DISTRIBUTION OF THE TETRACYCLINE GENES *tet*D AND *tet*E IN TETRACYCLINE-RESISTANT CLINICAL ISOLATES FROM MALAYSIAN HOSPITALS

By

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ABSTRACT

DISTRIBUTION OF THE TETRACYCLINE GENES *tet*D AND *tet*E IN TETRACYCLINE-RESISTANT CLINICAL ISOLATES FROM MALAYSIAN HOSPITALS

Lim Sze Yee

The extensive and widespread usage of tetracyclines by human has led to the emergence of resistance to these antimicrobial agents, thus limiting their efficacy as a treatment for infectious diseases. This present study was carried out to investigate the prevalence of *tet*D and *tet*E genes among tetracyclines resistant gram-negative clinical isolates in Malaysia using PCR method. The gram-negative clinical isolates were collected from Gribbles® Pathology Laboratories, Hospital Raja Permaisuri Bainun, Ipoh Specialist Hospital and Hospital Pantai-Putri Ipoh. A total of 53 tetracyclines resistant gram-negative isolates were selected for PCR detection of *tet*D and *tet*E genes. DNA extraction was performed using fast-boil method. The PCR amplification of 16S rRNA gene was carried out to determine the integrity of the extracted DNA. Besides PCR detection, optimisation of the annealing temperature for each pair of primers and PCR parameters for *tet*E primers were also undertaken. The specificity of the primers was also determined by direct sequencing. The sequences were then compared to those available on GenBank using Blast X. The findings of this study revealed that *tet*D was not widely distributed among different species of tetracyclines resistant gram-

negative clinical isolates. *tet*D was only detected in 4 out of 53 (7.55%) clinical isolates selected for this study. Nevertheless, this study did not manage to detect *tet*E in any of the isolates screened. Moreover, the optimisation of *tet*E primers was also unsuccessful in this study. In conclusion, the narrow distribution of *tet*D among different species of gram-negative bacteria observed in this study suggests that the isolates may carry one of the other known or novel *tet* genes.

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DECLARATION

I hereby declare that the project report 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 and concurrently submitted for any other degree at UTAR or other institutions.

_____

Lim Sze Yee

APPROVAL SHEET

This project report entitled **"DISTRIBUTION OF THE TETRACYCLINE GENES *tet*D AND *tet*E IN TETRACYCLINE-RESISTANT CLINICAL ISOLATES FROM MALAYSIAN HOSPITALS"** was prepared by **LIM SZE YEE** and submitted as partial fulfillment of the requirements for the degree of Bachelor of Science (Hons) Biomedical Science at Universiti Tunku Abdul Rahman.

Approved by:

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PERMISSION SHEET

It is hereby certified that **LIM SZE YEE** (ID No: 08ADB03611) has completed this final year project entitled **"DISTRIBUTION OF THE TETRACYCLINE GENES *tet*D AND *tet*E IN TETRACYCLINE-RESISTANT CLINICAL ISOLATES FROM MALAYSIAN HOSPITALS"** under supervision of Dr. Chew Choy Hoong (Supervisor) from the Department of Biomedical Science, Faculty of Science.

I hereby give permission to my supervisor to write and prepare manuscript of these research findings 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 authors for this article. Arrangement of the name depends on my supervisor.

Yours truly,

_____

(LIM SZE YEE)

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LIST OF ABBREVIATIONS

µg	Microgram
µl	Microlitre
µM	Micromolar
A_{260}	Absorbance at 260 nm wavelength
A_{280}	Absorbance at 280 nm wavelength
BLAST	Basic local alignment search tool
bp	Base pair
cc	Cubic centimeters
CLS	Cryogenic Liquid Systems
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleoside triphosphates
EDTA	Ethylenediaminetetraacetic acid
ETT	Endotracheal tube
FDA	Food and Drug Administration
g	Acceleration of gravity
g	Gram
HCl	Hydrochloric acid
kb	Kilobase
kPa	Kilopascal
L	Litre
M	Molar
mg	Milligram

min	Minutes
ml	Millilitre
mM	Millimolar
$MgCl_2$	Magnesium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
ng	Nanogram
nm	Nanometer
PCR	Polymerase Chain Reaction
PEG	Percutaneous endoscopic gastrostomy
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
S	Svedberg units
sec	Seconds
Taq	*Thermus aquaticus*
TAE	Tris-Acetate-EDTA
U	Unit
UV	Ultraviolet
v/v	Volume/volume
w/v	Weight/volume

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

In the 1940s, tetracyclines, a family of broad-spectrum antibiotics exhibiting activity against a wide range of microorganisms such as gram-positive and gram-negative bacteria, chlamydiae, mycoplasmas, rickettsiae, and protozoan parasites, were discovered (Chopra & Roberts, 2001). Tetracyclines show their effects by inhibiting the synthesis of protein through blocking the attachment of aminoacyl-tRNA to the ribosomal acceptor (A) site (Ophardt, 2003). Their effective antimicrobial properties as well as the absence of major adverse side effects have contributed to their extensive and widespread therapeutic use in human and animal infections (Chopra & Roberts, 2001). This class of antimicrobial agent can also be used for prophylaxis purpose to prevent malaria, traveler's diarrhoea, cholera and other diseases in addition to agricultural purposes (Foye, Lemke, & Williams, 2007).

However, due to the wide-range usage of tetracyclines by human beings, resistance to these antimicrobial agents has emerged in many countries, resulting in limitation on their efficacy in treatment of infectious diseases caused by many different microorganisms (Kwon et al., 2000). The emergence of such antibiotic resistance has brought about a lot of problems, including prolonged hospital stay, increasing the financial burden of the patient and the selective pressure for resistance to emerge among pathogens (Alanis, 2005). Furthermore, various new drugs and modified existing drugs, tigecycline, for

example, have also been developed in order to fight against tetracycline-resistant organisms (Foye et al., 2007).

At present, many tetracycline resistance determinants have been described but not all of these resistant determinants are well-studied, such as *tet*D and *tet*E. Generally, *tet* genes in the determinants can confer resistance to tetracyclines by encoding either one of these three mechanisms: tetracycline efflux, ribosome protection and tetracycline modification. The mechanism which is more abundantly found in gram-negative and gram-positive microorganisms seems to be tetracycline efflux and ribosome protection, respectively (Bryan, Shapir, & Sadowsky, 2004). Meanwhile, the rapid spread of the *tet* genes among bacterial species is often associated with conjugative or mobile elements, for instances transposons, plasmids and integrons (Alanis, 2005; Bryan et al., 2004).

So far, many studies have been carried out to investigate the prevalence of non-specific tetracycline resistance in humans and animals in Malaysia (Alhaj, Mariana, Raha, & Ishak, 2007; Son, Ansary, Salmah, & Maznah, 1995). However, limited epidemiological data about the prevalence of specific tetracycline resistance determinants especially *tet*D and *tet*E, can be found. Therefore, the aim of this study is to gain deeper understanding on this aspect. The overall objectives in this study are:

a) To determine the optimum temperature for the amplification of *tet*D and *tet*E genes using their gene-specific primers respectively;

b) To screen for the presence of *tet*D and *tet*E genes among tetracycline resistant clinical isolates using PCR method, and
c) To analyse the epidemiology data obtained from PCR screening of target genes.

Lastly, it is hoped that the updated epidemiological data on the prevalence of the *tet*D and *tet*E genes in Malaysia population from this study can further attract research on understanding the molecular mechanism behind their mode of action, which would indirectly contribute to solving and minimising problems of tetracycline resistance.

CHAPTER 2

LITERATURE REVIEW

2.1 Tetracycline antibiotics

2.1.1 Overview

Tetracyclines are a family of broad spectrum polyketide antibiotics, which are produced by a group of gram-positive bacteria, *Streptomyces* genus of Actinobacteria (Knox & Wishart, 2010). This family of antibiotics was discovered in the 1940s. They usually exert activity against a large variety of gram-positive and gram-negative bacteria as well as atypical organisms, for instance, chlamydiae, mycoplasmas, rickettsiae, and protozoan parasites (Chopra & Roberts, 2001). Tetracyclines consist of eight related antibiotics. Among these, tetracycline, chlortetracycline and doxycycline are the best known (Todar, 2011). The chemically and biologically simplest tetracycline is known as sancycline (Nelson, Greenwald, & Hillen, 2001).

Tetracycline antibiotics share a common structural element; a tetracycline nucleus which possesses four linearly fused 6-membered rings and is in the naphthacene ring family of organic polycyclic hydrocarbons (Figure 1.1). The major region responsible for the pharmacological properties of tetracycline family is the presence of highly dense oxygen functional group system in the structure. In addition, this group of antimicrobial agents has also been shown to exist as two different bioactive forms in equilibrium, based on solvent environment, the A zwitterions form and B unionised form (Nelson et al.,

2001) (Figure 1.2).

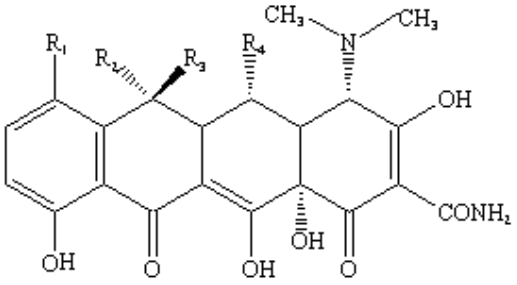

Figure 2.1: The tetracycline core structure (Adapted from Todar, 2011).

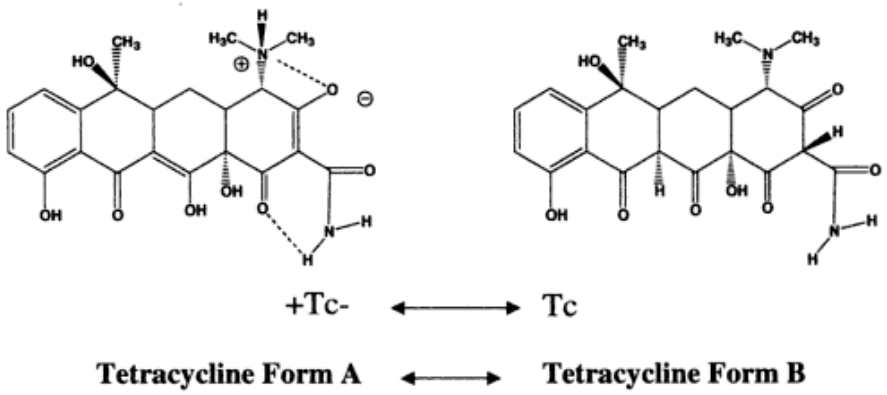

Figure 2.2: Two bioactive forms of tetracycline, the A zwitterions form and the B unionized form (Adapted from Nelson et al., 2001).

2.1.2 Mode of action

The bacteriostatic activity of tetracyclines is associated with the reversible inhibition of bacterial protein synthesis (Schnappinger & Hillen, 1996). In gram-negative bacteria, tetracyclines pass through the outer membranes via porin channels, OmpF and OmpC, and then accumulate in the periplasmic space. After that, they move across the cytoplasmic membrane by an energy-dependent process. These drugs bind reversibly to the prokaryotic 30S

ribosomal subunit once inside the bacterial cells to block the codon-specific attachment of aminoacyl-tRNA to the ribosomal acceptor (A) site. As a result, the ribosomal P site will be short of tRNA-amino acid substrates to form growing polypeptide chain. The aminoacyl transfer reaction is subsequently inhibited and the protein synthesis is then terminated (Roberts, 2003). There are 5 proteins such as protein S4, S7, S8, S14, S19 and some bases in 16S rRNA such as G_{693}, A_{892}, U_{1052}, C_{1054}, which have been identified to be important for tetracycline binding to the 30S ribosomal unit (Chopra & Roberts, 2001; Michalova, Novotna, & Schlegelova, 2004; Schnappinger & Hillen, 1996).

Other than the 30S ribosomal unit, tetracyclines can bind with the 70S ribosome in mitochondria and cause the inhibition of protein synthesis in that organelle. As for 80S ribosome of eukaryotic cells, tetracycline can only interact weakly with it and produce a rather weak inhibition of protein synthesis (Roberts, 2003).

2.1.3 Administration and dosage

Tetracyclines administration is different from other medicine, which is usually best to be taken after meal. Tetracyclines should be administered on an empty stomach to enhance its absorption (Arcangelo & Peterson, 2006). Route of administration used is preferably oral. Suitable dosage of tetracyclines for treatment is dependant on the types of disease, drug (tetracycline, doxycycline, oxytetracycline, etc.), patient's age and the manufacturer. For example, to treat acne problem in adults, 0.5-1 gram/day in 4 divided doses of tetracycline is

needed for 1-2 weeks or until clinical improvement occurs (UBM Medica, 2011).

However, tetracyclines are contraindicated for use in pregnant woman, infants and children under 8 years old due to tetracyclines may cause permanent brown discoloration of teeth. Enamel hypoplasia also has been reported. In addition, tetracyclines are inadvisable to be used in patients with known hypersensitivity to these drugs as well as those with pre-existing renal impairment (United States National Library of Medicine, 2005).

2.1.4 Adverse effects

Common adverse effects associated with tetracyclines therapy include nausea, vomiting, photosensitivity, diarrhoea, epigastric distress, hypersensitivity reaction, and many more. Some related rare problems, like hepatotoxicity, have also been reported. Tetracyclines have been reported to produce brown-black microscopic discoloration of thyroid glands when given over prolonged periods, but there are no abnormalities of thyroid function studies known (Arcangelo & Peterson, 2006; United States National Library of Medicine, 2005).

2.1.5 Applications

2.1.5.1 Human therapy and prophylactic uses

Tetracyclines can be used as low dose oral or topical therapy for many diseases, including acne, first course community-acquired urinary tract infection (main causative agent is *Escherichia coli*), sexually transmitted

diseases (particularly Chlamydia), rickettsial infections, mycoplasmal pneumonia and many more (Foye et al., 2007). Besides that, this class of antibiotics can be applied with other types of medicine to manage diseases. For example, tetracycline has been used as part of a quadruple therapy to manage peptic ulcer disease related with *Helicobacter pylori* (National Digestive Diseases Information Clearinghouse, 2010). Tetracyclines can be used for prophylaxis purpose as well. They can be utilised to prevent mefloquine-resistant *Plasmodium falciparum* malaria, traveler's diarrhoea, cholera, and *Enterobacter* infections (Chopra & Roberts, 2001; Foye et al., 2007).

2.1.5.2 Veterinary medicine

Compared to other types of antibiotic, the usage of tetracyclines in veterinary medicines are relatively high. Tetracyclines are largely used to treat gastrointestinal, respiratory and skin bacterial infections, infectious diseases of locomotive organs and genito-urinary tract in addition to systemic infection and sepsis. Beef cattle, pig, sheep, goat, horse, dog, cat, poultry, rabbit and even fish are the target animals of this application (Michalova et al., 2004). Furthermore, tetracyclines are also used in food animals in subtherapeutic amounts over long periods for improving the growth rate to feed intake ratio or growth promotion in certain countries. The mechanism responsible for growth promotion was yet to be fully elucidated. However, it seems to be related to vitamin production enhancement by gastrointestinal microbes, subclinical populations of pathogenic organisms' elimination as well as increased intestinal absorption of nutrients (Chopra & Roberts, 2001).

2.2 Mechanisms of resistance of tetracycline antibiotics

The effective antimicrobial properties and absence of major adverse side effects of tetracyclines have led to widespread therapeutic use in human and animal infections (Chopra & Roberts, 2001). Yet, the emergence of resistance to these antibiotics in many countries and the consequent limitation of their efficacy in infectious disease treatments is actually due to their widespread usage by human beings (Kwon et al., 2000). Many problems arise from the antibiotic resistance, such as prolonged hospital stay, increased the financial burden of the patient and increased the selective pressure for resistance to emerge among pathogens (Alanis, 2005).

Three different bacterial strategies of resistances have been described, which include reduction of the tetracyclines intercellular concentration (active efflux), protection of the ribosome as the antibiotic target (ribosomal protection) and antibiotics inactivation by modifying enzymes (enzymatic inactivation) (Michalova et al., 2004; Schnappinger & Hillen, 1996).

2.2.1 Active Efflux

So far, twenty-three classes of tetracycline resistance determinants encoding energy-dependent drug-specific efflux proteins are known. Most of these tetracycline resistance determinants (60%), which include *tet*D and *tet*E, are present in gram-negative bacteria. Efflux proteins act by exporting tetracycline out of the cells and therefore reducing the intercellular concentration of drugs and protecting the bacterial ribosome *in vivo*. These proteins, located in the

cytoplasmic membrane, exchange a proton for a tetracycline-cation complex against a concentration gradient (Roberts, 2005).

In gram-positive and gram-negative bacteria, the regulation of *tet* gene expression is different. In gram-negative bacteria, each determinant composes of two genes coding for one efflux protein and repressor protein. Both proteins originated in a divergent manner and share a central regulatory region. Without the presence of tetracycline, the repressor protein binds to the operator of the structural efflux gene and therefore inhibits its transcription and translation. On the other hand, when the tetracycline-cation complex in the cell binds to the repressor, it would induce conformation changes of the repressor, resulting in the release of repressor from the operator and then permitting the transcription and translation of the structural efflux gene. The repressor binds to the operator again when there is a decrease of intracellular amount of tetracycline (Michalova et al., 2004).

2.2.2 Ribosomal protection

This mechanism of tetracycline resistance was first discovered in Streptococci and appears to be more abundantly found in gram-positive organisms (Aarestrup, 2006; Bryan et al., 2004). Ribosomal protection proteins can be encoded by 11 *tet* genes and structurally similar in their N- terminal amino acid sequences to ribosomal elongation factor EF-G and EF-Tu (Roberts, 1996; Roberts, 2005). These cytoplasmic proteins have ribosome-dependent GTPase activity and confer resistance to tetracycline, doxycycline and minocycline (Aarestrup, 2006). Ribosomal protection can work *in vitro* and also *in vivo*

without requiring intact membranes to function, unlike efflux proteins (Chopra & Roberts, 2001).

The ribosomal protection proteins interact with the ribosome giving rise to an allosteric disruption of the primary tetracycline binding site(s). Such disruption then results in the release of tetracyclines from the ribosome. The ribosome returns to its original conformational state and the synthesis of protein can now be continued (Aarestrup, 2006; Roberts, 2005). Most of the work on this mechanism has been done on Tet(O) and Tet(M). These two proteins appear to release tetracycline from the ribosome and thereby inhibiting the effect of the drug and enabling the protein synthesis to continue (Connell, Tracz, Nierhaus, & Taylor, 2003).

2.2.3 Enzymatic activation

The genes *tet*X, *tet*37 and *tet*34 are the only three examples of tetracycline resistance due to the enzymatic modification and inactivation of the antibiotic. *tet*X was first identified by its ability to confer resistance in aerobically grown *Escherichia coli* (Speer, Shoemaker, & Salyers, 1992). The gene product is a cytoplasmic protein that chemically modifies and inactivates the tetracycline molecule in a reaction requiring both oxygen and NADPH. *tet*37 has been discovered from humans' oral cavity. It also needs oxygen to function, but bacterial host which carry this gene has yet to be identified. The third gene, *tet*34, is similar to xanthine-guanine phosphoribosyl transferase gene of *Vibrio cholerae* (Aarestrup, 2006).

2.3 Transfer of tetracycline resistance genes

tet genes are found in a variety of tetracycline-resistant bacteria isolated from humans, animals and the environment (Roberts, 1996). Their wide distribution among bacterial species is often associated with conjugative or mobile element, for instances, transposons, plasmids and integrons (Alanis, 2005; Bryan et al., 2004). The gram-negative *tet* efflux genes are found on transposons inserted into various groups of plasmids from a variety of incompatible groups, while gram-positive efflux genes are discovered to be associated with small plasmids. Meanwhile, ribosomal protection genes are found on conjugative plasmids or in the chromosome (Roberts, 1996). Nevertheless, there were no *tet* genes found within integrons yet although integrons have been identified in gram-negative genera and *Staphylococcus* species (Roberts, 2005).

2.4 Nomenclature of tetracycline resistance genes

DNA-DNA hybridisation has been used to classify different classes of tetracycline resistance genes and each class is designated by a different capital letter. It was recommended that the designation of Tet D, Tet E and so on, can be used to refer to a particular determinant. The first structural gene of any class would be designated as *tetA* and the subsequent ones would be *tetB*, *tetC* and so forth. Within each structural gene, it can be subdivided into different classes. For example, with the presence of class P, the first structural gene would be designated as *tetA*(P) and the second structural gene would be *tetB*(P). The protein product of the *tetA* is designated as TetA(A) or TetA. Meanwhile, the repressor gene of any class would be designated as *tetR* (Levy et al., 1989; Roberts, 1996).

2.5 New generation of tetracycline

In order to overcome the worsening bacterial resistance to the earlier tetracyclines, a new drug, tigecycline, was introduced after it was approved by FDA to treat various bacterial infection in 2005. Tigecycline was the first drug in the new generation of tetracycline, glycylcyclines. It was developed by modification of the chemical structure of the existing drug, minocycline. Tigecycline not only exhibits the same mode of action as the typical tetracyclines, it is also not affected by the two main tetracycline-resistance mechanisms, which are the active efflux and ribosomal protection mechanisms. This drug can now be used to treat diseases like complicated skin and skin-structure infections, as well as complicated intra-abdominal infections caused by various microbes. Moreover, it can be an alternative for patients with serious allergies to beta-lactam antibiotics (Wenzel, Bate, & Kirkpatrick, 2005).

2.6 Tetracycline resistance in Malaysia to date

In a study by Son et al. (1995), veterinary isolates of *Salmonella enteritis* were characterised by their susceptibility towards 10 antimicrobial agents using commercial discs. As a result, resistance to tetracycline in *Salmonella enteritis* appears to be the most common (89%) as compared to other antimicrobial agents.

In 2007, Alhaj et al. carried out an experiment to investigate the prevalence of antimicrobial resistance in *Escherichia coli* isolated from different sources (i.e. humans, marines, rivers, foods and animals). All seventy isolates were tested

for susceptibility towards 10 antimicrobial agents using the Kirby-Bauer disk diffusion method. From the findings, the isolates, apart from being equally resistant to kanamycin and tetracycline (81.4%), also displayed the highest resistance to these two antibiotics as compared to other antimicrobial agents.

In another study, Tan, Haresh, Chai, and Son, in 2009 tested 50 *Campylobacter jejuni* isolates collected from sushi retailed from different supermarkets in Malaysia, for their susceptibility to antibiotics through the Kirby-Bauer assay as well. They found that there was only low resistance rate to tetracycline (6%) in the 50 *Campylobacter jejuni* isolates.

Last but not least, Douadi, Thong, Watanabe, and Puthucheary, in 2010 have used forty-seven Malaysian *Salmonella Typhimurium* strains, isolated from local teaching hospital patients and animals, to test for the antimicrobial resistance using standard disk diffusion method. The presence of relevant resistance genes in these strains were also investigated with PCR method. They discovered that there was high resistance rate to tetracycline in these strains, where 33 out of 47 strains (70.2%) were tetracycline-resistant. Twenty-three *tet*A, 5 *tet*B and 4 *tet*C genes were detected among these 33 tetracycline-resistant strains. The remaining one tetracycline-resistant strain did not carry any of the four tetracycline-resistant genes (*tet*A, *tet*B, *tet*C, *tet*G).

Most of the studies above were carried out to investigate the prevalence of non-specific tetracycline in human, animals and the environment in Malaysia. There was only one study conducted to obtain epidemiological data about the

prevalence of specific tetracycline resistance determinants in clinical isolates and animals, including *tet*A, *tet*B, *tet*C and *tet*G, but none for *tet*D and *tet*E.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

The materials used in this project, their suppliers and country names are as summarised in Table 3.1.

Table 3.1: Materials used in the project, their suppliers and country names.

Materials	Suppliers and Country Names
Forward and Reverse primers (*tet*D, *tet*E, 16S rRNA)	1st Base, Singapore
Ethidium Bromide	Bio Basic, USA
Agarose	Cambrex, USA
DNA Loading Dye DNA Marker (100 bp & 1 kb)	Fermentas, USA
Ethanol	HmbG Chemicals, Malaysia
Taq Polymerase	i-DNA Biotechnology, USA
Tryptic Soy Broth	Merck, Germany
Deoxynucleoside Triphosphates (dNTPs) Mix Magnesium Chloride ($MgCl_2$) 5× PCR Buffer	Promega, USA
Agar-agar Powder Sodium Hydroxide	R&M, UK
Tetracycline	Sigma Aldrich, Germany
Glacial Acetic Acid Ethylenediaminetetraacetic Acid (EDTA) Sodium Acetate	Systerm®, Malaysia
Tris	Thermo Scientific, USA
Gel Extraction Kit	Viogene,USA

3.2 Equipments/apparatus used in this study

The equipments and apparatus used in this study are summarised in Table 3.2.

Table 3.2: Equipments/apparatus used in this study and their suppliers.

Materials	Suppliers and Country Names
5 ml/cc disposable syringe	Cellotron, Malaysia
Disposable plastic petri dishes	Plastilab, Canada
Single use filter unit	Gema Medical, Spain
Micropipettes	Vipro, Malaysia
Tips, microcentrifuge tubes, PCR tubes	Axygen Scientific, USA
PCR thermocycler	Bio-Rad, USA Eppendorf, Malaysia Biometra, Germany
UV transilluminator	UVP, USA
Glassware (measuring cylinder, conical flask, beaker)	GQ, China
Sterile scalpel	Albion, UK

3.3 Stock solution

Stock solution used was prepared according to Table 3.3.

Table 3.3: Solutions for electrophoresis of DNA.

Solution	Composition
25× TAE Buffer	60.5 g Tris, 14.3 ml 99.8% glacial acetic acid, 25 ml 0.5 M EDTA (pH 8.0)

CHAPTER 4

RESULTS

4.1 Clinical isolates

4.1.1 Bacterial strains

The gram-negative clinical isolates were selected based on their resistance towards tetracycline. The identities of the clinical isolates selected for this study and their sources are presented in Table 4.1.

4.2 Template DNA extraction

Template DNA was prepared from bacterial cell lysates as described in Section 3.6. Nanodrop 100 spectrophotometer (Implen) was used to measure the A_{260}/A_{280} ratio to determine the concentration of the DNA as well as to evaluate the purity of the extracted total DNA. As shown in Appendix A, the A_{260}/A_{280} ratio for all the samples falls between 1.8 and 2.1. This indicates that the extracted DNA contained minimal protein contamination.

4.3 Total DNA integrity test with 16S rRNA amplification

Total DNA isolated from the samples were subjected to 16S rRNA test to ensure that the extracted DNA was intact and could be used in the screening step as described in Section 3.7. The outcome of this test is shown in Figure 4.1, which shows that all samples were tested positive in this test.

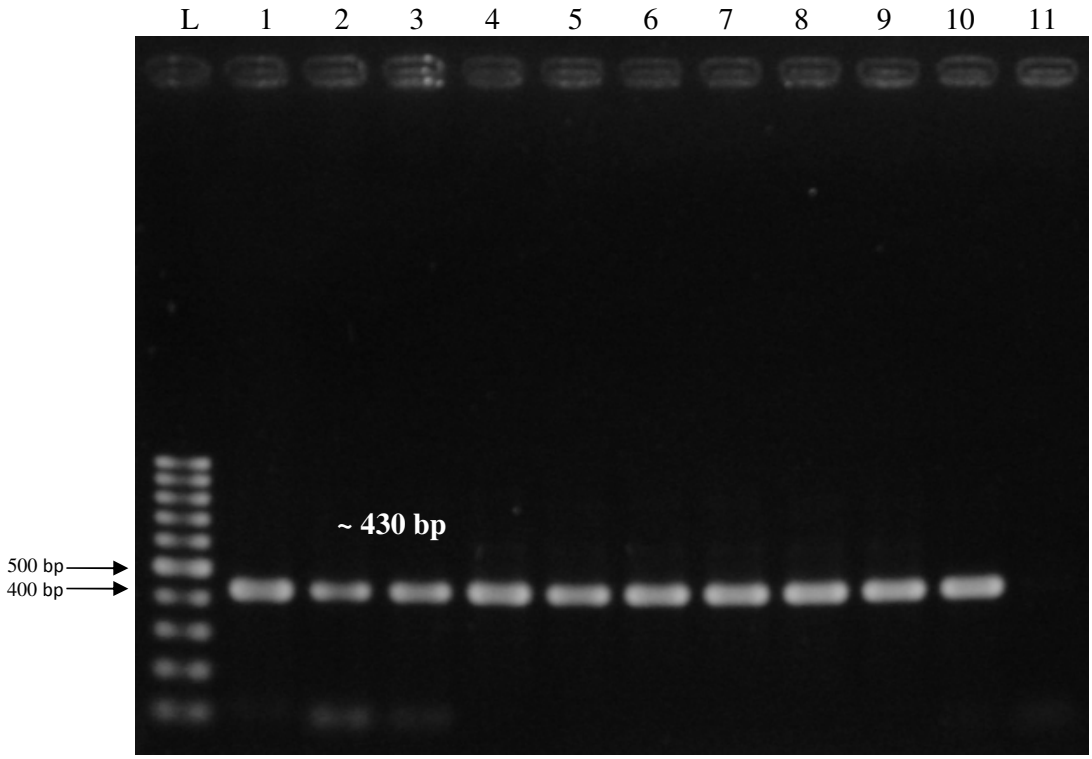

Figure 4.1: Representative image of the 16S rRNA test of samples.
Lane L was loaded with 100 bp DNA ladder while Lane 1 to 11 were loaded with amplicons from samples in the following order: H5, H49, K21, H13, H23, H33, G32, G40, G41, H16 and negative control. The size of the amplicons was approximately 430 bp, which corresponds well to the expected size of the PCR product (426 bp).

4.4 Preliminary PCR screening

Preliminary PCR screening was carried out on six randomly picked samples to obtain at least one positive sample for each putative gene (*tet*D/*tet*E) so that optimisation of the PCR conditions and direct sequencing could be carried out as described in Section 3.8. The outcome of the preliminary PCR screening is shown in Figure 4.2. There were two samples tested positive for putative *tet*D gene. One positive sample was selected for optimisation and direct sequencing. Meanwhile, for putative *tet*E gene, non-specific bandings were observed. The sample which had the amplicon size closest to the expected size of the PCR product of *tet*E gene (1198 bp) were chosen for optimisation and direct sequencing, since many bands were observed.

4.5 PCR optimisation

A total of five temperatures in the range of 50°C and 62°C were chosen for the optimisation of the PCR condition for each putative gene using the gradient function of the Eppendorf Thermal Cycler. As stated in Section 3.9, the five chosen temperatures for putative *tet*D and *tet*E gene in ascending order were: 50°C, 52°C, 55°C, 58°C, 60°C; and 52°C, 55°C, 58°C, 60°C, 62°C, respectively. Small aliquots (1 µl for putative *tet*D, 5 µl for putative *tet*E gene) of the resulting PCR products from different annealing temperatures were gel-electrophoresed in 1.5% agarose and analysed under UV after ethidium bromide staining. Figure 4.3 shows the gel images of the optimisation of the annealing temperatures of putative *tet*D and *tet*E genes. From Figure 4.3 (a), a specific band of the expected size of approximately 790 bp was obtained, but it can be observed that the amount of background smearing decreased with similar intensity of the PCR products as the annealing temperature for putative *tet*D gene amplification was raised. Meanwhile, for putative *tet*E gene, the intensity of the multiple bands (non-specific bandings) in each lane decreased with the increased annealing temperatures (Figure 4.3b). However, the amplification failed to produce a single PCR product for this case. The optimum annealing temperature for the PCR amplification for putative *tet*D gene was determined at 60°C where the amount of background smearing was minimal and the intensity of the PCR products was sharp. Yet, the optimum annealing temperature for putative *tet*E gene amplification could not be determined as many bands were obtained.

PCR amplification of putative *tet*E gene was also optimised using new PCR condition adapted from another journal (Akinbowale et al., 2007). Figure 4.4 shows the gel image of the optimisation of putative *tet*E gene amplification with new PCR condition. This figure shows that there was no band obtained from the new PCR condition.

4.6 Gel purification of PCR products

The PCR products were excised from the agarose gel and subjected to gel purification using the Viogene Gel Extraction Kit as described in Section 3.10. Only one band was excised from putative *tet*D positive sample, while from putative *tet*E suspected sample, two bands (x and y) which were the closest to 1 kb were excised and purified. Small aliquots of the purified PCR products were then subjected to spectrophotometric analysis using the Nanodrop 100 spectrophotometer (Implen) to have the A_{260}/A_{280} measured so that the DNA concentration and purity can be determined. Gel electrophoresis was also performed to confirm the presence of the purified PCR products. Table 4.2 shows the data of the spectrophotometric analysis on the purified PCR products. The PCR products recorded A_{260}/A_{280} ratio between 1.8 and 2.0, indicating a very low level of protein contamination. Figure 4.5 shows the outcome of gel electrophoresis of the purified PCR products. The products were of the expected sizes; approximately 790 bp for putative *tet*D and 1200 bp for putative *tet*E gene. Thus, the presence of purified PCR products were confirmed.

Table 4.2: The spectrophotometric analysis data of the purified PCR products.

The A_{260}/A_{280} and DNA concentration are as shown. The A_{260}/A_{280} ratio falls between 1.8 and 2.0, indicating very low levels of protein contamination.

Gene	Sample	A_{260}/A_{280} ratio	DNA concentration (ng/µl)
Putative *tet*D	H16	1.890	41.8
Putative *tet*E	H1-x	2.000	30.0
	H1-y	1.986	35.0

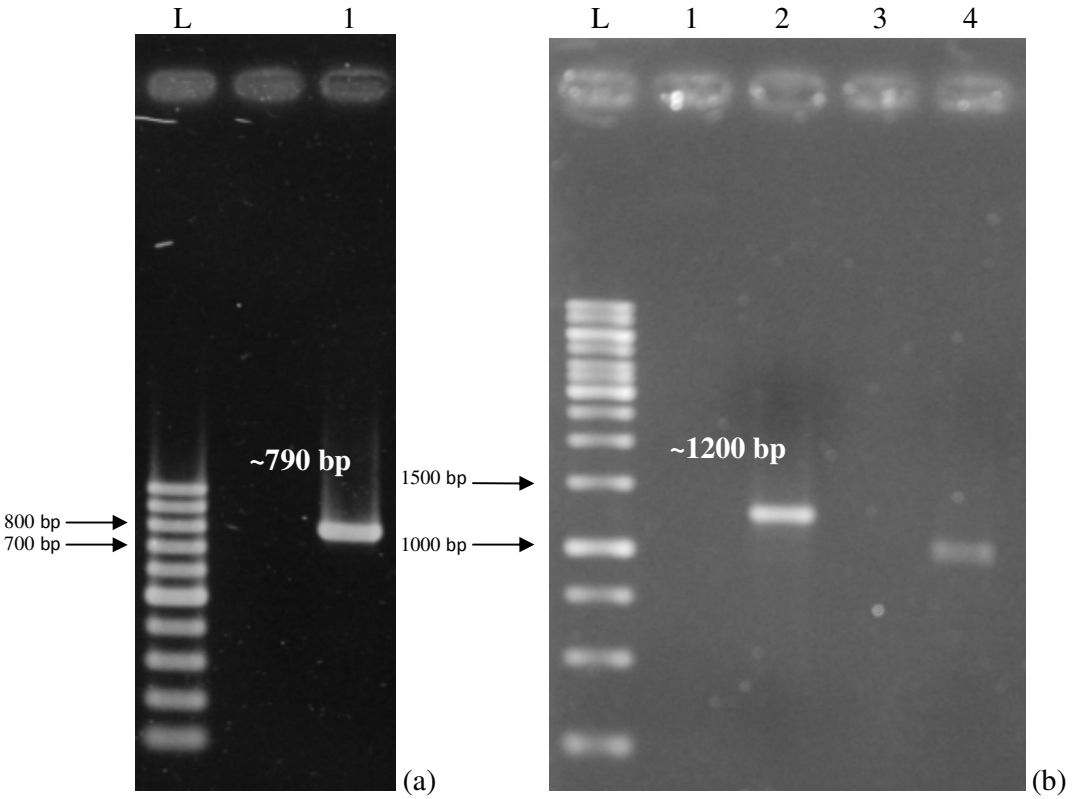

Figure 4.5: Gel electrophoresis of purified PCR products in 1.5% (w/v) agarose.

Lane L in (a) and (b) were loaded with 100 bp and 1 kb DNA ladders, respectively. Lane 1 in (a) was loaded with purified PCR product of putative *tet*D gene, which was amplified from the sample of H16. Meanwhile, Lane 2 and 4 in (b) were loaded with purified PCR products (x and y) of putative *tet*E gene, which were amplified from the samples of H1. The sizes of the purified PCR products were approximately 790 bp for putative *tet*D and approximately 1200 bp for putative *tet*E gene.

4.7 Direct sequencing and nucleotide alignment

The purified PCR products were sent to 1st Base for direct sequencing as described in Section 3.11. The sequences of the putative *tet*D and *tet*E purified PCR products obtained from direct sequencing were then compared with those available in GenBank database using the Blast X software available on NCBI website (http://www.ncbi.nlm.nih.gov/BLAST) as previously described in Section 3.12.

4.7.1 Nucleotide alignment of *tet*D

For putative *tet*D sequence comparison, one purified product of approximately 790 bp in size was sent for sequencing. Table 4.3 shows the top 3 highly similar matches obtained by comparing the sequence of the putative *tet*D purified PCR product with those available in GenBank. The alignment of the sequence of the putative *tet*D purified PCR product with the sequence that has the highest similarity is as shown in Figure 4.6. The sequence of the putative *tet*D purified PCR product was found to be 99% identical and similar (conserved amino acid) to that of class D tetracycline resistance gene of *Photobacterium damselae* subspecies piscicida (ref|YP_908601.1) available in GenBank. One mismatch amino acid residue was observed between the two sequences but there is no gap found. The E. value was 0.0, which indicates a high probability that these two genes are identical. With this, the specificity of the *tet*D primers in the amplification of Tet D was confirmed.

Table 4.3: The top 3 highly similar matches returned by Blast X for the putative *tet*D purified PCR product sequence query.

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
YP_908601.1	Class D tetracycline resistance gene [*Photobacterium damselae* subsp. piscicida]	337	337	98%	0.0	99%
BAC67147.1	*tet*D [*Citrobacter* sp. TA3]	335	335	98%	0.0	99%
P33733.1	Tetracycline resistance protein:ISOTYPE= class D	334	334	98%	0.0	98%

```
>ref|YP_908601.1| class D tetracycline resistance gene [Photobacterium damselae
subsp. piscicida]

GENE ID: 4549496 P91278ORF_003 | class D tetracycline resistance gene
[Photobacterium damselae subsp. piscicida] (10 or fewer PubMed links)

Score =  478 bits (1231),  Expect = 3e-133
 Identities = 247/248 (99%), Positives = 247/248 (99%), Gaps = 0/248 (0%)
 Frame = +3

Query  12   VCFAPLLGRWSDKLGRRPVLLLSLAGAAFDYTLLALSNVLWMLYLGRIISGITGATGAVA  191
            VCFAPLLGRWSDKLGRRPVLLLSLAGAAFDYTLLALSNVLWMLYLGRIISGITGATGAVA
Sbjct  55   VCFAPLLGRWSDKLGRRPVLLLSLAGAAFDYTLLALSNVLWMLYLGRIISGITGATGAVA  114

Query  192  ASVVADSTAVSERTAWFGRLGAAFGAGLIAGPAIGGLAGDISPHLPFVIAAILNACTFLM  371
            ASVVADSTAVSERTAWFGRLGAAFGAGLIAGPAIGGLAGDISPHLPFVIAAILNACTFLM
Sbjct  115  ASVVADSTAVSERTAWFGRLGAAFGAGLIAGPAIGGLAGDISPHLPFVIAAILNACTFLM  174

Query  372  VFFIFKPAVQTEEKPAEQKQESAGISFITLLKPLALLLFVFFTAQLIGQIPATVWVLFTE  551
            VFFIFKPAVQTEEKPAEQKQESAGISFITLLKPLALLLFVFFTAQLIGQIPATVWVLFTE
Sbjct  175  VFFIFKPAVQTEEKPAEQKQESAGISFITLLKPLALLLFVFFTAQLIGQIPATVWVLFTE  234

Query  552  SRFAWDSAAVGFSLAGLGAMHALFQAVVAGALAKRLSEKTIIFAGFIADATAFLLMSAIT  731
            SRFAWDSAAVGFSLAGLGAMHALFQAVVAGALAKRLSEKTIIFAGFIADATAFLLMSAIT
Sbjct  235  SRFAWDSAAVGFSLAGLGAMHALFQAVVAGALAKRLSEKTIIFAGFIADATAFLLMSAIT  294

Query  732  SGWMVNPV  755
            SGWMV PV
Sbjct  295  SGWMVYPV  302
```

Figure 4.6: Comparison between the sequence of the putative *tet*D purified PCR product and the sequence of the Class D tetracycline resistance gene of *Photobacterium damselae* subspecies piscicida available in GenBank (Accession Number YP_908601.1).
The sequence of the putative *tet*D purified PCR product was labelled as 'Query'.

4.7.2 Nucleotide alignment of *tet*E

For putative *tet*E sequence comparison, two purified products (x and y) of approximately 1 kb in size were sent for sequencing. Table 4.4 shows the top 2 highly similar matches obtained by comparing the sequence of the putative *tet*E (x) purified PCR product with those available in GenBank. The alignment of the sequence of the putative *tet*E (x) purified PCR product with the sequence that has the highest similarity is as shown in Figure 4.7. The sequence of the putative *tet*E (x) purified PCR product was found to be 93% identical and 98% similar (conserved amino acid) to that of putative chemotaxis transducer of *Aeromonas hydrophila* subspecies hydrophila ATCC 7966 (reflYP_855453.1) available in GenBank. There is no gap between the two sequences. However, one mismatch amino acid residue was observed. Even though the E. value was 0.0, the sequence of the putative *tet*E (x) purified PCR product (~1200 bp) was probably a non-specific amplification by the *tet*E primers. For the putative *tet*E (y) purified PCR product (~1000 bp), no significant similar sequences was found after comparing with those available in GenBank. Due to this, the specificity of the *tet*E primers has yet to be confirmed or optimised.

Table 4.4: The top 2 highly similar matches returned by Blast X for the putative *tet*E (x) purified PCR product sequence query.

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
YP_855453.1	Putative chemotaxis transducer [*Aeromonas hydrophila* subsp. hydrophila ATCC 7966]	113	113	96%	0.0	93%
YP_001143107.1	Methyl-accepting chemotaxis transducer [*Aeromonas salmonicida* subsp. salmonicida A449]	103	103	96%	0.0	84%

```
>ref|YP_855453.1|putative chemotaxis transducer [Aeromonas hydrophila subsp.
hydrophila ATCC 7966]

gb|ABK37058.1|putative chemotaxis transducer [Aeromonas hydrophila subsp.
hydrophila ATCC 7966]
Length=658

GENE ID: 4490252 AHA_0910 | putative chemotaxis transducer [Aeromonas
hydrophila subsp. hydrophila ATCC 7966] (10 or fewer PubMed links)

 Score =  113 bits (272),  Expect = 6e-24
 Identities = 54/58 (93%), Positives = 57/58 (98%), Gaps = 0/58 (0%)
 Frame = -2

Query  180  SLFEEMSAPFAELVGQVIVKRQSPDWDQANYLMATEIEPALGQLATQLAGQVTETRAE  7
            SLFEEMSAPFAELVGQVI KRQ+PDWDQAN+LMATEIEPALGQLATQLAGQVT+TRAE
Sbjct  230  SLFEEMSAPFAELVGQVISKRQAPDWDQANHLMATEIEPALGQLATQLAGQVTQTRAE  287
```

Figure 4.7: Comparison between the sequence of the putative *tet*E (x) purified PCR product and the sequence of the putative chemotaxis transducer of *Aeromonas hydrophila* subspecies hydrophila ATCC 7966 available in GenBank (Accession Number YP_855453.1).
The sequence of the putative *tet*E purified PCR product was labelled as 'Query'.

4.8 Sample Screening

Fifty-three samples were selected for each gene (*tet*D and *tet*E) for PCR screening. The outcome of the sample screening is shown in Figure 4.8 and 4.9. There were four samples tested positive for *tet*D gene, however, there was no positive sample detected for *tet*E gene. The outcome of PCR screening of *tet*D and *tet*E genes are as summarised in Table 4.10.

CHAPTER 5

DISCUSSION

5.1 Overview

The main objective of this study is to determine the prevalence of *tet*D and *tet*E genes among gram-negative clinical isolates resistant to tetracycline in Malaysia. A total of 53 isolates that were resistance towards tetracycline selected for PCR detection of *tet*D and *tet*E genes.

5.2 Bacterial strains

Escherichia coli accounted for 19 out of the 53 gram-negative clinical isolates (35.8%) that showed resistance towards tetracycline were selected for PCR screening of *tet*D and *tet*E genes as shown in Figure 5.1.

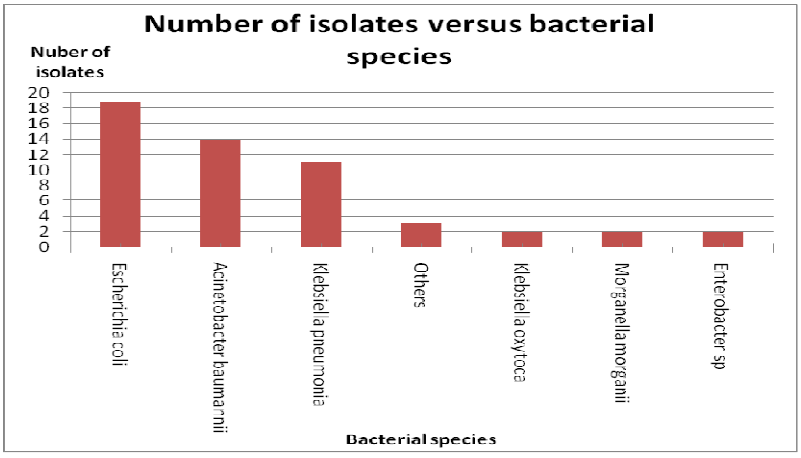

Figure 5.1: Distribution of the 53 tetracycline-resistant gram-negative clinical isolates selected for this study according to bacterial species.
The bacterial species of *Proteus* sp, *Providencia rettgeri*, and *Serratia marcescens* were included in the category of 'others'.

Escherichia coli, *Acinetobacter baumannii* and *Klebsiella pneumonia* are the top 3 prevalent bacteria detected in this study. The high prevalence of *Escherichia coli* agrees with the finding of past epidemiological studies in which *Escherichia coli* was identified as an important pathogen frequently involved in both community and nosocomial infections (World Health Organization [WHO], 2002). It has been known as the most common causative agent for urinary tract infection (UTI) (Zieve, Juhn, & Eltz, 2009). UTI is the second most common type of infection in the body as well as the most common hospital-acquired infections (National Kidney and Urologic Diseases Information Clearinghouse, 2005; WHO, 2002). *Escherichia coli* can also cause hospital-acquired pneumonia and other diseases, such as intra-abdominal infections, traveler's diarrhoea, and many more (Madappa & Go, 2010). This phenomenon was reflected by its isolation from various body sites like swab, urine and blood in this study as shown in Table 4.1.

In order to treat the diseases caused by *Escherichia coli*, antibiotics which include tetracycline are often utilised. As a consequence, the antibiotic-resistant *Escherichia coli* have evolved due to the overuse of antibiotics against these agents (Knight, 1999). Besides that, infections caused by *Escherichia coli* resistance strains may also develop due to the discontinuation of antimicrobial therapy of the patients (Lepelletier, Caroff, Reynaud, & Richet, 1999)

On the other hand, *Acinetobacter baumannii* and *Klebsiella pneumonia* constituted another 14 and 11 of the tetracycline-resistant gram-negative

isolates, respectively. This observation seems to agree with the findings of previous studies, where *Acinetobacter baumanni* was the most frequently detected bacteria in hospital-acquired infections, while *Klebsiella pneumonia* was also responsible for the large portion of nosocomial infections (Tolmasky, Roberts, Woloj, & Crosa, 1986; Villegas & Hartstein, 2003). Heavy use of antimicrobial drugs against these two organisms has enhanced the selection and propagation of antibiotics resistant strains in hospital environment (Madigan, Martiko, Dunlap, & Clarks, 2009).

5.3 Age as a risk factor for infection

In this study, it was observed that a large proportion of the samples that demonstrated tetracycline resistance phenotypes were isolated from people from advanced age. Among the 53 clinical isolates that demonstrated resistance towards tetracycline, 37 were recovered from patients aged 50 and above as shown in Figure 5.2.

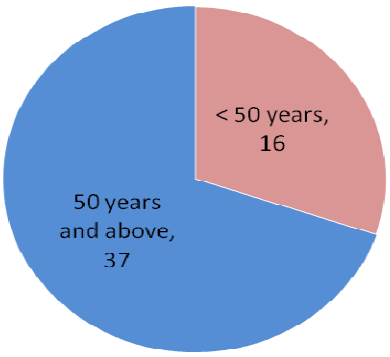

Figure 5.2: Distribution of the 53 tetracycline-resistant gram-negative clinical isolates selected for this study according to the age of patients from which they were isolated from.

Indeed, infectious diseases are more common in people over 50 years (Winn et al., 2006). This is probably due to two main reasons. The first reason is a decline in immune function of these people, leading to lower resistance in their body to combat infections. Secondly, age-associated anatomical changes in them may favour bacterial colonisation (Madigan et al., 2009).

5.4 Template DNA extraction

The method used in this study to extract template DNA was the fast boil method. In a previous study by Freschi, Carvalho, and Oliveira (2005), they stated that this method has similar effeciency to those obtained from commercial kits. However, DNA samples extracted by using this method can be degraded easily and cannot be stored at 4°C for long periods. As a consequence, the extracted DNA in the present study were only kept for at most two weeks old and fresh extraction would be done after that. This is to ensure the integrity of DNA remained intact. Fast boil method, nevertheless, is often selected for DNA template preparation due to its low cost, simple and fast to extract DNA (Freschi et al., 2005; Radji, Malik, & Widyasmara, 2010).

Furthermore, pure and high quality extracted DNA are also crucial for the success in PCR target gene amplification process and sequencing reactions. This is because substances present in the clinical samples, which are PCR inhibitors, can block the nucleic acid amplification process (Bergallo et al., 2006). In this case, Radji et al., in 2010 stated that centrifugation and washing steps in fast boiling technique were sufficient to get rid of PCR inhibitors in the culture medium.

A ratio of absorbance 260 to 280 (A_{260}: A_{280}) was measured on the extracted DNA to determine their concentration and purity. A pure preparation of nucleic acid should have a ratio between 1.8 and 2.0 (Bogner & Killeen, 2005). An A_{260}/A_{280} ratio lies in the range of 1.8 and 2.1 were obtained by the extracted product in this study (Appendix A). This indicates that there were low levels of protein contamination in the products. Although the absorbance method used in this study to determine the amount and purity of the nucleic acid was quick and easy, its accuracy remained limited (Phenix Research Products, 2003).

5.10 Future Studies

Follow-up studies are crucial to expand the understanding of the epidemiology of tetracycline resistance genes in Malaysia. For example, PCR optimisation on *tet*E gene amplification requires more studies, so that this particular gene can be amplified specifically in the absence of other non-specific bands. This optimisation process can be done by changing the concentration of the $MgCl_2$ or primers or the annealing temperature in the existing PCR programs. Prevalence study should also be carried out by using larger sample size, so that the prevalence of both *tet*D and *tet*E genes in Malaysia could be represented fairly. Finding novel antimicrobial drugs is especially important to combat diseases resulting from tetracycline-resistant microorganisms. For instance, pharmacological screening of potential useful inhibitors for prevalent types of tetracycline resistance determinants should be carried out.

CHAPTER 6

CONCLUSION

The main objective of this study is to determine the prevalence of *tet*D and *tet*E genes among gram-negative clinical isolates resistant to tetracyclines in Malaysia using PCR rapid detection method. This study successfully sorted out the distribution pattern of *tet*D that was responsible for tetracyclines resistance seen in some clinical isolates, but it failed to detect the presence of *tet*E in any of the samples. A low prevalence of *tet*D gene was detected among the tetracyclines resistant clinical isolates selected for this study. Out of the 53 gram-negative isolates screened for the presence of *tet*D, only 4 of them were found to be carrying the genetic determinant. In short, *tet*D was found narrowly dispersed among different species of gram-negative bacteria. As for *tet*E, no positive sample was detected among the 53 clinical isolates that show resistance to tetracyclines. The findings strongly suggest that other tetracyclines resistance determinants, instead of *tet*D and *tet*E gene, are responsible for the tetracyclines resistance in all the clinical isolates that were tested negative for both genes. The bacteria in these clinical isolates may carry one of the other known *tet* genes or may have a novel gene. Therefore, it is likely that *tet*D and *tet*E are not the prevalent type of tetracyclines resistance determinants in Malaysia.

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APPENDICES

Appendix A

Table 1: The spectrophotometric analysis data of extracted template DNA
The A_{260}/A_{280} ratio and DNA concentration are shown. The A_{260}/A_{280} ratio lies in the range of 1.8 and 2.1, indicating low levels of protein contamination.

Sample	A_{260}/A_{280} ratio	DNA concentration (ng/µl)
G7	2.017	1215.0
G32	1.995	1028.0
G40	1.986	1062.0
G41	1.938	780.0
G43	1.990	995.0
G55	2.042	1455.0
G58	1.809	783.0
G59	2.008	602.0
G64	1.925	1020.0
H1	2.083	755.0
H4	2.020	376.5
H5	1.968	1373.0
H9	1.958	930.0
H13	2.063	990.0
H15	2.067	542.0
H16	1.935	895.0
H23	2.009	1125.0
H25	2.030	1020
H29	1.828	795.0
H31	2.058	705.0
H33	2.135	593.0
H34	2.068	838.0
H35	1.934	1750.0
H36	2.006	893.0
H38	2.055	745.0
H39	1.899	1178.0
H40	2.019	818.0
H41	1.950	1062.0
H43	1.983	868.0
H44	1.972	522.0
H45	1.865	345.0
H46	1.993	688.0

Table 1 (continued)

H47	2.029	695.0
H48	1.933	1233.0
H49	2.008	642.0
H50	1.908	573.0
H51	1.890	770.0
H52	1.768	858.0
H53	2.031	1660.0
K6	1.894	895.0
K9	1.789	550.0
K10	1.950	590.0
K11	1.731	515.0
K12	1.874	1222.0
K14	1.862	1345.0
K16	1.914	775.0
K19	1.766	605.0
K21	1.951	703.0
K26	1.993	718.0
P8	1.966	1160.0
P9	1.946	1163.0
P11	2.000	945.0
P14	2.007	703.0

Appendix B

Putative *tet*D sequence obtained from direct sequencing (757 bp)

GTTGGGGTGCGGTCTGTTTTGCTCCGCTGCTGGGCAGATGGTCAGATAAG
CTGGGGCGCAGACCGGTGCTGCTGTTATCCCTGGCGGGTGCCGCGTTTGA
TTACACACTGCTGGCACTGTCCAATGTGCTGTGGATGTTGTATCTCGGGCG
GATTATCTCCGGGATCACTGGTGCCACCGGCGCGGTTGCGGCTTCGGTAG
TGGCGGACAGCACGGCGGTCAGCGAGCGTACCGCCTGGTTCGGCCGTCTC
GGTGCGGCCTTTGGTGCCGGGCTGATTGCCGGGCCGGCTATCGGCGGACT
GGCGGGGGATATCTCACCGCATCTGCCGTTTGTCATTGCGGCAATACTGA
ATGCCTGCACCTTTCTGATGGTCTTTTTTATCTTTAAACCGGCGGTACAGA
CAGAAGAAAAACCGGCGGAGCAGAAACAAGAAAGCGCAGGTATCAGCTT
TATCACACTGCTTAAACCTCTGGCGCTGTTGCTGTTTGTCTTTTTTACCGCG
CAGCTTATCGGGCAGATCCCGGCCACTGTCTGGGTATTGTTTACGGAGAG
CCGCTTTGCCTGGGACAGCGCGGCGGTCGGTTTTTCACTGGCGGGACTCG
GGGCGATGCATGCACTGTTTCAGGCGGTGGTTGCCGGGGCGCTGGCAAAA
CGGCTGAGTGAGAAAACCATTATTTTCGCCGGATTTATTGCCGATGCCAC
CGCGTTTTTACTGATGTCTGCTATCACTTCCGGATGGATGGTGAATCCGGT
CAA

Appendix C

Putative *tet*E sequences (x and y) obtained from direct sequencing

Putative *tet*E sequence (x) (187 bp)

GGCGGCTCTGTACTCGGCACGGGTCTCGGTCACCTGGCCGGCCAGCTGGG
TCGCCAGCTGGCCGAGGGCGGGTTCAATCTCGGTCGCCATCAGGTAATTG
GCCTGATCCCAGTCCGGGGACTGACGCTTCACGATCACCTGGCCCACCAG
CTCGGCAAAGGGGGCCGACATCTCCTCGAACAGCGAC

Putative *tet*E sequence (y) (1000 bp)

AGAGTTCTGAAGAATGAAGTGAGAATATTATGGTGGAAAACGTAAGCTA
GTGAAGAAAGTTGGTGTGTTCAGGTCGGTTGTTGTTGGTTGTTGNGGGTGT
GGNGGTGGGGGGGTTGGTTTTAGTGTGAAACCCCCTTTTAAAAAGAGAGT
NGNANATNNGNNGGAAAGGGGGGGTTATCTCTTAAAATAGGGAGAGTGG
AATGATAGATTATATTATTGTATTGAAGTAGTTGTGGGAGGGTGTGGGGA
TGGGGAGAGTGTGAAAAGAATAAGAGAAATGTTTAATGGGTATGGGGTG
ATAGGAGGGGATGAGGTGGTTGTGAGTGTGATTNAAGTGTTGAGGTGGAT
AAGAGAGAATGGAGTGAGATGGATTAGGGATGTGATTATAAAGTTGATG
TAGTGGAAATAGTGGTTATTGATATATTTTTATAAATATTATTGTTNTGTA
GTGTGGGTGTTTTTGGTGTTATATTTATGGAGTGTTAGTGTGTGAGTGATA
TTATGAGTTAATTATGATTTATATGGTGATGGATAATGATGAATGAGAGA
AGAGATGAATGGATGGGAATGTTTGATAGAGGATAATAATAGTGGTGTAT
ATGATATTAATTTGTTAATAGTGTTTTAGGTTAATTTGGGTGTTGTAGTTG
NTGGTGGAGATGTGATAGTGTGAGGTTATTGTTTGTTATGTGTTATGTGAG
TTGATTGGTTGAGTTATAATGAAATGTTATTTGATAGAATGGAAAAATAT
AATAGTTTTGAATTGGAAGGTTTTTTGTGATAATTTATAATATATTTTTAA
GTTTTGAGGTAAATTATATGTTTANTGATAAGGTGTTATTATAGTAATNAT
GTTGTTATTTTTTGTAGAATTAATAATGATTGTAATGGATAGATAAAGAGT
TGAGGTAATAATATAAAAGATTATAAGGATAANGGGAAGAGGGGATGGA
GGAGGAAAAGGAGATGAAGAAAGTGGAAGAGGTGATGTAGGGTTTTT