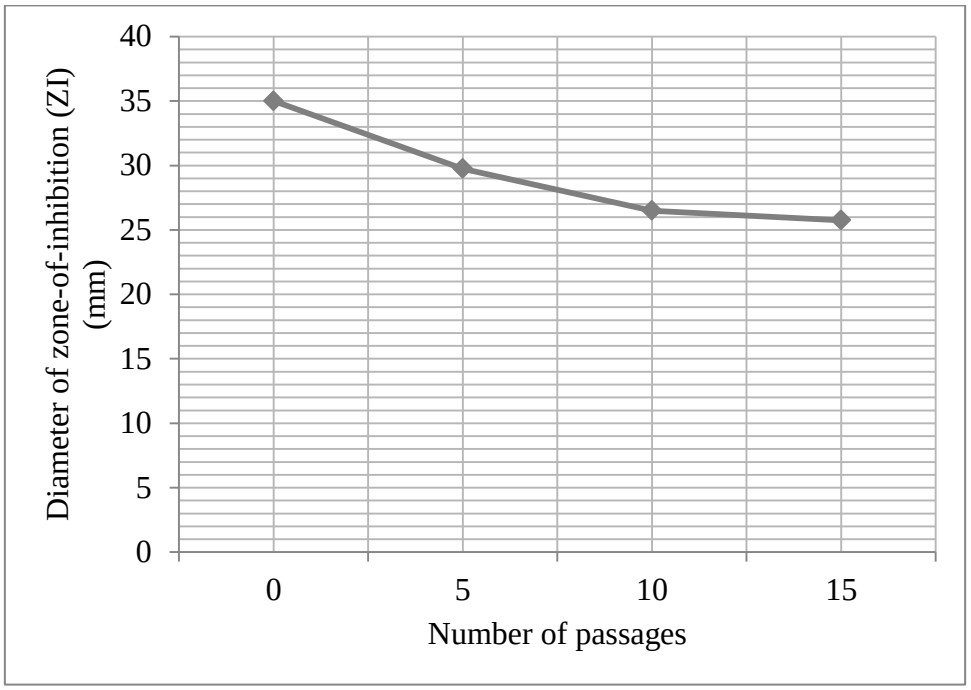



**Figure 4.12:** Trends of antibiotic susceptibility of *E. coli* ATCC 25922 to trimethoprim.

**Table 4.6:** Diameter of zone-of-inhibition (ZI) of trimethorpim to *K. pneumoniae* ATCC 13883.

| Number of passages | Average reading on the diameter of inhibition zone (mm) | Indication |
|---|---|---|
| 0 | 30.00 | Susceptible |
| 5 | 27.50 | Susceptible |
| 10 | 15.50 | Resistant |
| 15 | 0 | Resistant |

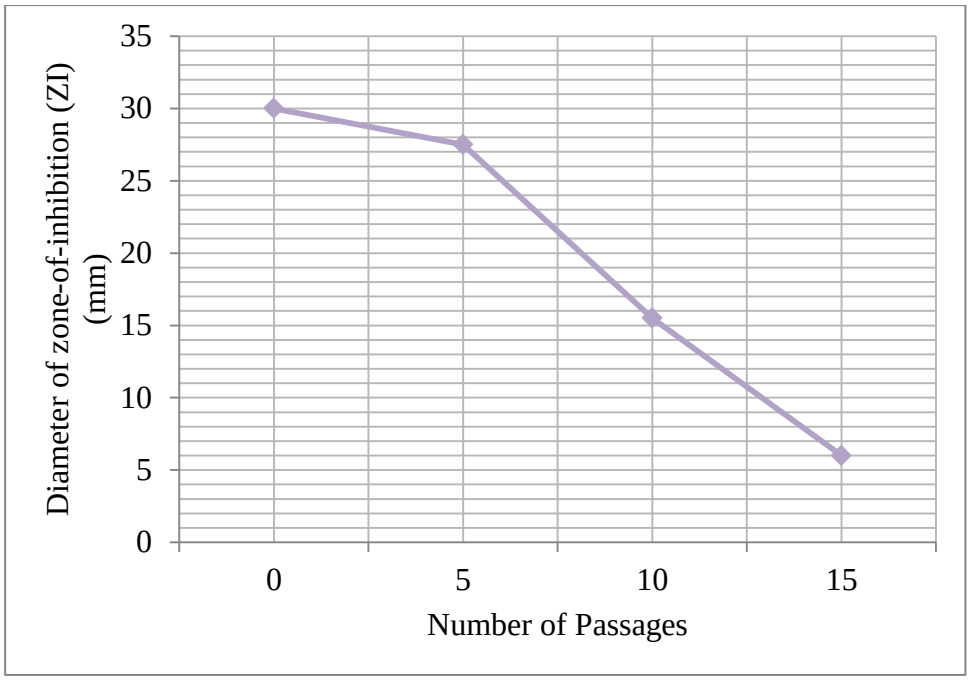



**Figure 4.13:** Trends of susceptibility of *K. pneumoniae* ATCC 13883 to trimethoprim .

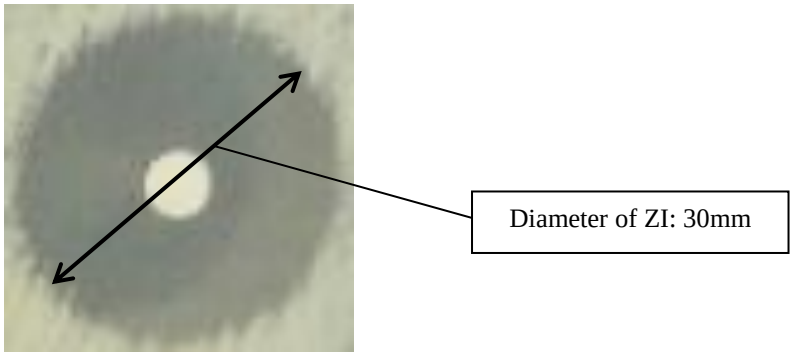



**Figure 4.14:** Zone-of-inhibition (ZI) on *K. pneumoniae* ATCC 13883 at Passage 0 (Disk content: 5 µg).

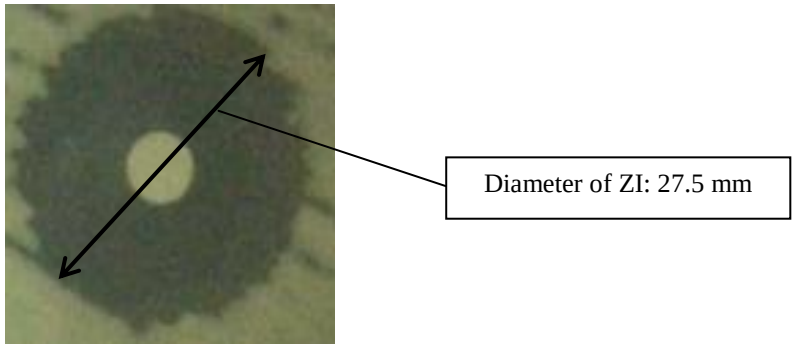



**Figure 4.15:** Zone-of-inhibition (ZI) on *K. pneumoniae* ATCC 13883 at Passage 5 (Disk content: 5 µg).

**Figure 4.16:** Zone-of-inhibition (ZI) on *K. pneumoniae* ATCC 13883 at Passage 15 (Disk content: 5 µg).

## 4.3 Transfer of antibiotic resistance from *Escherichia coli* 594370 to *Klebsiella pneumoniae* 594394

Susceptible, clinical isolate *K. pneumoniae* 594394 that served as the recipient bacteria was co-incubated with resistant, clinical isolate *E. coli* 594370 that served as the donor bacteria. The diameters of ZI of ciprofloxacin, gentamicin and moxifloxacin that were measured on the recipient bacteria throughout the 30 passages were tabulated in Table 4.7, Table 4.8 and Table 4.9, respectively. Based on Table 4.7, ciprofloxacin susceptibility of the recipient bacteria interchanged between "Intermediate" and "Susceptible" after co-incubation with donor bacteria for 30 passages. The recipient bacteria remained susceptible to gentamicin and intermediate to moxifloxacin (Table 4.8 and Table 4.9). Based on Figure 4.17, there were minor fluctuations in the ZI diameter measured and this indicated that there was little or no gain of antibiotics resistance throughout the 30 passages of co-incubation.

**Table 4.7:** Diameter of zone-of-inhibition (ZI) of ciprofloxacin to clinical isolate *K. pneumoniae* 594394.

| Number of passages | Average reading on the diameter of inhibition zone (mm) | Indication |
|---|---|---|
| 0 | 21.00 | Susceptible |
| 5 | 17.50 | Intermediate |
| 10 | 19.50 | Intermediate |
| 15 | 21.25 | Susceptible |
| 20 | 20.00 | Intermediate |
| 25 | 21.50 | Susceptible |
| 30 | 22.00 | Susceptible |

**Table 4.8:** Diameter of zone-of-inhibition (ZI) of gentamicin to clinical isolate *K. pneumoniae* 594394.

| Number of passages | Average reading on the diameter of inhibition zone (mm) | Indication |
|---|---|---|
| 0 | 19.00 | Susceptible |
| 5 | 19.25 | Susceptible |
| 10 | 18.50 | Susceptible |
| 15 | 18.00 | Susceptible |
| 20 | 19.00 | Susceptible |
| 25 | 19.00 | Susceptible |
| 30 | 18.50 | Susceptible |

**Table 4.9:** Diameter of zone-of-inhibition (ZI) of moxifloxacin to clinical isolate *K. pneumoniae* 594394.

| Number of passages | Average reading on the diameter of inhibition zone (mm) | Indications |
|---|---|---|
| 0 | 18.25 | Intermediate |
| 5 | 17.75 | Intermediate |
| 10 | 17.00 | Intermediate |
| 15 | 18.00 | Intermediate |
| 20 | 19.00 | Intermediate |
| 25 | 19.00 | Intermediate |
| 30 | 18.50 | Intermediate |

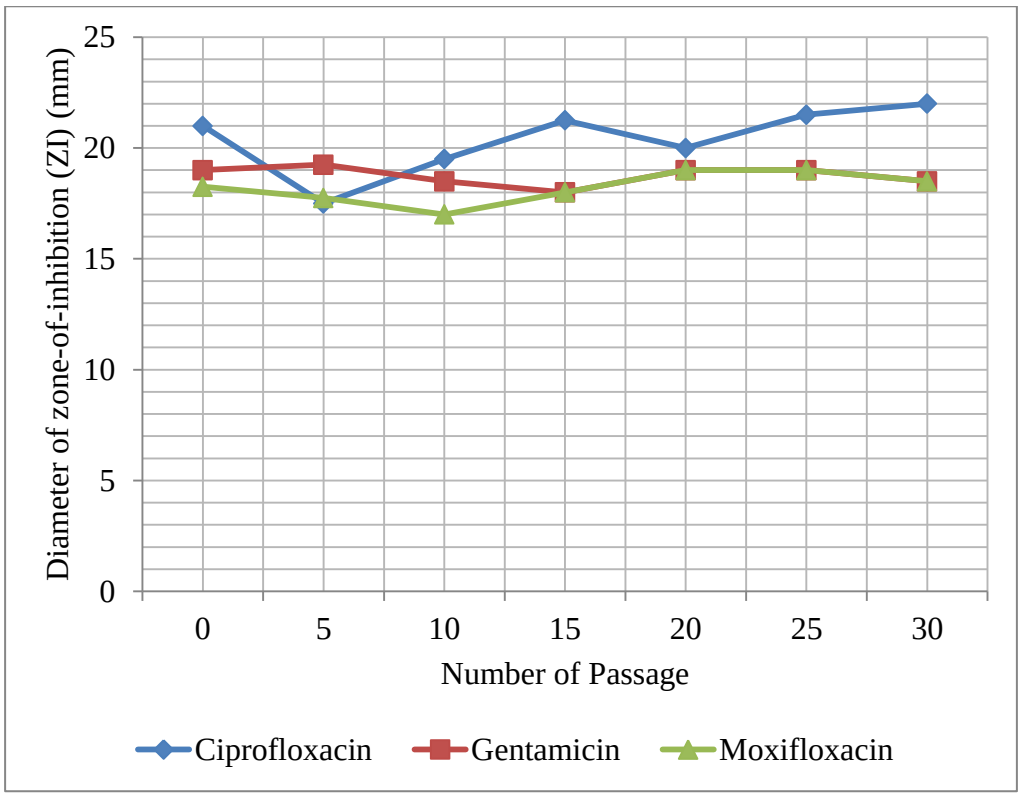



**Figure 4.17:** Trends of antibiotic susceptibility of clinical isolate *K. pneumoniae* 594394 to ciprofloxacin, gentamicin and moxifloxacin.

# CHAPTER 5

# DISCUSSION

## 5.1 Losing of antibiotic resistance

As shown in Section 4.1, there was no noticeable increase in the diameter of zone-of-inhibition (ZI) observed when ciprofloxacin, gentamicin, moxifloxacin and trimethoprim were tested on clinical isolate *E. coli* 594370. Reverse resistance of the bacteria to ciprofloxacin, gentamicin, moxifloxacin and trimethoprim was not observed throughout the 85 passages. Various factors may affect the losing of antibiotic resistance: number of passages, biological cost of resistance and type of resistance mutation. These factors are discussed in Section 5.1.1, Section 5.1.2 and Section 5.1.3.

### 5.1.1 Number of passages

More passages may be required for the assessment of losing antibiotic resistance. Courvalin and Triou-Cuot (2001) mentioned that it is possible to reverse antibiotic resistance but the reversibility occurs very slowly. This finding is also supported by the study from De Gelder et al. (2004) which reported that withdrawal of antibiotics pressure resulted in 6.9% increase in the fraction of tetracycline-sensitive bacteria population after 500 generations. Thus, clinical isolate of *E. coli* 594370 may possibly loss its resistance to ciprofloxacin, gentamicin, moxifloxacin and trimethoprim by increasing the

number of passages. In addition to *in vitro* experimental settings, epidemiological studies of antibiotic resistance in community setting also suggested that the reverse in antibiotic resistance occurs at very slow rate, which may take months or years even in the absence of antibiotic pressure (Sundqvist et al. 2009). Johnsen et al. (2009) also mentioned that there is no study that reported complete reversal of antibiotic resistance.

### 5.1.2 Biological cost of resistance

On the other hand, fitness cost is another factor that affects the loss of antibiotic resistance in resistant bacteria population. In normal condition, antibiotics that target on essential functions of bacterial cell will induce biological fitness cost (Guo et al., 2012; Andersson and Hughes 2010; Olosson and Cars 2007). For example, mutation in *rpoB* gene which encodes for β subunit of RNA polymerase confers to disruption in the rate of gene transcription (Reynolds 2000). Thus, resistant mutants with greater fitness cost are expected to be outcompeted by susceptible bacteria population in the absence of antibiotic pressure (Andersson and Hughes 2011).

As the cost of resistance increases, the time required to reduce the amount of resistant bacteria decreases, and vice versa (Andersson and Hughes 2010). Persistence of antibiotic resistance is attributed to the compensatory evolution that occurs throughout the serial passages of resistant bacteria (Andersson 2006). In fact, the fitness cost caused by the resistance mutations can be

reduced by compensatory evolution (Andersson and Hughes 2010). Types of compensatory mechanisms as mentioned by Maisnier-Patin and Andersson (2004) include restoration of structure and function of altered RNA or proteins by intragenic mutations; up regulation of gene expression, intergenic mutations in a multi-subunit molecule and bypass mechanism in which the mutated function is substituted by alternative pathway. Therefore, the fitness can be restored by reducing the need for the resistance protein and substitute the affected function of resistance protein with alternative function (Andersson and Hughes 2010). Thus, the persistence resistance in clinical isolate *E. coli* 594370 may be associated to the minimal fitness cost and/or the presence of compensatory evolution throughout the 85 passages.

### 5.1.3 Chromosomal drug resistance

Sander et al. (2002) mentioned that it is very difficult to remove the cost-neutral, chromosomal resistance mutations, as these mutations have very minimal fitness cost. Point mutation that occurs in quinolone resistance determining region (QRDR), *parC* and *parE* are examples for the chromosomal resistance mutations (Alekshun and Levy 2007; van Hoek et al., 2011; Guan et al., 2013). Thus, it will be difficult to reverse quinolone resistance caused by these chromosomal mutations. In this study, the resistance to ciprofloxacin and moxifloxacin in clinical isolate *E. coli* 594370 may not be reduced easily as these antibiotics are classified as second generation and third generation of quinolone, respectively (Oliphant and Green 2002).

## 5.2 Induction of antibiotic resistance in *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 13883

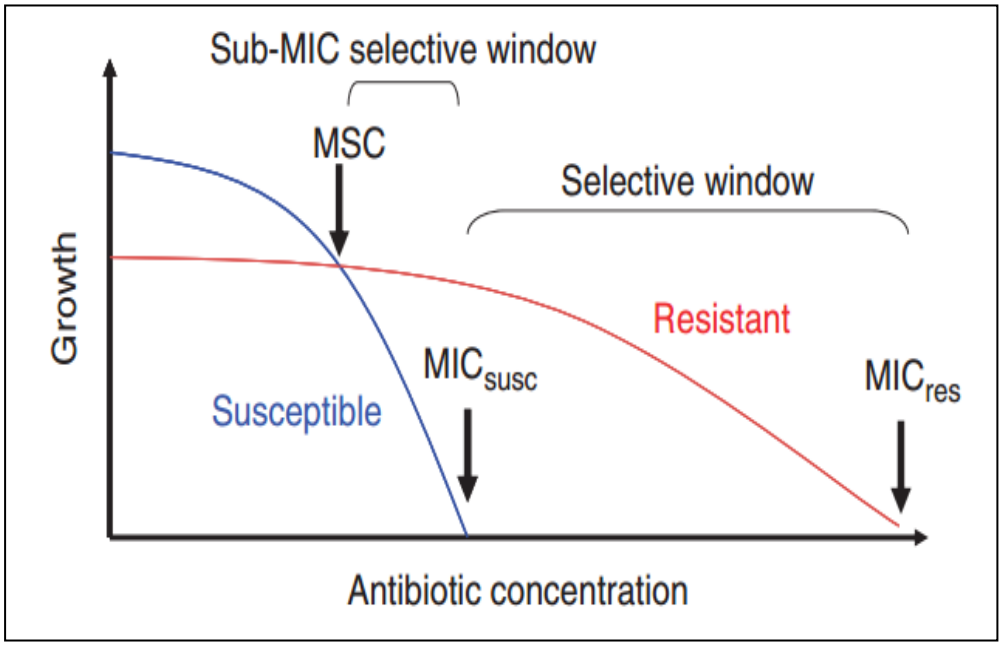
After 15 passages in the presence of 12 µg/ml trimethoprim, there was observable reduction in susceptibility to trimethoprim in both *K. pneumoniae* ATCC 13883 and *E. coli* ATCC 25922. This inducible phenotypic resistance was in line to the study from Drlica and Zhao (2007) and Andersson and Hughes (2011). Both of these studies indicated that resistant bacteria subpopulation will be selected in the presence of antibiotics with sub-lethal concentration. In addition, Courvalin and Trieu-Cuot (2001) also suggested that antibiotics exert the selective force for the dissemination of resistant bacteria. In the presence of antibiotic pressure, the resistant subpopulation with better survival will dominate the bacteria population (Drlica and Zhao 2008). This selection is further discussed below.

### 5.2.1 Traditional Selective Window

In Figure 5.1, resistant strain outcompete the susceptible strain in the presence of antibiotic with the concentration that falls within sub-MIC selective window and selective window (Andersson and Hughes 2011). According to Clinical and Laboratory Standards Institute (CLSI), minimal inhibitory concentration of the susceptible strain ($MIC_{susc}$) for trimethoprim is 8 µg/mL and the minimal inhibitory concentration of resistant strain ($MIC_{res}$) is 16 µg/mL. These reference ranges are applied to Enterobacteriaceae. In this project, both of the ATCC strains were subjected to the resistance induction using trimethoprim at 12 µg/ml. This antibiotic concentration falls within the

traditional selective window, which is also known as mutant selection window concentration (Drlica and Zhao 2007). As a consequence of the serial passages with the presence of trimethoprim at this concentration, both *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 13883 reduced its susceptibility to trimethoprim.

**Figure 5.1:** Schematic diagram of bacteria growth rate in response to the antibiotic concentration.




(Andersson and Hughes, 2011, p. 903)

As suggested by Townsend, Bøhn and Nielsen (2012), directional selection can be possibly exerted using antibiotic with concentrations below minimum inhibitory concentration (MIC). One problem of that study was that the experimentally achieve sub-lethal concentrations of antibiotics may reduce the viability of overall bacterial population. In fact, the viability of both of the ATCC strains of bacteria were reduced throughout the study, as the $OD_{650}$

reading measured after each passages reduced. This problem was then overcome by consistent assessment on the bacteria growth throughout the study. Every 5 passages, both of the ATCC strains of bacteria were cultured on EMB agar and MacConkey agar, followed by checking the bacteria viability. Both of the ATCC strains of bacteria were able to survived even in the presence of trimethoprim at 12 μg/ml. This also means that both the bacteria were able to grow on this antibiotic concentration.

### 5.2.2 Clinical significance in antibiotic dosing strategies

This study confers a profound implication in antibiotic dosing strategies. In clinical settings, traditional dosing approach using antibiotics concentration within mutant selection window eradicates the susceptible bacteria population and gives rise to the emergence of resistant subpopulation (Liu et al. 2005; Drlica and Zhao 2007; Zhao and Drlica 2008). Study from Olofsson et al. (2007) proposed that the susceptible *E. coli* populations were eliminated by the approved, clinical dosing fluoroquinolone regimens, and eventually develop resistance to the antibiotic. Somehow, the effective dose of antibiotics prescribed by clinicians may not kill all the bacteria and exerts selective pressure on the susceptible bacteria population. Eventually, the susceptible bacteria may mutate and gain resistance over the antibiotics.

## 5.3 Assessment on the transfer of antibiotics resistance from clinical isolate *Escherichia coli* 594370 to clinical isolate *Klebsiella pneumoniae* 594394

In this aspect of study, resistant clinical isolate *E. coli* 594370 served as the donor bacteria while susceptible, clinical isolate *K. pneumoniae* 594394 served as the recipient bacteria. Based on the result, the recipient bacteria did not gain any of the antibiotic resistance after 30 passages of co-incubation with the donor bacteria. Thus, the transfer of antibiotic resistance from resistant *E. coli* to susceptible *K. pneumoniae* was not observed. This may be due to the insufficient number of passages conducted in this study. As suggested by Townsend, Bøhn and Nielsen (2012), horizontal gene transfer (HGT) events may not take place over a short time frame. 30 passages may not be sufficient for transfer of antibiotic resistance from donor bacteria to recipient bacteria. Moreover, the transfer of antibiotic resistance may be driven by the presence of antibiotics, which is elaborated in Section 5.3.1.

### 5.3.1 Role of antibiotic in transfer of antibiotic resistance

The transfer of antibiotic resistance from resistant bacteria to sensitive bacteria may be promoted by the presence of antibiotics at very low concentration. As suggested by Courvalin and Trieu-Cuot (2001), antibiotics can act as sex pheromones that induce the conjugative transfer of resistance gene from resistant bacteria to susceptible bacteria.

However, one may argue that the resistance acquired in the recipient may be induced by the mutation under selective pressure of antibiotic instead. As shown in the study by Gullberg et al. (2011), ciprofloxacin with 1/10 of the minimum inhibitory concentration (MIC) was able to induce the resistance in susceptible strains of *E. coli*. In that study, the ciprofloxacin resistance in *E. coli* was only induced after 600 generations of growth. As so, antibiotic concentration less than 1/10 MIC may be essential to mediate the transfer of antibiotic resistance from donor bacteria to recipient bacteria. In fact, it's not the antibiotics that induce the mutation for the resistance in recipient bacteria strains in this case, as this minimal amount of antibiotics will only induce the antibiotic resistance after few hundreds of generations. Thus, this minimal amount of antibiotics may be used to promote the antibiotic resistance transfer from donor bacteria to recipient bacteria as the transfer of antibiotic resistance may occur before the induction of antibiotic resistance which requires few hundreds of passages. Thus, the transfer of antibiotic resistance may be occurred much faster than the mutation conferring resistance in the bacteria population.

Many of the horizontal gene transfer (HGT) events that involved integration of genetic materials into bacteria chromosome are relatively non-beneficial and may confer to fitness cost (Martínez, 2012; Townsend, Bøhn and Nielsen, 2012). However, the acquisition of resistant genes in the recipient bacteria by HGT may be promoted in the presence of antibiotic pressure as the resistant gene transferred are essential for survival of bacteria population (Townsend, Bøhn and Nielsen, 2012).

### 5.3.2 Clinical significance of the transfer of antibiotic resistance

As described by Jernberg et al. (2010), human intestine serve as an optimal site for the transfer of resistant genes, as the abundance in nutrients with moist and warm environment of the intestine favor the colonization diverse Enterobacteriaceae. Reservoir hypothesis suggested that human intestinal bacteria may serve as the harbor for antibiotic resistant genes (Salyers, Gupta and Wang, 2004; Marshall, Ochieng and Levy 2009).

Presence of antibiotics in food contaminants and oral antibiotic therapy may promote the transfer of antibiotic resistance from donor bacteria to recipient bacteria even though in minute amount (Salyers, Gupta and Wang 2004; Schjørring and Krogfelt 2010). A survey conducted by McNulty et al. (2007) revealed the public attitude on the consumption of antibiotics. From the survey, some of the respondents failed to complete the full course of the antibiotic treatment, and even recycle the left-over antibiotics. The potency of these "left-over" antibiotics may reduce over time. Thus, the antibiotic concentration in the body may not reach the minimum inhibitory concentration (MIC), leading to the induction of antibiotic resistance in susceptible bacteria population. This create an environment with sub-lethal concentration of antibiotics, favors the transfer of antibiotic resistance from resistant pathogenic bacteria to susceptible commensal flora, as well as the induction of antibiotic resistance as shown in Section 5.2. Thus, public attitude is one of the driven force that promotes the transfer and induction of antibiotic resistance from the pathogenic bacteria to the susceptible intestinal flora.

## 5.4 Future Study

There were several methodological limitations in this project, and these limitations are expected to be solved in future study. More number of passages is required to assess the losing of antibiotic resistance and transfer of antibiotic resistance. Hereby, the reversal and transfer of antibiotic resistance required longer duration as discussed in Section 5.1 and Section 5.3 respectively. On the other hand, *in vivo* models and cell culture methods can be conducted concurrently with the *in vitro* experimental settings. The results obtained by this approach may be more conclusive and provide a better picture on the various aspects of antibiotic resistance. Furthermore, application of molecular biology methods may provide a better understanding on the type of resistance genes that are disseminated. In addition, bioinformatics tools and mathematical models may be used as a guide in assessing the evolutionary analysis of the bacteria.

# CHAPTER 6

# CONCLUSION

In conclusion, the losing of antibiotic resistance in clinical isolate *Escherichia coli* 594370 did not occurred due to the limited duration in this project. However, this reversibility is possible if the amount of passages is increased. On the other hand, reduction of susceptibility to trimethoprim was observed in both *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 13883. Thus, this indicates that trimethoprim resistance is inducible using trimethoprim with sub-lethal concentration, which falls under the category of traditional selective window. Thus, this indicates that the losing of antibiotic resistance may require more duration as compared to the induction of the antibiotic resistance. Last but not least, the transfer of antibiotic resistance from donor bacteria, clinical isolate *E. coli* 594370 to recipient bacteria, clinical isolate *K. pneumoniae* 594394 was not observed. It was discussed that the transfer of antibiotic resistance from donor bacteria to recipient bacteria may require longer duration and presence of antibiotics may be required.

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## APPENDIX A

### Chemicals and media used

| Chemicals/Media | Manufacturer |
|---|---|
| Ciprofloxacin disk | Becton Dickinson and Company, Sparks, USA |
| Dimethyl sulphoxide | Lab-Scan Ltd., Ireland |
| Eosin methylene blue (EMB) agar | Laboratorios Conda S.A., Spain |
| Ethanol (95%) | Copens Scientific (M) Sdn. Bhd., Malaysia |
| Gentamicin disk | Oxoid LTD. Basingstoke, Hampshire, England |
| MacConkey agar | Laboratorios Conda S. A., Spain |
| Moxifloxacin disk | Benex Limited Shannon, Country Clare, Ireland (USA) |
| Trimethoprim powder | Nacalai Tesque, Japan |

## APPENDIX B

### Equipment and lab wares used

| Lab Wares/Equipment | Manufacturer |
|---|---|
| Conical flask (250 ml) | Schott Duran, Germany |
| Falcon tube (15 ml, 50 ml) | BD Falcon™, USA |
| Pipetter (10 µL, 100 µL, 1000 µL) | Vipro, Germany |
| Pipette tips | Axygen Scientific, CA |
| Schott bottle (100 ml, 500 ml, 1000 ml) | Schott Duran, Germany |
| Biosafety Cabinet (Level 2) | TELSTAR Industrial S. L., Spain |

## APPENDIX C

### Preparation of trimethoprim disks

Autoclaved paper disks with diameter of 6 mm were used for the preparation of trimethoprim. According to Antonio-Velmote, Gonzaga and Darvin (1988), it was assumed that 20 µl of antibiotic solution was absorbed by each paper disk. The disk content for trimethoprim as recommended by CLSI is 5 µg, in which the trimethoprim solution prepared must contain 250 µg of trimethoprim in 1 ml of solution. 1 ml of working solution (250 µg/ml) was prepared from the stock solution (10 mg/ml) with the calculation shown as followed.

$$M1\ V1 = M2\ V2$$

$$(10\ mg/ml)\ (V1) = (250\ \mu g/ml)\ (1\ ml)$$

$$(10 \times 10^{-3}\ g/ml)\ (V1) = (250 \times 10^{-6}\ g/ml)\ (1ml)$$

$$V1 = 0.025\ ml \text{ or } 25\ \mu l$$

∴ In order to prepare 1 ml of working solution of trimethoprim (250 µg/ml), 25 µl of stock solution (10 mg/ml) was diluted with 975 µl of dimethyl sulfoxide (DMSO).

**Preparation of 12 µg/ml trimethoprim solution**

Working solution of trimethoprim with the concentration of 12 µg/ml was used in the induction of antibiotic resistance in Section 3.2. 10 ml of working solution (12 µg/ml) was prepared from the stock solution (10 mg/ml) with the calculation shown as followed.

$$M1\ V1 = M2\ V2$$

$$(10\ mg/ml)\ (V1) = (12\ \mu g/ml)\ (10\ ml)$$

$$(10 \times 10^{-3}\ g/ml)\ (V1) = (12 \times 10^{-6})\ (10\ ml)$$

$$V1 = 0.012\ ml \text{ or } 12\ \mu l$$

∴ In order to prepare 10 ml of working solution (12 µg/ml), 12 µl of stock solution (10 mg/ml) was diluted with 988 µl of dimethyl sulfoxide.

## APPENDIX D

The duplicated reading for measurement of the diameter of zone-of-inhibition (ZI) in Section 4.1, Section 4.2 and Section 4.3 was tabulated as followed.

Zone-of-inhibition (ZI) diameter of ciprofloxacin to 594370 *E. coli*.

| **Number of passages** | **Zone-of-inhibition Diameter (mm)*** | | |
|---|---|---|---|
| | **Reading 1** | **Reading 2** | **Average reading** |
| 0 (Initial ASP) | 0 | 0 | 0 |
| 5 | 8 | 9 | 8.5 |
| 10 | 10 | 10 | 10 |
| 15 | 8 | 9 | 8.5 |
| 20 | 8.5 | 8.5 | 8.5 |
| 25 | 8.5 | 8.5 | 8.5 |
| 30 | 8.5 | 8.5 | 8.5 |
| 35 | 7.5 | 8 | 7.75 |
| 40 | 9 | 9 | 9 |
| 45 | 0 | 0 | 0 |
| 50 | 8 | 8.5 | 8.25 |
| 55 | 9 | 8 | 8.5 |
| 60 | 0 | 0 | 0 |
| 65 | 0 | 0 | 0 |
| 70 | 9 | 9.5 | 9.25 |
| 75 | R | R | R |
| 80 | 10 | 9 | 9.5 |
| 85 | 8 | 9 | 8.5 |

Zone-of-inhibition (ZI) diameter of gentamicin to 594370 *E. coli*.

| **Number of passages** | **Zone-of-inhibition Diameter (mm)*** | | |
|---|---|---|---|
| | **Reading 1** | **Reading 2** | **Average Reading** |
| 0 (Initial ASP) | 8 | 9 | 8.5 |
| 5 | 9 | 9 | 9 |
| 10 | 9 | 9 | 9 |
| 15 | 8 | 8 | 8 |
| 20 | 0 | 0 | 0 |
| 25 | 8 | 7.5 | 7.75 |
| 30 | 8.5 | 8 | 8.25 |
| 35 | 7.5 | 8 | 7.75 |
| 40 | 8 | 8 | 8 |
| 45 | 7.5 | 7 | 7.25 |
| 50 | 7.5 | 7 | 7.25 |
| 55 | 0 | 0 | 0 |
| 60 | 0 | 0 | 0 |
| 65 | 0 | 0 | 0 |
| 70 | 10 | 10 | 10 |
| 75 | 7 | 8 | 7.5 |
| 80 | 10 | 10 | 10 |
| 85 | 0 | 0 | 0 |

Zone-of-inhibition (ZI) diameter of moxifloxacin to 594370 *E. coli*.

| **Number of passages** | **Zone-of-inhibition Diameter (mm)** | | |
|---|---|---|---|
| | **Reading 1** | **Reading 2** | **Average Reading** |
| 0 (Initial ASP) | 10 | 9 | 9.5 |
| 5 | 11 | 10.5 | 10.75 |
| 10 | 12 | 12 | 12 |
| 15 | 10 | 9.5 | 9.75 |
| 20 | 9 | 8.5 | 8.75 |
| 25 | 9 | 10 | 9.5 |
| 30 | 10.5 | 10 | 10.25 |
| 35 | 9.5 | 10 | 9.75 |
| 40 | 9.5 | 9.5 | 9.5 |
| 45 | 9 | 8.5 | 8.75 |
| 50 | 9 | 9 | 9 |
| 55 | 8 | 7.5 | 7.75 |
| 60 | 8.5 | 8 | 8.25 |
| 65 | 8.5 | 8 | 8.25 |
| 70 | 10 | 9 | 9.5 |
| 75 | 9 | 8 | 8.5 |
| 80 | 10 | 9 | 9.5 |
| 85 | 9 | 9.5 | 9.25 |

Zone-of-inhibition (ZI) diameter of trimethoprim to clinical isolate *E. coli* 594370.

| **Number of passages** | **Zone-of-inhibition Diameter (mm)*** | | |
|---|---|---|---|
| | **Reading 1** | **Reading 2** | **Average Reading** |
| 0 (Initial ASP) | 0 | 0 | 0 |
| 5 | 0 | 0 | 0 |
| 10 | 0 | 0 | 0 |
| 15 | 0 | 0 | 0 |
| 20 | 0 | 0 | 0 |
| 25 | 0 | 0 | 0 |
| 30 | 0 | 0 | 0 |
| 35 | 0 | 0 | 0 |
| 40 | 0 | 0 | 0 |
| 45 | 0 | 0 | 0 |
| 50 | 0 | 0 | 0 |
| 55 | 0 | 0 | 0 |
| 60 | 0 | 0 | 0 |
| 65 | 0 | 0 | 0 |
| 70 | 0 | 0 | 0 |
| 75 | 0 | 0 | 0 |
| 80 | 0 | 0 | 0 |
| 85 | 0 | 0 | 0 |

Zone-of-inhibition (ZI) diameter of trimethorpim to ATCC 25922 *E. coli*.

| Number of Passages | Zone-of-Inhibition (ZI) Diameter (mm) | | |
|---|---|---|---|
| | **Reading 1** | **Reading 2** | **Average Reading** |
| 0 | 35 | 35 | 35 |
| 5 | 30 | 29.5 | 29.75 |
| 10 | 26 | 27 | 26.5 |
| 15 | 25.5 | 26 | 25.75 |

Zone-of-inhibition (ZI) diameter of trimethorpim to ATCC 13883 *K. pneumoniae.*

| Number of Passages | Zone-of-Inhibition (ZI) Diameter (mm)* | | |
|---|---|---|---|
| | **Reading 1** | **Reading 2** | **Average Reading** |
| 0 | 30 | 30 | 30 |
| 5 | 27 | 28 | 27.5 |
| 10 | 15 | 16 | 15.5 |
| 15 | 10 | 9 | R |

Zone-of-inhibition (ZI) diameter of ciprofloxacin to clinical isolate *K. pneumoniae* 594394.

| Number of Passages | Zone-of-Inhibition (ZI) Diameter (mm) | | |
|---|---|---|---|
| | **Reading 1** | **Reading 2** | **Average Reading** |
| 0 | 21 | 21 | 21 |
| 5 | 17 | 18 | 17.5 |
| 10 | 20 | 19 | 19.5 |
| 15 | 21.5 | 21 | 21.25 |
| 20 | 20 | 20 | 20 |
| 25 | 21 | 22 | 21.5 |

| | | | |
|---|---|---|---|
| 30 | 22 | 22 | 22 |

Zone-of-inhibition (ZI) diameter of gentamicin to clinical isolate *K. pneumoniae* 594394.

| **Number of Passages** | **Zone-of-Inhibition (ZI) Diameter (mm)** | | |
|---|---|---|---|
| | **Reading 1** | **Reading 2** | **Average Reading** |
| 0 | 19 | 19 | 19 |
| 5 | 19 | 19.5 | 19.25 |
| 10 | 18 | 19 | 18.5 |
| 15 | 18 | 18 | 18 |
| 20 | 19 | 19 | 19 |
| 25 | 19 | 19 | 19 |
| 30 | 19 | 18 | 18.5 |

Zone-of-inhibition (ZI) diameter of moxifloxacin to clinical isolate *K. pneumoniae* 594394.

| **Number of Passages** | **Zone-of-Inhibition (ZI) Diameter (mm)** | | |
|---|---|---|---|
| | **Reading 1** | **Reading 2** | **Average Reading** |
| 0 | 18 | 18.5 | 18.25 |
| 5 | 18 | 17.5 | 17.75 |
| 10 | 17 | 17 | 17 |
| 15 | 18 | 18 | 18 |
| 20 | 19 | 19 | 19 |
| 25 | 19 | 19 | 19 |
| 30 | 19 | 18 | 18.5 |