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INTERRELATIONSHIP BETWEEN *HELICOBACTER PYLORI* AND P-GLYCOPROTEIN: POTENTIAL ROLE IN DRUG RESISTANCE AND CARCINOGENICITY

School of Pharmacy

Interrelationship between *Helicobacter pylori* and P-glycoprotein: Potential role in drug resistance and carcinogenicity

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This thesis is presented for the Degree of
Doctor of Philosophy
of
Curtin University

April 2013

Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

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Abstract

Prior to the 1980`s, stress and lifestyle issues were thought of as the main causes of peptic ulcer disease and gastritis. In 1982, Marshall and Warren discovered that *Helicobacter pylori* were present in patients with chronic gastritis and gastric ulcers. Since that time it has been found that *H. pylori* infection occurs in up to 15 to 20% of the population and that the bacteria is also linked to development of duodenal ulcers and gastric cancer world-wide. Current treatment guidelines for the eradication of *H. pylori* infection recommend the combination of a proton pump inhibitor and antibiotics which has successfully treated up to 90% of *H. pylori*-positive cases. However, cases of *H. pylori* resistance have been reported world-wide. Bacterial resistance and other issues such as poor compliance, low gastric pH and high bacteria load contribute to the increasing episodes of eradication failure.

P-glycoprotein is believed to be associated with *H. pylori* eradication therapy failure. This drug efflux protein is present along the intestinal epithelial lining and functions as a biological barrier by pushing toxins and xenobiotics out of cells. As most of the drugs used to treat *H. pylori* infection are involved with P-glycoprotein pathway, their pharmacodynamic and pharmacokinetic may be affected by alterations of P-glycoprotein expression. The variability of P-glycoprotein gene polymorphisms, particularly *MDR1 C3435T* may potentially influence the therapy outcomes. In addition, P-glycoprotein expression may be manipulated to prevent *H. pylori* attachment to the human intestinal epithelium which may limit its potential to cause gastrointestinal disease.

This study was divided into two phases: – **Phase 1** (*Helicobacter pylori* and P-glycoprotein expression) and **Phase 2** (*Helicobacter pylori* attachment to gastrointestinal cell lines). Phase 1 of the study aimed to evaluate the P-glycoprotein level expressed in human antral and duodenal tissue in the presence of *H. pylori*, to examine the relationship between P-glycoprotein expression and its genetic variant, particularly *MDR1 C3435T* as well as to assess the level of P-glycoprotein expression and the potential influence of *CYP2C19* polymorphisms in subjects who experienced resistance towards *H. pylori* eradication therapy. Phase 2 of the study was designed to investigate the extent of *H. pylori* attachment to the human

gastrointestinal cell lines, namely LS174T and Caco-2, in the presence of a potent inducer and a potent inhibitor for P-glycoprotein.

In Phase 1 of the study, 91 participants who were referred for upper gastroendoscopy at the Endoscopy Unit, Sir Charles Gairdner Hospital, Nedlands, Western Australia from October 2010 to July 2011 were recruited. Participants were required to answer two questions prior to the endoscopy procedure and their medical records were reviewed to get further information regarding their past medical and medication history. Antral and duodenal biopsies were collected during the endoscopies and their level of P-glycoprotein expression was quantified using Western Blot analysis. The *H. pylori* infection status was determined through various methods including bacterial culture and polymerase chain reaction. Participants` venous blood were also analyzed to determine their *MDR1 C3435T* polymorphisms. In addition, a subgroup of participant (n =11) who had failed *H. pylori* eradication therapy also had antibiotic sensitivity testing and *CYP2C19* polymorphisms undertaken.

This study had a total of 33 participants who were classified as *H. pylori* –positive including 11 whom had been diagnosed with failure of *H. pylori* eradication therapy. The remaining 58 participants were *H. pylori* – negative. Dyspepsia was the most common indications for endoscopy in this study followed by anaemia and epigastric pain. In this study, gender and age were found to have no association with human gastrointestinal P-glycoprotein. P-glycoprotein expression was significantly higher in the duodenum than the antrum and interestingly, a significant difference was found in P-glycoprotein expression between *H. pylori*-positive and *H. pylori*-negative subjects ($p = 0.028$).

For *MDR1 C3435T* polymorphisms, the *3435TT* genotype appeared to have a significantly lower P-glycoprotein expression than *3435CC* genotype in the antrum ($p=0.041$). The homozygous *3435TT* subjects who were *H. pylori* –positive also demonstrated a significant difference in their P-glycoprotein expression compared to those who were *H.pylori* – negative ($p = 0.029$). In the resistance group, the subjects also demonstrated higher relative antral P-glycoprotein expression levels compared to those in *H.pylori* – negative group ($p = 0.036$). Most subjects who had failed eradication therapy demonstrated resistance to clarithromycin (72%), metronidazole

(63.6%) or both (54.5%). About 90% of the resistance subjects were cured after being treated with a second-line regimen consisting of rifabutin, amoxicillin and rabeprazole.

In the second phase of the study, Caco-2 and LS174T cell lines were used to assess bacterial attachment. Two strains of *H. pylori* causing gastric cancer, namely *G27* and *J99* were used to see how well they attached to the gastrointestinal cell lines within certain incubation periods. *Escherichia coli* W and *Staphylococcus aureus* were used as positive controls in this study. A potent P-glycoprotein inhibitor, valspodar (PSC-833) was used to evaluate whether inhibition of P-glycoprotein would have any influence on bacterial attachment. Additionally, the level of P-glycoprotein expression in the LS174T cell lines was induced with rifampicin for the bacterial attachment study.

Generally, all bacteria used in the study showed an increasing pattern of attachment to the Caco-2 cells over a 4 hour study period. *H. pylori G27* demonstrated significantly higher bacterial attachment to Caco-2 cells at 30, 90, 180 and 240 minutes when PSC-833 was introduced compared to the control cells ($p < 0.05$). *H. pylori J99* was also observed to have higher bacterial attachment to Caco-2 cells, significantly at 90 and 180 minutes in the presence of the P-glycoprotein inhibitor compared to the control Caco-2 cells ($p < 0.05$). Increased bacterial attachment to Caco-2 cells over a 4 hour study period was also observed with *E. coli* and *S. aureus.*

On the other hand, the bacterial attachment of both *H. pylori G27* and *J99* strains to LS174T cell lines were found to be unaffected by the levels of P-glycoprotein expression. In contrast, *E. coli* W demonstrated significantly more bacterial attachment to the LS174T cells that had been pre-incubated with PSC-833 at 90, 120, 180 and 240 minutes than the control cell line ($p < 0.001$). *S. aureus* showed significantly less bacterial attachment to the LS174T cells that had been pre-incubated with rifampicin than the control cell lines at 90, 120, 180 and 240 minutes ($p < 0.001$). *S.aureus* also demonstrated higher bacterial attachment to the LS174T cells that had been pre-incubated with PSC-833 than the control cell line at all time points ($p < 0.001$).

Phase 1 of this study provides evidence that increased P-glycoprotein expression in the upper gastrointestinal tract is associated with *H. pylori* infection. Further to that, the *3435TT* homozygotes have the capacity to increase P-glycoprotein expression to a greater extent than *3435CC* and *3435CT* homozygotes when being exposed to *H. pylori* which may explain of why *3435TT* subjects have lower risk of gastric cancer. It is postulated that this potential protective mechanism of P-glycoprotein in the human intestinal tract may be exploited through the use of P-glycoprotein inducers, such as rifabutin as part of the eradication regimen.

The second phase of this study demonstrated that manipulating P-glycoprotein expression by the use of inducers and inhibitors may influence the bacterial attachment to gastrointestinal cell lines of *H. pylori* and other bacterial pathogens. Based on these findings, the role of P-glycoprotein expression in *H. pylori* infection may not be dismissed. *H. pylori* infection induces P-glycoprotein in the gastrointestinal tract and genetic polymorphism does influence its protein expression. With high P-glycoprotein expression, *H. pylori* attachment to the gastrointestinal cell lines may be limited, subsequently reducing the risk of the development of gastrointestinal diseases.

Acknowledgements

I would like to express my deepest gratitude to my supervisor, Professor Jeffery Hughes, who has provided me with guidance, encouragement and advice to complete my doctoral programme. At many stages in the course of this programme I benefited from his advice, particularly so when exploring new ideas. I would also like to thank my co-supervisor, Dr Andrew Crowe, whose helpful guidance enabled me to complete my laboratory work and provided constructive criticism in writing my thesis.

Special thanks to Dr. Hooi Ee and his helpful staff from Department of Gastroenterology/Hepatology, Sir Charles Gairdner Hospital, Nedlands without which I would not be able to collect the needed samples for my project. I would like to thank Professor Barry Marshall and his staff, particularly Dr Chin Yen Tay and Ms Fanny Thirriot from *Helicobacter pylori* Research Laboratory, University of Western Australia, Nedlands for the training and permission to utilize their facilities during my research.

I would like to acknowledge Dr. Richard Parsons who provided me with statistics consultation. I would also like to express my thanks to Dr. Simon Fox and Erin Bolitho who facilitated my laboratory work in the first phase of my study. Staff from School of Pharmacy, Curtin University also deserves my sincere thanks, their assistance has meant more to me that I could not express.

I thank Ministry of Higher Education, Malaysia and National University of Malaysia for the generous sponsorship and support for my study. Finally, I would like to thank my husband, my parents and family for all the support and encouragement that they had given me to complete my doctorate.

Communications related to the thesis

1. Publication

- P-glycoprotein expression in *Helicobacter pylori*-positive patients: The influence of *MDR1* C3435T polymorphism. Omar M, Crowe A, Parsons R, Ee H, Tay CY, Hughes J. J Dig Dis 2012;13;414–420

2. Manuscript (under review)

- Potential exploitation of P-glycoprotein expression levels in treatment - resistant *Helicobacter pylori* subjects

3. Conferences

- Oral presentation "P-glycoprotein expression in *Helicobacter pylori*-positive patients". 7th conference on New Frontiers in Microbiology and Infection on *Helicobacter pylori* - from basic science to clinical issues. Villars sur Ollon, Switzerland (2- 6 October 2011)

- Poster presentation " Do *Helicobacter pylori* and *MDR1 [C3435T]* polymorphism influence P-glycoprotein expression in human ?". Keystone Symposia on Proteomics, Interactomes, Stockholm, Sweden (7-12 May 2012)

- Poster presentation "P-glycoprotein expression level in treatment - resistant *Helicobacter pylori* patients". Joint ASCEPT-APSA 2012 Conference, Sydney Australia (2-5 December 2012).

4. Seminar

- Poster presentation "P-glycoprotein expression in *Helicobacter pylori*-positive patients". Mark Liveris Health Science Research

Student Seminar, Faculty of Health Sciences, Curtin University Western Australia (14 November 2011)

- Poster presentation "P-glycoprotein expression in *Helicobacter pylori*-positive patients". The Inaugural Curtin Health Innovation Research Institute [CHIRI] Conference, Curtin University Western Australia (24 November 2011)

- Oral presentation "P-glycoprotein expression level in treatment - resistant *Helicobacter pylori* patients". Mark Liveris Health Science Research Student Seminar, Faculty of Health Sciences, Curtin University Western Australia (9 November 2012)

CHAPTER 1

INTRODUCTION

CHAPTER 1 INTRODUCTION

1 Overview

Helicobacter pylori has been reported to be associated with a number of gastrointestinal diseases in humans and this bacterial infection may occur in up to 15 to 20% of the population [1]. The combination of a proton pump inhibitor and antibiotics successfully treats up to 90% of *H. pylori*-positive cases. However, there have been numerous reports worldwide observing an increasing incidence of antibiotic resistance which is linked to failed *H. pylori* eradication [2]. Among the common reasons for therapy resistance are poor patient compliance [3], molecular bacteria resistance [4], low gastric pH [5] and a high bacterial load [6]. Other mechanisms have also been proposed for multidrug resistance associated with *H. pylori* eradication failure including reduced permeability of the cell membrane to certain drugs, active efflux of the drug from resistant cells and reduced binding of the drugs to their intracellular targets [7].

P-glycoprotein (also known as multidrug resistance protein 1, *MDR1* or ATP-binding cassette sub-family B member 1, *ABCB1*) is a large transmembrane glycoprotein (170kDa) that belongs to a large super family of highly conserved ATP-binding cassette (ABC) transporters [8]. In humans, P-glycoprotein is expressed on the luminal surface of the intestinal epithelial, the renal proximal tubule, bile canalicular membrane of the hepatocytes, the placenta and the blood brain barrier where it acts as a defence mechanism towards harmful xenobiotics [9]. Proton pump inhibitors such as omeprazole, pantoprazole and lansoprazole are P-glycoprotein substrate and their absorption from gastrointestinal tract might be affected by alterations of P-glycoprotein expression. The variability of P-glycoprotein gene polymorphism, such as *MDR1 C3435T* has been proposed in the literature as one of the reasons for different treatment outcomes in various diseases [10]. However, little literature exists pertaining to *H. pylori* infection and its eradication therapy and *MDR1 C3435T* polymorphisms. Assuming that P-glycoprotein expression will vary depending on its polymorphism and *H. pylori* as Class 1 carcinogen could lead to gastric cancer, there has been discussions as to whether a particular allele may have an effect on the risk

of gastric cancer [11-12]. There are also other genetic polymorphisms that have been linked to different outcomes in *H. pylori* eradication therapy, for example in CYP2C19 and CYP3A4. The inter-individual differences in those genes activity could produce changes in drug pharmacokinetics and pharmacodynamics, thus they may potentially affect the success of treatment given.

Meanwhile, the attachment of *H. pylori* to the human gastric epithelium is a crucial step in its carcinogenicity [13]. *H. pylori* can penetrate the protective gastric mucosa lining, producing urease that damages the mucus layer which neutralises gastric acid [14]. The gastric tissue is thus prone to the damaging effects of acid and pepsin, leading to the ulcer formation in the stomach or duodenum. If then, increases in P-glycoprotein expression levels in gastric mucosa tissue influences the extent of *H. pylori* binding and manipulation of these levels could potentially prevent the development of *H. pylori*-associated gastrointestinal disease, such as gastric cancer.

1.1 Research questions

Does P-glycoprotein expression influences the outcome of *H. pylori* eradication therapy and is *H. pylori* attachment to the gastrointestinal mucosa influenced by P-glycoprotein expression levels?

1.2 Research hypotheses

a) *H. pylori* induces P-glycoprotein expression in the human gastrointestinal tract, contributing to the eradication therapy failure.

b) P-glycoprotein polymorphism influences the *H. pylori* eradication therapy outcomes.

c) P-glycoprotein expression influences *H. pylori* attachment gastrointestinal epithelial cell lines.

1.3 Focus of dissertation

There are plentiful studies on influence of drug efflux transport protein, P-glycoprotein on the drug-drug interactions, yet little is known about the effect of P-glycoprotein in *H. pylori* eradication therapy. The main goal of this dissertation was to study the effect of *H. pylori* on P-glycoprotein expression and to determine whether P-glycoprotein expression plays a role in *H. pylori* eradication therapy outcomes.

1.4 Research objectives

a) To evaluate the level of P-glycoprotein expressions in human antral and duodenal tissues in relation to the *H. pylori* infection status.

b) To examine the relationship between P-glycoprotein expressions and its genetic variant, particularly *MDR1 C3435T*.

c) To assess the level of P-glycoprotein expression and the potential influence of CYP2C19 polymorphism in subjects who experienced resistance towards *H. pylori* eradication therapy.

d) To investigate the extent of *H. pylori* attachment to the human gastrointestinal cell lines, namely LS174T and Caco-2, in the presence of a P-glycoprotein potent inducer or inhibitor.

1.5 Research significances

This study demonstrated the changes in P-glycoprotein expression levels in human gastrointestinal tract in the presence of *H. pylori*. This study also highlighted the association between P-glycoprotein expression and the prevalence of *MDR1 C3435T* and is the first to illustrate the prevalence of *MDR1 C3435T* polymorphisms in the Australian population. In this study, subjects who experienced resistance towards *H.*

pylori eradication therapy showed elevated P-glycoprotein levels in the presence of *H. pylori* irrespective of their *MDR1 C3435T* polymorphism. Finally, this study also demonstrated the extent of *H. pylori* attachment to the human gastrointestinal cell lines in regard to the P-glycoprotein expression levels.

CHAPTER 2

LITERATURE REVIEW

CHAPTER 2 LITERATURE REVIEW

2 Overview

The adenosine triphosphate (ATP)-binding cassette (ABC) transporter super family is the biggest transporter gene family that is essential to transport specific substances including amino acids, sugars, inorganic ions, polysaccharides and peptides across the extra and intracellular lipid membranes [15]. ABC transporters employ the energy released from ATP hydrolysis to pump substrate across the membrane against a concentration gradient [16]. Classic ABC transporters usually consist of two transmembrane domains (TMD) and two nucleotide-binding domains (NBD) encoded by a single polypeptide [17].

ABC transporters are found in bacteria, yeast, protozoa, insects and animals [18]. In humans, there are 48 members of the ABC super family that are grouped into seven families based on their structure, namely *ABCA, ABCB, ABCC, ABCD, ABCE, ABCF* and *ABCG* [19] as shown in **Table 2-1**. The ABC transporters play significant roles in human, from bile acid homeostasis to the protection of tissue from cytotoxic substances [79]. Impairment of this mechanism has been associated with certain diseases. For instance, cystic fibrosis which occurs due to a mutation in the encoding gene on chromosome 7 that impairs cystic fibrosis transmembrane regulator protein (CFTR) synthesis, a member of the multidrug-resistance-associated-protein (MRP) subfamily [80].

Only a few human ABC transporters are known to be involved in xenobiotic transport. For example, *ABC1* which are located in the intestine, liver, kidney and blood brain barrier have their substrates such drugs as digoxin, cyclosporine and loperamide [81]. A list of ABC transporters with their drug substrate is shown in **Table 2-2**.

Table 2-1: List of ABC transporters, location, function and disease associated with the genes

Subfamily	Member	Function	Associated Disease	Reference
ABCA	*ABCA1*	Cholesterol and phospholipids transport	Tangier Disease	[20-21]
			Dementia	[22]
			Alzheimer's Disease	[23]
			Atherosclerosis	[24]
	ABCA2	Drug resistance	Small cell lung cancer	[25]
		Cholesterol and phospholipids transport	Alzheimer`s Disease	[26-28]
	ABCA3	Drug resistance	Childhood acute myeloid leukemia	[29]
	ABCA4	Transport prorogated N-retinylide- phosphotidylethanolamine	Stargardt macular dystrophy	[30-31]
		Lipid transporter in myelination process	Unknown	[32]
	ABCA7	Peripheral phospholipids metabolism	Unknown	[33]
	ABCA12	Transport glucosyl-ceramides	Lamellar ichthyosis	[34]
	ABCA13	Cholesterol and phospholipids transport	Schizophrenia, bipolar disorder, depression	[35]
ABCB	*ABCB1*	Drug resistance	Parkinsonism	[36]
	ABCB2	Peptide transport	Immune deficiency	[37]

Subfamily	Member	Function	Associated Disease	Reference
	ABCB3	Peptide transport	Immune deficiency	[38]
	ABCB4	Bile-acid transport	Cholelithiasis, sclerosing	[39]
			cholangitis, cirrhosis	[40]
	ABCB5	Drug resistance	Unknown	[41]
	ABCB6	Iron transport	Unknown	[42]
	ABCB7	Hematopoiesis	X-linked sideroblastic anemia	[43]
			with ataxia	
	ABCB8	Drug resistance	Unknown	[44]
	ABCB9	Drug resistance	Unknown	[45]
	ABCB10	Drug resistance	Unknown	[46]
	ABCB11	Bile-acid transport	Hypercholesterolemia	[47]
ABCC	*ABCC1*	Drug resistance	Unknown	[48]
	ABCC2	Drug resistance	Unknown	[49]
		Bile-acid transport	Dubin-Johnson Syndrome	[50]
			Non-alcoholic fatty liver	[51]
	ABCC3	Bile-acid transport	Hepatotoxicity	[52]
	ABCC4	Platelet adenine nucleotide storage	Unknown	[53]

Subfamily	Member	Function	Associated Disease	Reference
	ABCC5	Fetus protection	Abnormal placenta development	[54]
	ABCC6	Unknown	Pseudoxanthome elasticum	[55]
	ABCC7	Chloride ion channel	Cystic fibrosis	[56-57]
	ABCC8	Insulin receptor	Persistent infancy hypoglycemia	[58]
	ABCC9	Chloride ion channel	Heart disease	[59]
	ABCC10	Drug resistance	Unknown	[60]
	ABCC11	Drug resistance	Abnormal axillary odor	[61]
	ABCC12	Drug resistance	Unknown	[62]
ABCD	*ABCD1*	Fatty acid transport	X-linked adrenoleukodystrophy	[63]
	ABCD2	Fatty acid transport	Unknown	[64]
	ABCD3	Fatty acid transport	Unknown	[65]
	ABCD4	Fatty acid transport	X-linked adrenoleukodystrophy	[66]
ABCE	*ABCE1*	Eukaryotic post-termination ribosomal recycling	Unknown	[67]
ABCF	*ABCF1*	Cellular immunity	Unknown	[68]
	ABCF2	Drug resistance	Breast cancer	[69]
	ABCF3	Tumorigenesis	Melanoma	[70]
ABCG	*ABCG1*	Apoptosis in macrophage	Unknown	[71]

Subfamily	Member	Function	Associated Disease	Reference
		Cholesterol transport	Unknown	[72]
	ABCG2	Drug resistance	Unknown	[73]
	ABCG4	Cholesterol transport	Changes in metabolic pathway	[74]
	ABCG5	Sterol transport	Sitosterolemia	[75-76]
	ABCG8	Sterol transport	Sitosterolemia	[77-78]

Table 2-2: List of ABC transporters, primary tissues distribution and substrates

Gene	Protein	Primary tissue distribution	Substrate
ABCB1	*MDR1*, P-glycoprotein	Kidney, liver, intestine, brain, lymphocytes	Digoxin, cyclosporine, paclitaxel, vinca alkaloids, loperamide, erythromycin
ABCB11	*BSEP*	Liver	Vinblastine, tamoxifen citrate
ABCC1	*MRP1*	Ubiquitous	Vinca alkaloids, methotrexate, etoposides
ABCC2	*MRP2*	Liver	Vinca alkaloids, methotrexate, pravastin, irinotecan, cisplatin
ABCC3	*MRP3*	Liver, kidney, small intestine	Doxorubicin, vincristine, methotrexate, cisplatin
ABCC4	*MRP4*	Prostate, liver, brain, kidney	Nucleoside analogues (9-(2-phosphonylmethoxyethyl) adenine), nucleobase analogues (6-mercaptopurine, methotrexate)
ABCC5	*MRP5*	Brain, heart, placenta	Nucleoside analogues (cladribine, gemcitabine), nucleobase analogs (5-fluorouracil, 6-mercaptopurine)
ABCC6	*MRP6*, *MOAT-E*	Liver, kidney	Pentapeptide
ABCG2	*MXR*, *BRCP*, *ABCP*	Placenta, liver, small intestine	Mitoxantrone, doxorubicin, topotecan, methotrexate, irinotecan

Reference: [82-83]

2.1 P-glycoprotein

2.1.1 Introduction

The most widely studied human ABC transporter is *ABCB1*, which encodes P-glycoprotein. P-glycoprotein is a 170-180 kDa adenosine triphosphate (ATP)-dependent efflux protein that was first identified by Juliano and Ling in 1976 in Chinese hamster ovary cells which exhibited colchicine resistance [84]. P-glycoprotein belongs to a large super-family of ATP- binding cassette (ABC) transporter proteins that is composed of two homologous halves; each consists of a nucleotide binding domain and a transmembrane domain separated by a flexible linker region (**Figure 2-1)** which is important for the proper interaction of the two halves or ATP sites [85, 86]. P-glycoprotein has a large hydrophobic area in the transmembrane domain and drug binding domain located within the membrane.

Although the mechanism of P-glycoprotein transport is not well understood, it is believed that mediated action is energy dependent [87]. It has been hypothesized that the transport pathway of P-glycoprotein is initiated by the binding of drug and ATP. The drug binds to the protein membrane on the inward facing high-affinity site (cytoplasmic) and is pushed out through a conformational change that transforms the protein membrane to a low-affinity outward facing site (extracellular). P-glycoprotein is believed to have more than one binding sites not including that two ATP-binding domains. During the catalytic cycle, ATP binding is stimulated by P-glycoprotein substrates causing dimerization in the nucleotide binding domain leading to large structural changes [88]. This results in an outward facing conformation that allows drug movement to the extra-cellular space. Thus, inhibition of P-glycoprotein transport of a drug can be triggered by either competition for drug binding and ATP-hydrolysis binding sites or from the blockage of ATP-hydrolysis process.

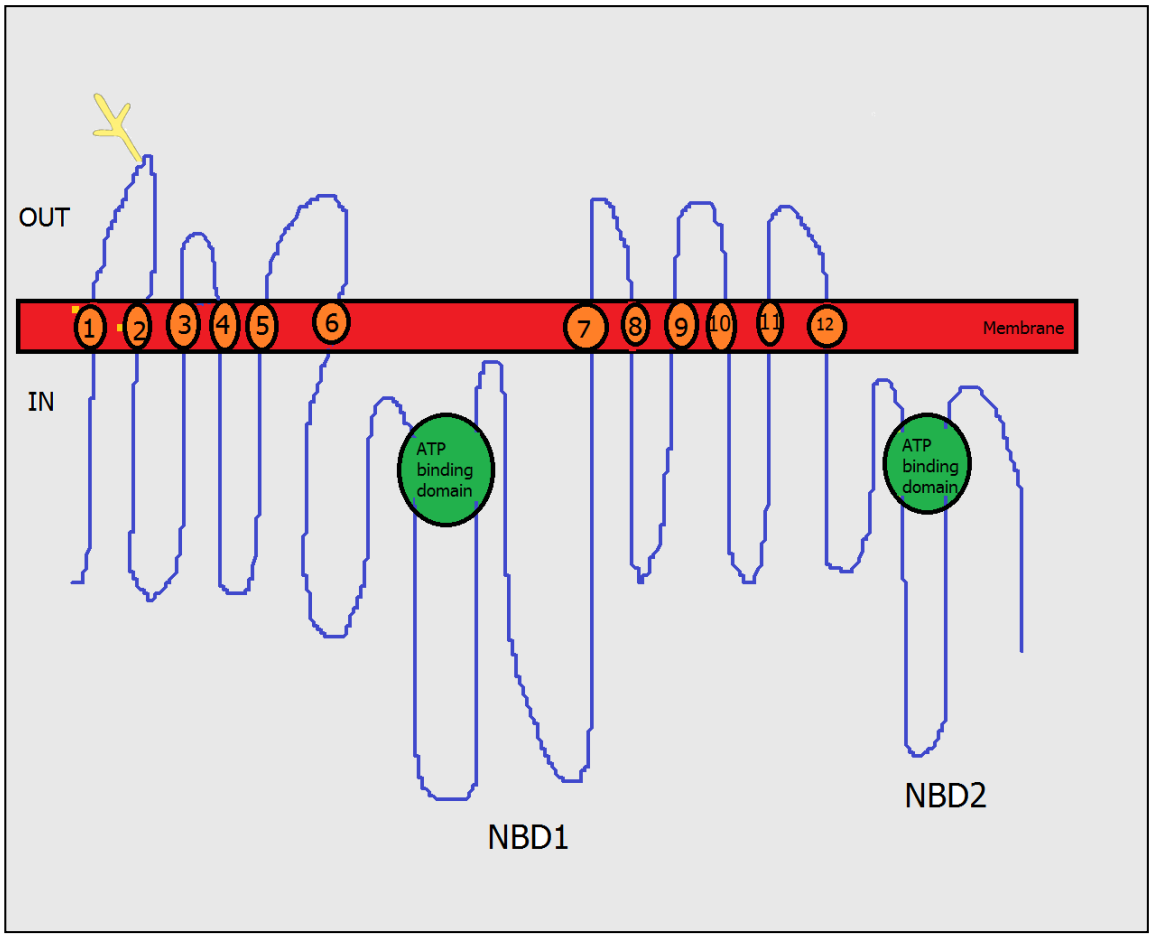

Figure 2-1: Schematic structural organization of P-glycoprotein. Each half contains a highly hydrophobic domain with six transmembrane-α – helices and a hydrophilic domain located at the cytoplasmic face of the membrane, nucleotide binding domain 1 (NBD1) and 2 (NBD2), containing an ATP-binding site. (adapted from [85], drawing by M. Omar)

Paine et al [89] reported there were no difference between healthy men and women found for P-glycoprotein expression. However, changes in P-glycoprotein expression according to the age group have been seen. According to Pilarski et al [90], the efflux of R123 in B-cells increased in early childhood and reached maximum levels in adulthood before decreasing with age. This finding was supported by Machando et al [91] who measured the R123 efflux from $CD4^+$ and CD8+ T cells in subjects aged between 0 to 80 years. The P-glycoprotein function in both cells were highest in cord blood, then markedly decreased with age and reached the lowest levels in the 70 - 80 years age group.

In another study, the elderly group (i.e. those aged between 70 to 90 years old) tended to show a higher expression of *ABCB1* gene from $CD4^+$ and *CD8+* T cells and lower intracellular concentration of R123 compared to the young group age between 22 to 26 years old [92]. A progressive increase in P-glycoprotein activity as a function of age was also observed in the 35 to 50 years age group based on R123 efflux from normal human bone marrow *CD34+/CD45+* stem cells [93]. The activity of P-glycoprotein in the 50-82 years age group was lower, however not significantly lower than the other age groups. In another study using a group of kidney transplant recipients, Lown et al [94] found that P-glycoprotein expressions in the intestinal cells were not significantly correlated with age. The differences between the various studies` findings might be contributed by the differences in methodologies, types of cell studied, sample size and age group distribution.

2.1.2 Location of P-glycoprotein

P-glycoprotein is present in various locations in the human body including gastrointestinal tract epithelium, renal proximal tubule, bile canalicular membrane of the hepatocytes, adrenal gland, testes, placenta and blood-brain barrier [95]. **Figure 2-2** illustrates the organ distribution of P-glycoprotein found in human tissues. In the liver, P-glycoprotein has been detected in a highly polarized fashion on the biliary canalicular surface of hepatocytes and on the apical surface of small biliary ducts [96]. High

polarized P-glycoprotein has also been found on the apical surface of columnar epithelial cells of the colon and jejunum. Similarly, the apical surface of small pancreatic ducts also expressed high polarized P-glycoprotein levels. As opposed to other tissues, P-glycoprotein in the adrenal gland was not present in a polarized distribution but being detected in both the adrenal cortex and medulla.

It has been reported that P-glycoprotein expression is not uniform along the gastrointestinal tract. The *MDR1* mRNA expression has been found to progressively increase from stomach to the colon when measured in normal tissues which supports P-glycoprotein`s role as access barrier in the gastrointestinal tract [97]. Mouly and Paine [98] reported that "among individuals, the relative P-glycoprotein levels varied 2.1 fold in the duodenal /proximal jejunal region, 1.5- to 2.0- fold in the middle/distal jejuna region and 1.2-fold to 1.9-fold in the ileal region" which indicated that the differences between P-glycoprotein expression in the intestine may influence bioavailability of drugs in human. Similarly, *mRNA* expression has also been found to increase from stomach and duodenum with the highest level was recorded in left descending colon [99]. The increased of P-glycoprotein seen in that study occurred without influence of gender, age or medication taken by the subjects. On the other hand, Ueda et al [100] demonstrated that over expression of P-glycoprotein is associated with an increase in transcripts of a gene called *MDR1* (Multi Drug Resistance) which has been shown to be the gene product of *MDR1*. Another member of the P-glycoprotein gene family that exists in humans is *MDR3;* the other three members of the family are present in mice (*mdr1a, mdr1b* and *mdr2*). Mice have two genes encoding P-glycoprotein namely the *mdr1a* gene which is predominantly expressed in the intestine, liver and brain as well as testis, and the *mdr1b* gene that is be abundantly found in the adrenals, placenta and ovaries [101]. In mice, *mdr1a* is expressed at relatively high levels in the intestines, intermediate levels in the brain and testes and at lower levels in the kidney but not in the adrenal gland, pregnant uterus or placenta [102]. In contrast, mouse *mdr1b* is expressed at high level in the adrenals, pregnant uterus, placenta and kidney and at a relatively lower level in the brain and testes but is not expressed in the intestines.

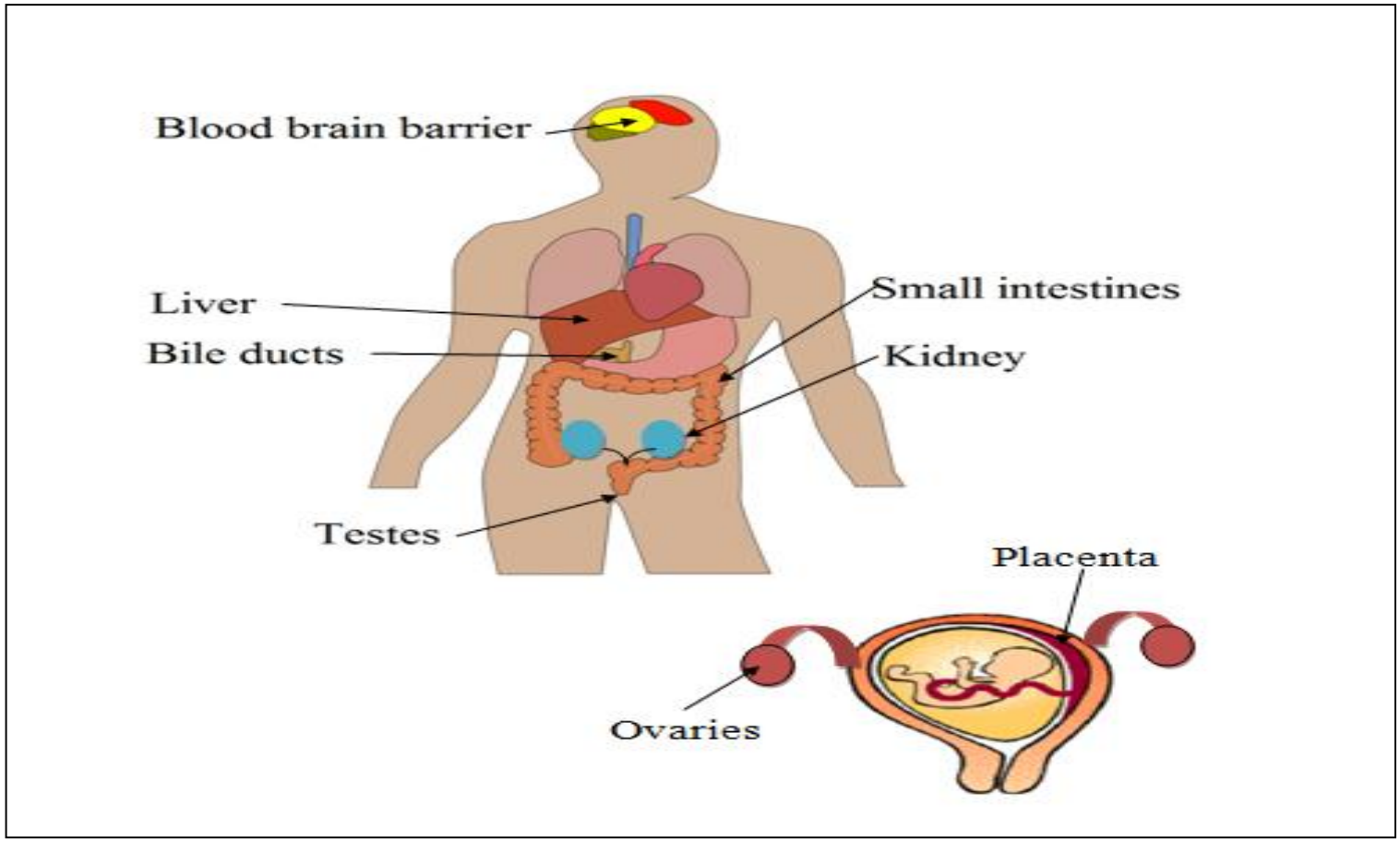

Figure 2-2: Illustration of the organ distribution of P-glycoprotein in humans (original by M. Omar)

2.1.3 Role of P-glycoprotein

Both human *MDR1* and mouse *mdr1a/1b* genes act as drug transporter by pushing out substrates of the cells [101]. P-glycoprotein is also involved in active transport of drugs back to the lumen after passive absorption into the enterocytes. Because of its localization, P-glycoprotein appears to have a greater impact on limiting cellular uptake of drugs from the blood circulation into the brain and from the intestinal lumen into epithelial cells than on enhancing the excretion of drugs out of hepatocytes or the renal tubules into the adjacent luminal space [102].

Due to the abundant expression of P-glycoprotein in gastrointestinal tract, it is believed that the protein acts as a body defence mechanism against toxic xenobiotics and drugs in humans by excreting those compounds into the intestine lumen [103-104]. P-glycoprotein also acts to excrete those compounds into the bile and urine, as well as preventing their accumulation in brain and testes. Further, over expression of P-glycoprotein has often been associated with drug resistant in cancer patients where it acts as xenobiotic pump which makes certain cancer cell resistant to selected chemotherapeutic agents.

P-glycoprotein expression has also been associated with altered secretion of cytokines and progressive T cell-mediated immune deficiency when tested in T-lympocytes in mice, human T –lympocytes and liver, intestine, kidney, lymphocytes and epithelium in mice [105-107]. There are various P-glycoprotein substrates ranging from small organic cations, carbohydrates, amino acids and antibiotics to macromolecules such as polysaccharides and proteins [108]. There is also a broad range of overlapping substrate specificities and tissue distribution for *CYP3A* and P-glycoprotein where both proteins act synergistically as a protective barrier to the orally administered drugs [109].

P-glycoprotein plays a major role as a drug efflux transporter and influences the regulation of drug absorption, distribution and elimination [110]. In addition to anti-neoplastic agents, P-glycoprotein also transports saquinavir, colchicines, dexamethasone, cortisol, rhodamine 123, digoxin, cimetidine, morphine and cimetidine [111]. A list of common P-glycoprotein substrates as well as inducers and

inhibitors is shown in **Appendix I** [112-116]. The P-glycoprotein inhibition will result in an increase of substrate plasma levels and tissue distribution. On the other hand, induction of P-glycoprotein will cause lower drug plasma levels, which may lead to therapy failure. Both inhibition and induction of P-glycoprotein may lead to drug interactions in human, particularly for drugs with a narrow therapeutic index [112].

It is difficult to assess the mechanism of P-glycoprotein inhibition when its substrates and inhibitors are given simultaneously. For instance, a daily dose of 160 mg verapamil caused a 40% increased in digoxin plasma concentration while 240 mg verapamil caused a 60 to 80% increase; suggesting a dose-dependent P-glycoprotein inhibition [117]. The interaction resulting in increased digoxin absorption and decreased elimination which in turn leads to accumulation of digoxin in cardiac tissue. Interestingly, Fenner et al [118] found only four co-medications namely valspodar, quinidine, amiodarone and cyclosporin A significantly increased levels of digoxin. The dissimilarities of the findings could be explained by the different mechanisms that are responsible for P-glycoprotein inhibition. For example, verapamil inhibits the transport function in a competitive manner without interrupting the cyclic activity of ATP hydrolysis of P-glycoprotein while cyclosporin A, on other hand, inhibits transport function by interfering with both substrate recognition and ATP hydrolysis [119-120].

Additionally, some herbal ingredients commonly used in complementary and alternative medicines and dietary phytochemicals have been reported to vary P-glycoprotein expression and/or activity [112]. Constituents such as piperine, ginsenosides, silymarin found in milk thistle has been reported to inhibit P-glycoprotein activity *in vitro,* whereas curcumin, curcuminoids and several catechins from green tea are reported to reduce P-glycoprotein expression and activity *in vitro*. Similarly, constituents of grapefruit and orange juice were also found to inhibit P-glycoprotein expression and/or activity.

Although it is difficult to prove directly the effect of P-glycoprotein in humans, a clinical study has shown that quinidine, a brain and intestinal P-glycoprotein inhibitor significantly increased oral morphine maximum plasma concentrations up

to two-fold but had no influence its elimination rate [121]. In cancer patients, the oral bioavailability of paclitaxel has been found to be increased by 50% when cyclosporin A was co-administered [122]. Similarly, another study reported that the bioavailability of docetaxel increased up to 88% when given in combination with cyclosporin A [123]. These results suggested that there is involvement of P-glycoprotein as barrier to the absorption of anti-cancer drugs.

P-glycoprotein expression can be up-regulate by certain drugs, hence reducing the intracellular concentration of co-administered P-glycoprotein substrate as well as limiting their pharmacological efficacy. For example, using a Caco-2 cell line, Silva et al [124] demonstrated that P-glycoprotein expression and activity was significantly increased after doxorubicin exposure which subsequently reduced paraquat cytotoxicity. Antiretroviral agents such as non-nucleoside HIV-1 reverse transcriptase inhibitors (NNRTIs) have been shown to increase P-glycoprotein expression [125-126]. Further, the chronic morphine administration has been found to induce P-glycoprotein expression in the brain and proposed as one of the mechanisms of morphine tolerance as more morphine is required as more is effluxed out from the brain [127].

Apart from drugs, certain diseases have been reported to increase P-glycoprotein expression. Tsujimura et al [128] observed that P-glycoprotein was over-expressed on rheumatoid arthritis lymphocytes compared with normal lymphocytes. P-glycoprotein over-expression on lymphocytes therefore may cause efflux of corticosteroids and disease-modifying antirheumatic drugs from lymphocytes, resulting in drug resistance in patients with highly active rheumatoid arthritis. Likewise, P-glycoprotein expressions in the peripheral lymphocytes of the systemic lupus erythematosus patients were observed to be significantly higher compared with that of the healthy controls [129]. In that study, there was a positive correlation observed between disease activity and P-glycoprotein expression levels among systemic lupus erythematosus patients suggesting that the high expression levels of P-glycoprotein in the peripheral lymphocytes in this group of patients may lead to poor disease control by systemic corticosteroids.

2.1.3.1 Role of P-glycoprotein in drug pharmacokinetics

2.1.3.1.1 Role of P-glycoprotein in drug absorption and distribution

Most orally administered drugs will be absorbed in the small intestine where the presence of *CYP3A4* and P-glycoprotein may reduce the intracellular concentration of drug substrates by metabolism and transmembrane efflux, respectively [130]. **Figure 2-3** illustrates the intestinal disposition of substrate by P-glycoprotein.

The colon carcinoma cell line, Caco-2 is often used as an *in vitro* model for absorption studies since it expresses abundant P-glycoprotein but less oxidizing cytochrome P450, particularly the isoform *CYP3A4* [131]. When this model is used, the study drugs are added to one side of the monolayer and the rate of transport or diffusion to or from either media compartment is measured. Functional P-glycoprotein is expressed along the apical surface of Caco-2 cell monolayers and has been shown to mediate efflux from the basolateral to apical media compartment. Hunter et al [133] first demonstrated by using the Caco-2 cell line that the transport of vinblastine and docetaxel were higher when measured from basolateral-to-apical compared to apical-to-basolateral. This indicated that drug-efflux at the apical surface renders the epithelium relatively impermeable to the substrate. However, apical-to-basolateral transport was enhanced significantly in presence of verapamil that blocked P-glycoprotein efflux function, suggesting P-glycoprotein plays significant role in drug absorption. Drug absorption after oral administration was found to increase by P-glycoprotein inhibition and be further enhanced when *CYP3A4* was suppressed [134]. The efflux function of intestinal P-glycoprotein was further demonstrated by Hochman et al [135] who showed that indinavir metabolites were selectively secreted into the apical compartment in the absence of cyclosporin A in the Caco-2 cell lines. The metabolites then were released into both basolateral and the apical compartment suggesting that it actively been effluxed by the P-glycoprotein.

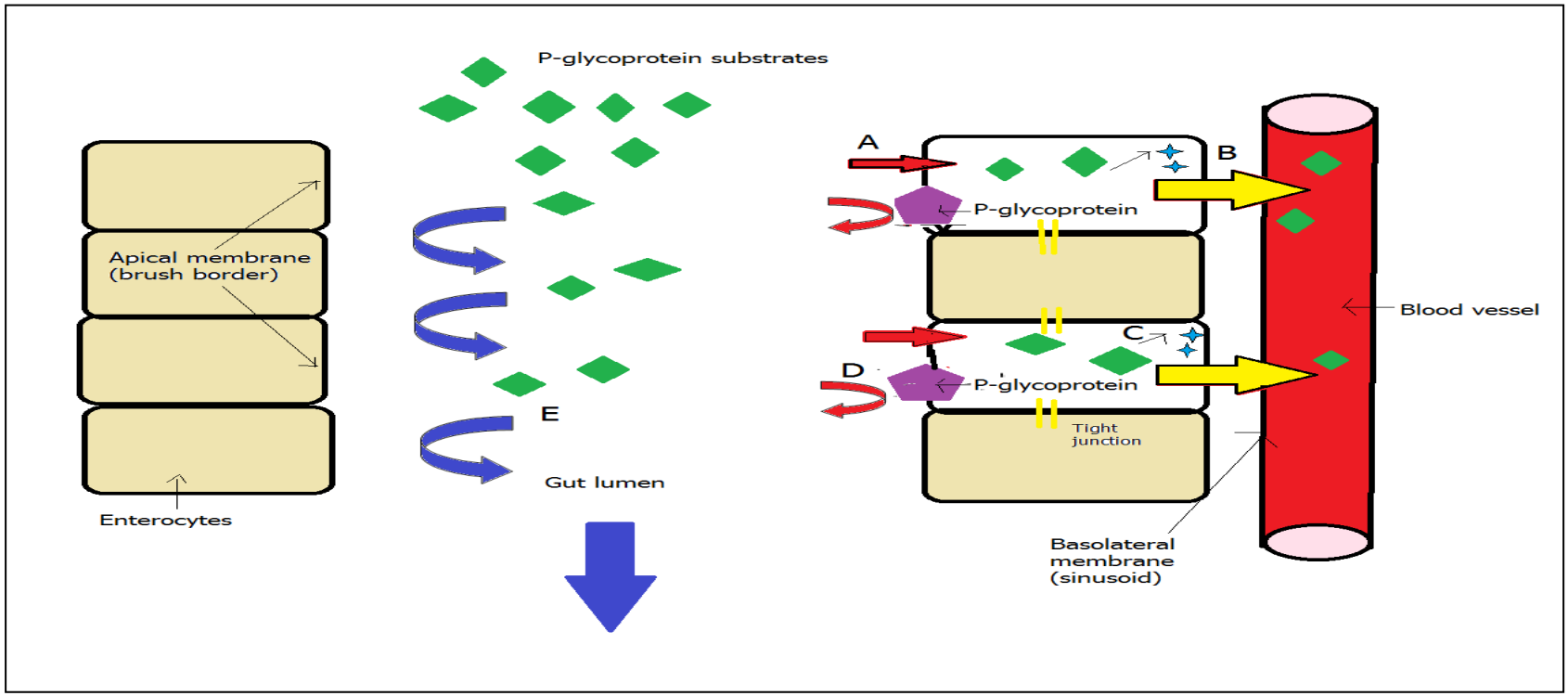

Figure 2-3: Intestinal disposition of substrates by P-glycoprotein. (A) P-glycoprotein substrates are absorbed from the intestinal lumen into enterocytes. (B) Absorption of substrates from enterocytes into the circulation. (C) Substrates are metabolized in the enterocytes. (D) Substrates are secreted back into the intestinal lumen facilitated by P-glycoprotein. (E) Movement of substrates through the intestinal lumen for elimination in feces (adapted from [132], drawing by M. Omar)

Fricker et al [136] reported that cyclosporin A permeation basolateral to apical was greater than apical to basolateral. Further, the authors found the P-glycoprotein-mediated transport *in vitro* was inhibited in the presence of vinblastine and daunomycin. When they administered cyclosporin A enterally to a group of healthy volunteers, they found that the absorption of cyclosporin A was dependent on the location of drug absorption, greatest from stomach, then the jejunum/ileum and finally the colon. This decrease in absorption exhibited a marked correlation ($r = 0.994$) to the expression of mRNA for P-glycoprotein over the gastrointestinal tract (stomach < jejunum < colon). This finding did support the fact that difference in intestinal P-glycoprotein expression influence the rate of drug`s bioavailability. Similarly, the absorption of talinolol, a P-glycoprotein substrate was found to be lower at the distal site of the small intestine compared to the proximal site [137]. The drug bioavailability after distal administration was only half of that proximal intestinal site. This site-dependent characterization absorption provides strong support that difference levels of P-glycoprotein expression may contribute to the variability of drug absorption.

Meanwhile, Greiner et al [138] demonstrated that concomitant rifampin therapy affects digoxin disposition in humans by induction of P-glycoprotein in a group of eight healthy volunteers who received a single-dose of 1 mg orally and intravenously of digoxin before and after co-administration of rifampin (600 mg/day for 10 days). The plasma concentration of oral digoxin was significantly lower during rifampin treatment which was accompanied by an increased in intestinal P-glycoprotein of 3.5 ± 2.1–fold. However, that effect was less observed when subjects received digoxin intravenously. This lead to the idea that P-glycoprotein may act as a drug efflux protein transporter that forced out foreign compounds from enterocytes into the intestinal lumen when they begin to be absorbed across the epithelial cells. This process will be repeated along the intestinal tract and since those discarded xenobiotics can be reabsorbed into the enterocytes before another cycle of extrusion by P-glycoprotein, the exposure to the drug-metabolizing enzymes will be increased [139].

Ito et al [134] found that drug absorption after oral administration was increase by P-glycoprotein inhibition and further enhanced when *CYP3A4* was suppressed. This demonstrated the importance of both drug efflux and metabolism in drug oral bioavailability. P-glycoprotein as mentioned above prolongs drug absorption time due to repeated cycles of absorption and efflux, and in doing so it increases the parent drug's exposure to *CYP3A4*. P-glycoprotein may also remove *CYP3A4* metabolites from inside cells further enhancing the extent of *CYP3A4* metabolism [134,140].

2.1.3.1.2 Role of P-glycoprotein in drug metabolism and excretion

P-glycoprotein in the liver has been suggested to be critical to the metabolism and elimination of certain drugs which are P-glycoprotein substrates, such as tacrolimus. As demonstrated by Chiou et al [141], the mean hepatic intrinsic clearance of tacrolimus was reduced by 10-fold in P-glycoprotein knockout mice compared to the normal mice, suggesting that P-glycoprotein influences the access of substrates to the site of elimination.

Interestingly, Sparreboom et al [143] found that P-glycoprotein could limit the oral uptake of paclitaxel and mediates direct excretion of the drug from the systemic circulation into the *mdr1a* ($^{-/-}$) mice intestinal lumen. The drug plasma concentration was higher in that group compared to wild-type mice after drug administration, both oral and intravenous. The oral bioavailability of paclitaxel was increased from 11% in wild-type mice to 35% in knock-out mice. Furthermore, the total faecal excretion in *mdr1a* ($^{-/-}$) mice was significantly lower compared to wild-type mice although no significant difference in term of biliary excretion was found in both groups.

2.1.3.2 Role of P-glycoprotein in drug pharmacodynamics

There is growing evidence indicating that P-glycoprotein also contributes to the biochemical or physiological effects of drugs in the human body. P-glycoprotein does influence the distribution of drugs and metabolites to the site of action but not in the relationship between substrate concentration and effect. For example, Sadeque et al [144] found that loperamide produced no respiratory depression when administered alone, but respiratory depression occurred when the drug was co-administered with quinidine, a known inhibitor of P-glycoprotein. Loperamide is a substrate for P-glycoprotein and is not associated with central opiate effects such as respiratory depression at usual or even high clinical doses, reflecting the fact that loperamide can not gain access to the brain in the present of P-glycoprotein.

Furthermore, using Visual Analogue Scales (VAS), subjective self-assessment of feelings or mood states was measured by Kharasch et al [145] in 12 subjects who received methadone after a dose of quinidine. Although the quinidine increased the plasma concentrations of oral methadone, the study found that quinidine had no effect on methadone pharmacodynamics, suggesting that P-glycoprotein did not appear to be a determinant of the access of methadone to the brain.

In a study involving 10 healthy volunteers, pre-treatment with rifampicin (600mg daily for 5 days) was found to significantly reduce both the total absorption and peak concentration of glyburide and to shorten its elimination half-life [146]. These changes in the pharmacokinetics of the glyburide were accompanied by a reduction in its blood glucose lowering effects. In the case of glipizide, the rifampicin pre-treatment was found to reduce the its overall absorption, although to a less extent than glyburide (AUC reduction 22% vs. 39% for glyburide) and its elimination half-life by over an hour (3.0 hours to 1.9 hours). These changes were however not accompanied by significant changes in blood glucose concentrations. In the case of glyburide, the interaction is probably due to induction of either *CYP2C9* or P-glycoprotein or both. The increased systemic elimination of glipizide is likely to be due to *CYP2C9* induction.

2.1.4 P-glycoprotein (*ABCB1*) polymorphisms

ABCB1 was among the first genes to be fully sequenced in the coding and regulatory regions [147-148]. Majority of the single nucleotide polymorphisms (SNPs) involved in non-coding regions and do not affect the P-glycoprotein amino acid sequence. The SNPs can be linked to altered oral bioavailability, drug resistance and susceptibility to human disease. As for *C3435T* SNPs, the Japanese demonstrated similar C-allele frequency to the other Asian populations while higher frequency of the C-allele was found in the African population [149].

2.1.4.1 P-glycoprotein polymorphisms and diseases predisposition

Polymorphism in P-glycoprotein has been associated with predisposition to certain clinical conditions although there were contradicting results reported, for example in inflammatory bowel disease. Inflammatory bowel disease constitutes of chronic diseases of the gastrointestinal tract in which ulcerative colitis and Crohn's disease are the two main diseases [150]. Genome-wide association studies have been performed to identify genetic factors that may contribute to these disorders.

Schwab et al [151] reported a strong association between *MDR1 C3435T* polymorphisms and ulcerative colitis in a group of German subjects although no effect for that particular gene polymorphism was observed in Crohn`s disease. Similarly, Ho et al [152] reported that in Scottish subjects, the *3435TT* genotype was found more commonly in subjects with ulcerative colitis, however there was no association seen with *G2677T* and inflammatory bowel disease. In another study, Iranian subjects with ulcerative colitis also showed higher frequency of the *3435T* allele [153]. On the other hand, an association between *MDR1 C3435T* with Crohn`s disease was observed in a group of Spanish subjects, suggesting that the *MDR1* gene may be a risk factor for inflammatory bowel disease [154].

In contrast, there was no association reported for neither *MDR1 C3435T* nor *G2677T/A* with overall disease susceptibility although *C3435T* polymorphism was

found significantly increased in subjects with ileoclonic Crohn`s disease [155]. Another genotyping study conducted amongst Hungarian subjects also showed that the *MDR1 C3435T* and *G2677T/A* alleles were not associated with the inflammatory bowel disease [156]. Dudarewick et al [157] reported that although the *3435CC* genotype was present more frequently among Polish patients with inflammatory bowel disease than matched controls, there was no indication that *C3435T* gene is a risk factor for inflammatory bowel diseases such as ulcerative colitis and Crohn's disease. Similarly, although the 3435*CT* allele was the most frequently found in Greek subjects with ulcerative colitis, there was no association found with the *C3435T* polymorphism and ulcerative colitis [158].

Other examples of P-glycoprotein *(ABCB1)* gene polymorphisms and diseases disposition are shown in **Table 2-3.** Of all *ABCB1* genes, *3435C/T, G2677T/A* and *C1236T* have been the most studied single-nucleotide polymorphisms. Overall, it does appear that *ABCB1* gene polymorphisms may predispose people to certain diseases such as mental and behavioral disorders. Variability in findings between disease predisposition and gene polymorphisms, especially in carcinoma however suggest that the genetic polymorphism may be influenced by other factors such as ethnic differences and geographic locations.

Table 2-3: P-glycoprotein *(ABCB1)* polymorphisms associated with disease predisposition

Targeted gene polymorphism	Study Population	Disease	Finding	Reference
G2677 A/T	French subjects (207 cases and 482 controls)	Parkinson`s disease	No association between disease and gene polymorphism	[159]
1236 C/T *2677 A/G/T* *3435 C/T*	288 Swedish patients and 313 control subjects	Parkinson`s disease	*1336 C >T* associated with Parkinson`s disease	[160]
3435 C/T	599 European patients and controls	Parkinson`s disease	Significant association with disease risk due to pesticide exposure	[161]
G2677 A/T	182 Spanish white men, 86 cancer-free controls and 96 lung cancer patients	Lung cancer	*3435 TT* allele has significant association with lung cancer risk	[162]
3435C/T	Turkish subjects (79 non-small cell lung cancer patients)	Non-small cell lung cancer	No association between disease and gene polymorphism	[163]
3435C/T *G2677T/A* *C1236T*	Caucasian subjects (177 small cell lung cancer patients)	Small cell lung cancer	*G2677T>A, 3435CT, 3435TT* and *2677T/A-3435T* haplotype had a longer progression-free survival rate	[164]
rs418737 *rs1128503* *rs10276036*	102 osteosarcoma patients	Osteosarcoma	Significant association between *ABCB1* polymorphism and survival rate	[165]

Targeted gene polymorphism	Study Population	Disease	Finding	Reference
3435 C/T	Epidemiological studies (3,829 cases and 6,193 controls)	Breast cancer	*3435 TT* allele has significant association with high risk of breast cancer	[166]
3435C/T	39 samples of breast cancer tissues	Breast cancer	No association between disease and gene polymorphism	[167]
3435C/T	106 patients and 77 controls	Breast cancer	No association between disease and gene polymorphism	[168]
3435C/T G2677T/A C1236T	Chinese subjects (1173 breast carcinoma patients and 1244 controls)	Breast carcinoma	*C3435T, G2677T/A* variations and haplotype *3435T-1236T-2677T* related to the risk and clinical outcomes of breast carcinoma	[169]
1236 C/T 2677 A/G/T 3435 C/T	309 patients from the Australian Ovarian Cancer Study	Ovarian cancer	*2677G* allele associated with progression-free survival	[170]
1236C/T 3435C/T	Brazilian population (109 patients)	Acute myeloid leukemia	*CC* allele associated with better prognostic	[171]
rs3789243	Norwegian population (167 carcinomas, 990 adenomas, 400 controls)	Colorectal adenoma and colorectal cancer	No association between disease and gene polymorphism	[172]
rs1202168, rs868755	3,662 German subjects (1809 colorectal cancer cases and 1853 controls)	Colorectal cancer	*rs1202168T* and *rs868755T* carriers had an increased of colorectal cancer	[173]

Targeted gene polymorphism	Study Population	Disease	Finding	Reference
2677G/T	Iranian subjects (60 cancer patients and 60 controls)	Colorectal cancer	*2677GG* associated with high P-glycoprotein expression in cancerous region	[174]
3435C/T	Iranian population (118 patients and 137 controls)	Colorectal cancer	C allele associated in decreased susceptibility to colorectal cancer	[175]
3435 C/T	Bulgarian subjects (146 patients and 160 controls)	Sporadic colorectal cancer	No association between disease and gene polymorphism	[176]
3435 C/T	Japanese subjects (150 *H. pylori*- positive gastritis, 292 gastric cancer, 215 gastric ulcer and 163 duodenal ulcer patients)	Gastric cancer and peptic ulcer disease	No association between disease and gene polymorphism	[177]
3435 C/T	102 patients with surgically resected gastric cancers	Gastric cancer	*3435TT* and *3435CT* were significantly associated with a shorter progression-free survival and overall survival	[178]
3435C/T	48 gastric cancer patients	Gastric cancer	*3435TT* allele associated with high incidence of gastric cancer	[179]
3435 C/T	Chinese subjects (284 controls and 244 patients with end stage renal disease)	End stage renal disease	*3435TT* allele has significant association with progression of end stage renal disease	[180]

Targeted gene polymorphism	Study Population	Disease	Finding	Reference
1236 C/T *2677 A/G/T* *3435 C/T*	116 Swedish subjects	Psychosis	*3435TT* allele has significant association with social and clinical needs	[181]
129 C/T *1236 C/T*	Iranian subjects (200 healthy controls and 332 epileptic patients)	Epilepsy	High risk of disease resistance in *CC* and *CT* allele carriers	[182]
3435C/T *G2677T/A* *C1236T*	Chinese children's population (91 patients and 368 controls)	Epilepsy	*3435TT* associated with increased risk of having epilepsy syndrome	[183]
rs2188524 *3435C/T* *C1236T* *G2677A/T*	Japanese subjects (631 major depressive disorders patients and 1100 controls)	Major depressive disorders	*3435TT* allele was significantly more common in patients than in the controls	[184]
3435C/T	Caucasian subjects (40 cases, 40 controls)	Cannabis dependence	*3435 CC* allele is independently associated with cannabis dependence	[185]
3435 C/T	97 patients with Behçet's disease	Behcet`s disease	No association between disease and gene polymorphism	[186]
3435 C/T	104 patients with Behcet`s disease	Behcet`s disease	*3435 TT* allele has significant association with treatment response	[187]

2.1.4.2 P-glycoprotein polymorphism affecting drug pharmacokinetics

Digoxin is a well-known probe for the activity of P-glycoprotein *in vivo* and *in vitro* [188]. Several variants in the *ABCB1* have been found to influence P-glycoprotein expression in subjects who taking cardiac glycosides. For example, the relationship between the *ABCB1* gene with digoxin pharmacokinetic was first reported by Hoffmeyer et al [189] where a significant correlation was found between polymorphism in exon 26 (*C3435T*) with P-glycoprotein expression and function in duodenum. The homozygous *TT* subjects were associated with lower P-glycoprotein expression levels compared with homozygous *CC* and recorded the highest digoxin plasma levels. In a subsequent study, Johne et al [190] reported that homozygous *TT* showed faster and more complete absorption of digoxin as compared to *CC* genotype carriers. In addition, *C3435T* was found to significantly affect digoxin concentrations with homozygous *TT* subjects presenting with higher digoxin plasma concentrations than *CC* and *CT* subjects [191]. Similarly, Sakaeda et al [192] reported that the serum concentrations of digoxin was higher in the Japanese subjects whom carried T-allele at exon 26 of the *MDR1* gene after single oral of drug administration as compared to *CC* and *CT* subjects. Kurata et al [193] also reported that after a single oral dose of 0.5 mg digoxin in healthy Japanese subjects, a 70% higher digoxin exposure was detected in *TT* subjects compared to *CC* subjects. However, Gerloff et al [194] using 1 mg of digoxin dose, demonstrated that saturation of the transport capacity of intestinal P-glycoprotein, and suggested this would lead to the lack of effect of *MDR1* on digoxin absorption.

Chowbay et al [195] reported that in Caucasian and Japanese subjects, there was no major influence of the *C3435T* on levels of digoxin although maximum concentration for digoxin were lower in *3435CC* subjects compared with *3435TT* genotypes and the oral availability of digoxin appeared to be lower in wild-type (*CC*) Caucasian populations compared with wild-type Japanese subjects. However, it is not only digoxin which demonstrates pharmacokinetic variations in association with *ABCB1* polymorphisms, the pharmacogenetic studies of cyclosporine also showed conflicting results. The cyclosporine intracellular and blood concentration in the

3435T carriers were found to be increased up to 1.7-fold and 1.2-fold respectively in 64 stable organ transplant recipients suggesting that *ABCB1* polymorphisms could influence drug immunosuppressive activity [196]. The homozygous *TT* carriers also tend to develop more cyclosporine nephrotoxicity as compared to the *CC* genotype [197-198]. On the other hand, no association was demonstrated between *ABCB1* polymorphisms and cyclosporine absorption in Japanese kidney transplant recipients [199]. In addition, Haufroid et al [200] reported that no association was found between trough cyclosporine blood concentrations or dose requirement with *ABCB1* genotype in stable renal transplants subjects. The variations discussed have implications for the therapeutic efficacy of many drugs especially for P-glycoprotein substrates that could explain inter-individual variability in dosing requirements and pharmacological response. Other examples of the influence of polymorphism in *ABCB1* on number of drug pharmacokinetics are shown in **Table 2.4.**

2.1.4.3 P-glycoprotein polymorphism affecting drug pharmacodynamics

Most studies on *MDR1* genotype focused more on pharmacokinetics; however there have been few studies conducted aimed at determining the influence of *MDR1* genotype on drug pharmacodynamics, particularly on P-glycoprotein substrates. Among different *MDR1* variants, the *C3435T* genotype has been extensively studied to determine any association with pharmacodynamic effect of certain drugs **(Table 2.5).** For instance, the inhibition of P-glycoprotein may allow loperamide to cross the blood-brain barrier and subsequently exerts the central opioid effects which could be contributed by a particular genotype [225]. The authors also demonstrated that *TT* genotype was associated with the significant increased of the miotic effects of loperamide when quinidine was co-administered. On the other hand, another study reported that there was no association found between *MDR1 C3435T* and the respiratory response in a group of healthy subjects who received 16 mg of loperamide alone, although minimal respiratory response was recorded [226]. Nevertheless, the different observations in those clinical responses could be attributed by pharmacokinetic changes which alter the dose-response relationship rather than involving the pharmacodynamic pathway.

Table 2-4: *ABCB1 (C3435T)* polymorphism associated with drug pharmacokinetics

Polymorphism	Study drug	Population	Parameter	Outcome	Reference
3435 C> T	Fluvoxamine	62 psychiatric patients	Fluvoxamine C_{ss}	*CC* had significantly higher concentration to dose ratio at the 200 mg/dose	[201]
3435 C> T	Tacrolimus	32 renal transplant recipients	Tacrolimus body-weight-based dose	No significant association	[202]
3435 C> T	Cyclosporine	106 renal transplant patients	Cyclosporine C_p and AUC	*TT* carriers had association with lower cyclosporine dose	[203]
3435 C> T	Sirolimus	105 healthy volunteers and 50 renal transplant patients	Sirolimus concentration/dose ratio	No significant association	[204]
3435 C> T	Tacrolimus	63 Caucasian renal transplant recipients	Tacrolimus dnC(0), dnAUC (0-12) and dnC(max)	No significant association	[205]
3435 C> T	Efavirenz	67 HIV-positive subjects	Efavirenz C_p	No significant association	[206]
3435 C> T	Tacrolimus	103 Chinese renal transplant recipients	Tacrolimus AUC	*ABCB1* had significant association with dose requirement	[208]

Polymorphism	Study drug	Population	Parameter	Outcome	Reference
3435 C> T	Lopinavir	113 HIV-infected men	Lopinavir C_p	No significant association	[209]
3435 C> T	Tacrolimus	104 full liver transplant patients	Tacrolimus C_p	No significant association	[210]
3435 C> T	Tacrolimus	118 Chinese renal transplant patients	Tacrolimus dose-adjusted concentration (ng/mL per mg/kg/d)	No significant association	[211]
3435 C> T	Cyclosporine	50 renal stable transplant patients	Cyclosporine dose-adjusted trough concentrations	No significant association	[212]
3435 C> T	Prednisolone	95 renal transplant recipients	Prednisolone C_{max}	*CC* carriers had higher prednisolone plasma concentrations	[213]
3435 C> T	Atazanavir	118 Caucasians HIV-infection subjects	Atazanavir Cp	*TT* carriers had lower atazanavir plasma concentrations	[214]

Polymorphism	Study drug	Population	Parameter	Outcome	Reference
3435 C> T	Tacrolimus	50 Chinese liver transplant patients	Tacrolimus concentration/dose ratio	*CC* carriers had significantly low ratio	[215]
3435 C> T	Tacrolimus	44 renal transplant recipients	Tacrolimus C_p	*TT* carrier had significant lower drug blood concentrations	[216]
3435 C> T					
3435 C> T	Tacrolimus	106 renal transplant Chinese recipients	Tacrolimus C_0	*TT* carriers required lower dose compared to CC subjects	[217]
3435 C> T					
3435 C> T	Carbamazepine	84 epilepsy subjects	Carbamazepine C_{ss}	*TT* carriers had a significantly lower drug plasma concentration	[218]
3435 C> T	Efavirenz	121 healthy subjects	Efavirenz C_L	Higher drug bioavailability in *CC* carriers	[219]
3435 C> T	Phenytoin	96 healthy Turkish subjects	Phenytoin C_p	*CC* carriers had significant lower drug level	[220]
3435 C> T	Paclitaxel	10 prostate cancer subjects	Paclitaxel C_L	No significant association	[221]

Polymorphism	Study drug	Population	Parameter	Outcome	Reference
3435 C> T	Tipifarnib	28 Caucasians with advanced solid tumors subjects	Tipifarnib AUC	*TT* allele associated with high AUC	[222]
3435 C> T	Amlodipine	26 healthy subjects	Amlodipine C_p	*CC* carriers had higher drug levels	[223]
3435 C> T	Vincristine	52 childhood acute lymphoblastic leukemia	Vincristine AUC	No significant association	[224]

Abbreviations:

- dose-normalized trough levels (dnC[0])
- dose-normalized area under the curve (dnAUC[0-12])
- dose-normalized maximum concentration (dnC[max])

Table 2-5: *ABCB1 (C3435T)* polymorphism associated with drug pharmacodynamics

Polymorphism	Study drug	Population	Tested Parameter	Outcome	Reference
3435 C> T	Morphine	145 Italian subjects	Pain relief response	*TT* carriers had best response towards morphine	[227]
3435 C> T	Nelfinavir and efavirenz	67 subjects on long term HIV treatment	Rise in CD4-cell count	*TT* carriers had higher rise after 6 month of therapy	[228]
3435 C> T	Nelfinavir and efavirenz	504 subjects on HIV treatment	Decrease in plasma HIV-1 RNA level	*TT* genotype was associated with decreased likelihood of virologic failure	[229]
3435 C> T	Vincristine	34 survivors of acute lymphoblastic leukemia	Motor performance	No association	[230]
3435 C> T	Paclitaxel	10 prostate cancer and 6 advanced transitional cell carcinoma subjects	Level of neutrophils and platelets after drug administration	No association	[231]
3435 C> T	Etoposide and cisplatin	54 small cell lung cancer subjects	Response to chemotherapy	*CC* carrier had better response to chemotherapy	[232]
3435 C> T	Morphine	194 subjects underwent abdominal surgery	Clinical analgesia and side effects	No association	[233]

Polymorphism	Study drug	Population	Tested Parameter	Outcome	Reference
3435 C> T	Carbamazepine	210 Japanese epileptics	Presence of seizure	*TT* carriers had higher number of drug-resistance cases	[234]
3435 C> T	Clopidogrel	60 subjects	Platelet function	No association	[235]
3435 C> T	Efavirenz	59 HIV-infected subjects	HDL-cholesterol concentrations	Increased HDL-cholesterol in *CC* carriers	[236]
3435 C> T	Phenobarbital	60 subjects with idiopathic epilepsy	Seizure frequency	Increased seizure frequency in *CC* carriers	[237]
3435 C> T	Risperidone	59 subjects with schizophrenia	Akathisa and dystonia occurrence	No association	[238]
3435 C> T	Morphine	631 pregnant women underwent elective caesarean section	Incidence of persistence pain post surgery	*TT* carrier had higher incidence of chronic pain after surgery	[239]
3435 C> T	First line antiepileptic	329 epileptic subjects	Drug response	No association	[240]
3435 C> T	Bromperidol	31 acute exacerbated schizophrenic subjects	Schizophrenic symptoms	*TT* carriers had poor symptoms improvement	[241]

2.2 *Helicobacter pylori* and P-glycoprotein expression

2.2.1 *Helicobacter pylori*

Helicobacter pylori is a Gram-negative microaerophilic non invasive spiral bacillus (**Figure 2-4)** that colonizes the gastric mucosa [242]. *H. pylori* has urease activity that catalyze the hydrolysis of urea to ammonia in order to survive in the surrounding gastric acid [243]. This bacteria was initially known as *Campylobacter pylori* before being included in a new genus, *Helicobacter* and renamed as *Helicobacter pylori* in 1989 [244]. The principal reservoir for *H. pylori* infection appears to be the human stomach, especially the antrum [245]. *H. pylori* colonizes the gastric antrum of more that 95% of patients with duodenal ulcer disease. The overall seroprevalance of *H. pylori* infection was reported to be 15.1% in 2002 with seropositively rates increasing progressively with age. These range between 4% in the 1 to 4 years old to about 23.3% in 50 to 59 years old. *H. pylori* infection and use of non-steroidal anti-inflammatory drugs are the predominant causes of peptic ulcer disease, accounting to 48% and 24% of the cases respectively.

Peptic ulcer disease is characterized by mucosal damage secondary to pepsin and gastric acid secretion which usually occurs in the stomach and proximal duodenum [246]. The necrotic mucosa defects that extend through the muscular mucosa into the submucosa will occur when the epithelial cells are damaged by acid and pepsin [247]. Worldwide ulcer prevalence differs according the region with duodenal ulcers dominating in Western populations and gastric ulcers being more frequent in Asian populations [248]. *H. pylori* infection is mainly acquired during childhood and is usually asymptomatic [249]. Around 15 to 20% of people infected will progress to develop peptic ulcer disease or gastric ulcer usually in later life. Modes and risk factors of transmission vary between developing and developed countries [250]. As such, low socioeconomic status constitutes the main risk factor for asymptomatic infection amongst children in developing countries. Whereas the prevalence of *H. pylori* infection in developed countries varies considerably with ethnic background and within each population with age. **Table 2-6** illustrates the prevalence of *H. pylori* infection in selected populations in Australia and New Zealand.

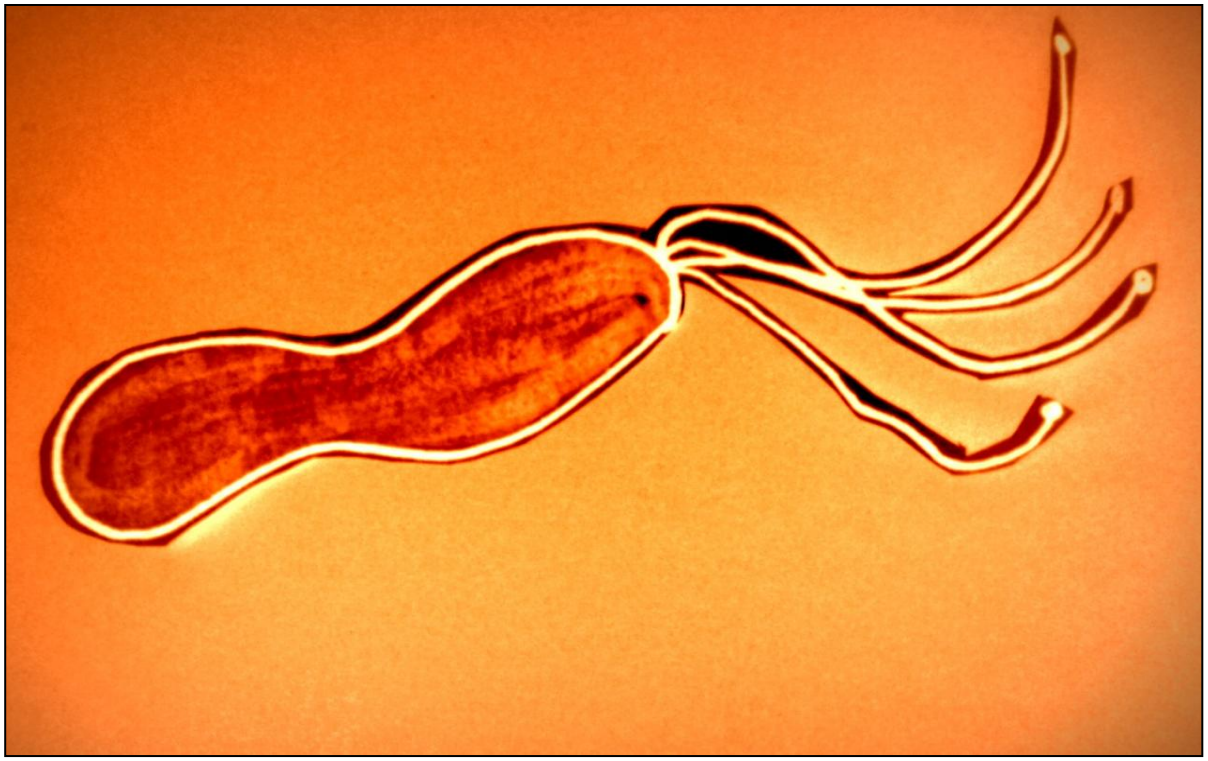

Figure 2-4: Illustration of *Helicobacter pylori* (original by M.Omar)

Table 2-6: Prevalence of *H. pylori* infection in selected populations in Australia and New Zealand

Country	Number of subject	Population	Age range (years)	Diagnostic test	Percentage of positive test	Reference
Australia	1355	Australian adults	18–79	Serology	15.5%	[251]
	520	Indigenous Australian	2-90	^{13}C-Urea breath test	76%	[252]
	273	Random population	20-80	Serology	38%	[253]
	112	Australian adults	19-47	Serology	75%	[254]
	273	Caucasian (controls)			23%	
New Zealand	792	Pacific Islander	12-16	Serology	49%	[255]
		Maori			26.7%	
		Asian			24.7%	
	579	New Zealand`s adults	40-64	Serology	56%	[256]
	190	European			36%	
	195	Maori			57%	
	194	Pacific Islander			73%	

Previous studies have shown that *H. pylori* is present in more than 90% of patients with duodenal ulcer and in 60 to 90% of patients with gastric ulcer [257-259]. The infection is acquired by oral ingestion of the bacterium and is mainly transmitted within families who become the main source of transmission [260]. Once acquired, *H. pylori* evades the host immune system and causes chronic inflammation by reducing the thickness of the mucus gel layer as well as diminishing mucosal blood flow and interacting with the gastric epithelium throughout all stages of the infection [261]. Urease protects the *H. pylori* from the bactericidal effects of gastric acid and helps to enhance the bacterial adherence [262]. Further, gastric acid secretion is increased due to antigens produced, virulence factors and soluble mediators. Chronic gastritis induced by *H. pylori* increases the risk for gastric and duodenal ulcer, distal adenocarcinoma and gastric mucosal lymphoproliferative diseases such as non-Hodgkin's lymphoma.

There is no single test that can be considered the gold standard for the diagnosis and the sensitivity of all endoscopic and non-endoscopic tests that identify active *H. pylori* infection can be reduced by the recent consumption of proton pump inhibitors, bismuth or antibiotics [263]. A 'test and treat' approach is recommended in adult patients under the age of 45 years presenting in primary care with persistent dyspepsia [264]. However, the prevalence of *H. pylori* infection in the elderly is higher than younger patients, accounted about 70% of people with *H. pylori* pathologies and over 50% in asymptomatic patients [265]. It has been reported that only 40 to 60% of elderly patients who were hospitalized for peptic ulcer disease were tested for *H. pylori* infection and that eradication therapy was given to only 50 to 73% of infected patients [266].

The most severe clinical outcome after *H. pylori* infection is gastric cancer which usually occurs in the elderly and for this reason, eradication of the anaerobe in high risk patients is requisite [267]. For elderly patients with dyspepsia, the 'test and treat' strategy may be misleading and potentially dangerous [268-269]. Upper gastrointestinal endoscopy is always indicated for elderly patients with new abdominal symptoms because of the high prevalence of advanced gastric diseases. It is recommended to obtain gastric biopsies at least from both the antrum and the body

of the stomach. A second test for *H. pylori* should be performed in high risk elderly patients if a urease-based or histological test is negative [268].

Therapy with bismuth subsalicylate tablets, 525 mg four times a day; metronidazole, 250 mg or 500 mg three times a day and either tetracycline or amoxicillin, 500 mg four times a day for two weeks has been found to eradicate *H. pylori* in up to 65% to 90% of peptic ulcer disease`s patients [270]. Use of standard triple therapy containing of proton pump inhibitor, clarithromycin and amoxicillin or metronidazole is more successful if extended to more than 7 days [271]. The proton pump inhibitor-clarithromycin-amoxicillin or metronidazole is the first choice treatment in populations with less than 15 to 20% clarithromycin resistance. In populations with less than 40% metronidazole resistance, proton pump inhibitor-clarithromycin-metronidazole is preferable. A sequential treatment consisting of 5 days of a proton pump inhibitor plus amoxicillin followed by 5 additional days of a proton pump inhibitor plus clarithromycin plus tinidazole has been shown to be better than the combination of a proton pump inhibitor plus amoxicillin and clarithromycin for 7 days. *H. pylori* infection is successfully treated in about 90% of all cases with the remaining 10% continuing to harbor gastrointestinal *H. pylori* [272]. **Figure 2-5** summarizes the suggested *H. pylori* eradication regimens.

The common causes of treatment failure in *H. pylori* eradication therapy are patients' non-compliance and antimicrobial resistance of the infecting bacterial strain [273]. Resistance to clarithromycin and metronidazole is one of the major reasons for treatment failure [274]. The prevalence rate of metronidazole resistance among *H. pylori* strains has been reported as high as 30% in North America, in Australia it is around 17% and varies from 5 to 50% in European countries [275]. Resistance to clarithromycin will reduce treatment efficacy by an average of 55.4% [276]. For adults in Northern Europe, the global prevalence of clarithromycin resistance is less that 5% while in Southern Europe it is as high as 20%. Overall, resistance to amoxicillin is the least reported.

First line therapy		
Proton pump inhibitor (standard dose, twice daily), clarithromycin (500 mg, twice daily) and amoxicillin (1000 mg, twice daily) for 1 week	or	Proton pump inhibitor (standard dose, twice daily), clarithromycin (500 mg, twice daily) and metronidazole (400 or 500 mg, twice daily) for 2 weeks

If fail

Second line therapy				
Proton pump inhibitor (standard dose, twice daily), bismuth (120 mg, four times daily) metronidazole (500 mg, three times daily) and tetracycline (500 mg, four times daily) for 1-2 weeks	or	Proton pump inhibitor (standard dose, twice daily), metronidazole (500 mg, two times daily) and tetracycline (500 mg, four times daily) for 1-2 weeks	or	Proton pump inhibitor (standard dose, twice daily) metronidazole (500 mg, two times daily) and amoxicillin (1 g, two times daily) for 1 week

If fail

Third line therapy		
Empiric rescue therapy	or	Treatment tailored to individual antibiotic sensitivity

Figure 2-5: Summary of the suggested *Helicobacter pylori* eradication regimens (adapted from Malfertheiner et al [271])

In Australia, Grove and Koutsouridis [277] reported a 4-fold increase in proportion of isolates of *H. pylori* that were resistant to clarithromycin over 5 year period. Strong associations between *H. pylori* infection and gastric lymphoma as well as gastric ulcer have been reported [278]. The risk of ulceration is higher with more virulent strains with expression of active forms of vacuolating cytotoxin (*VacA*) and possession of cytotoxin associated gene, *cagA* that stimulates the host inflammatory response. The *VacA* gene is present in all *H. pylori* strains where its subtype *s1, m1* genotype may produce higher levels of cytotoxin activity than other genotypes. Meanwhile, *cagA* gene is a marker for a pathogenicity island which is not present in every *H. pylori* strain but is associated with more severe clinical outcomes [279].

2.2.2 *Helicobacter pylori* eradication therapy resistance

The aim of *H. pylori* eradication is to cure peptic ulcer disease and reduce the lifetime risk of gastric cancer [280]. In Australia, the first-line standard regimen for the *H. pylori* eradication is a 7 day course consisting of two antibiotics namely amoxicillin (1 g twice daily) and clarithromycin (500 mg twice daily) together with proton pump inhibitor to increase antibiotic efficacy [281]. The standard eradication regimen is useful in preventing gastric ulcer and may eradicate *H. pylori* in up to 70 % of the infected people. It has been recommended to increase the dose of proton pump inhibitor if the first treatment regimen fails and patients should be given second attempt with longer therapy duration, for example 10 to 14 days followed by an antimicrobial sensitivity test if two eradication attempts have failed [282].

The Maastricht III Consensus Report in 2007 also recommended that the Triple Therapy using a proton pump inhibitor with clarithromycin and amoxicillin or metronidazole should remain as the first choice therapy to eradicate *H. pylori* [283]. The bismuth-containing quadruple regimen would be the best second choice if available. Whereas the combination of proton pump inhibitor-amoxicillin or tetracycline and metronidazole are recommended if bismuth is not available. There is geographic variability in the efficacy of proton-pump inhibitors in the treatment of peptic ulcer disease, due to differences in body weight, *CYP2C19* genetic polymorphisms and drug response [283].

With the wide use of antibiotics among patients for various other conditions such as pulmonary infections, there are increasing reports worldwide of increased antibiotic resistance and subsequent failure of *H. pylori* eradication therapy [284]. Antibiotic resistance is a key factor in the failure of eradication therapy and recurrence of *H. pylori* infection [283]. Fischbach and Evans [285] have reported that standard proton pump inhibitor-based therapy fail in up to 30% of patients and that eradication rates have fallen to 70% over the last few years, mostly contributed by resistance towards clarithromycin. The prevalences of clarithromycin, metronidazole and levofloxacin resistance have been significantly increased in Europe, Asia, America and Africa since 2006 [286]. Among the main reasons for resistance to *H. pylori* therapy are poor patient compliance [287], molecular bacteria resistance [288-290], low gastric pH [291-292] and a high bacterial load [293-294].

Overall, in populations with less than 15 to 20% of clarithromycin resistance, proton pump inhibitor-clarithromycin-amoxicillin or metronidazole treatment is the recommended first choice [282]. Whereas, in populations with less than 40% metronidazole resistance, proton pump inhibitor-clarithromycin-metronidazole regimens are preferred. As for second choice treatment, bismuth based quadruple therapy remains the preferred option (if available) and if rescue treatment is needed, patients should be treated based on antimicrobial susceptibility testing.

The use of fluoroquinolones, furazolidone or rifabutin as an alternative to the standard eradication therapy also have been studied, however their efficacy has been limited by the increased incidence of fluoroquinolone resistance over the years [295]. Several other possible mechanisms for multidrug resistance associated with *H. pylori* eradication have been proposed such as reduced permeability of the cell membrane to the certain drugs, active efflux of the drug from resistant cells and reduced binding of the drugs to their intracellular targets as well as a role for P-glycoprotein [296].

2.2.3 Role of P-glycoprotein in *Helicobacter pylori* eradication therapy

The induction of P-glycoprotein expression in the gastrointestinal tract as part of the xenobiotic defence has been demonstrated by *Helicobacter spp*. as these bacteria has been used in various studies to examine the pathogenenesis of bacteria-induced intestinal disorders [297-298]. It is believed that *H. pylori* plays a significant role in pathogenic mechanisms such as damage to the intestinal mucosa [299] and abnormal activation of immune competent cell [300].

Panwala et al [301] reported that *mdr1a* (-/-) mice have been shown to be more susceptible to develop inflammatory bowel disease. However, the high incidence of colitis in these knockout mice could be prevented by treatment with antibiotics. Therefore, it has been suggested that P-glycoprotein could prevent the accumulation of inflammation-inducing bacteria and bacterial products. *Helicobacter bilis* and *H. hepaticus* are commonly found in the rodent liver and intestine [302]. *H. hepaticus* has been shown to induce hepatocellular carcinomas in susceptible strains of mice that are not associated with mutation in p53 or Ras genes while colorectal cancer has been induced in RAD2-deficient mice by infection with *H. hepaticus.*

Meanwhile, *H. bilis* has been shown to induce the development of inflammatory bowel disease [303]. Significantly, the *mdr1a* (-/-) mice infected by *H. bilis* was found to develop severe diarrhoea, weight loss and inflammatory bowel disease. Infection with *H. hepaticus* infection of *mdr1a* (-/-) mice did not accelerate the development of colitis rather it delayed it and lessened it severity. These authors suggested that "*Helicobacter spp*. may provide a useful tool to explore the pathogenesis of microbial-induced inflammatory bowel disease in a model with a presumed epithelial cell "barrier" defect." Subsequently Maggio-Price et al [302] reported that *Helicobacter spp.* infection results in increase of *c-myc*, IL-1 and COX-2 expression in *mdr1a* [-/-] mice. As a result, the increased inflammation that is associated with *Helicobacter* infection further elevates oxidative damage, leading to changes in cell survival.

P-glycoprotein is generally undetectable in normal gastric mucosal tissues but it is over-expressed in up to 50 % of gastric cancer patients [303]. *H. pylori*- dependent induction of COX-2 is associated with enhanced production of P-glycoprotein and *Bcl-xL* that may contribute to gastric tumorigenesis and resistance to the drug therapy. Preliminary data from Babic et al [305] suggested that *H. pylori* might stimulate P-glycoprotein expression in patients with peptic ulcer disease. The author assessed P-glycoprotein activity in gastric mucosa biopsies from *H. pylori* - positive subjects who were treated with antibiotics plus a proton pump inhibitor and compared these with control subjects with normal endoscopic findings. In their study, stronger gastric biopsies extrusion of rhodamine dye was recorded in most patients who were *H. pylori* positive. The patients were then randomly chosen to assess their pre- and post-therapeutic P-glycoprotein activity regardless of therapeutic success. The intensity of rhodamine dye extrusion by gastric biopsies from patients who were *H. pylori* positive was found to be higher than the control group.

2.2.4 P-glycoprotein polymorphisms (*MDR1 C3435T*) and *Helicobacter pylori* eradication therapy

P-glycoprotein (*MDR1*) polymorphism has been studied to evaluate the association between this polymorphism and the risk of gastric cancer and its influence on *H. pylori* eradication therapy outcomes. Tahara et al [306] examined the influences of *MDR1* gene polymorphism on the risk of gastric cancer in the Japanese populations. The frequency of *MDR1 3435TT* genotype was found to be significantly higher in the control group compared to gastric cancer patients. There was no significant difference in the *CT* and *CC* genotype frequencies between gastric cancer patients and control group. Their study suggested that *3435TT* polymorphism of *MDR1* was associated with a reduced risk of gastric cancer in the Japanese population.

Nevertheless, Sugimoto et al [307] found that no significant difference existed in frequencies of *MDR1 C3435T* polymorphisms between *H. pylori*-negative controls and *H. pylori*-positive gastritis patients and that the *MDR1 3435T* allele carriage did not affect the risk of gastric cancer or peptic ulcer development. The age- and sex-

adjusted odds ratios of *MDR1 3435T* allele carriers relative to the *3435CC* genotype group for gastric cancer, gastric ulcer and duodenal ulcer risk were 0.96 (95%CI: 0.56–1.66), 1.16 (95%CI: 0.72–1.84) and 1.00 (95%CI: 0.61–1.62), respectively.

Recently, Salagacka et al [308] suggested that having the *3435CT* and *3435TT* genotype increased the likelihood of *H. pylori* infection compared to the *3435CC* genotype. In their study, a trend towards a higher incidence of the *3435TT* genotype in a subgroup of men with peptic ulcer was also observed. Additionally, Gawronska-Szklarz et al [309] reported that there was a significantly higher prevalence of subjects with *3435TT* genotype in Caucasian group of patients who has successful *H. pylori* eradication after the first cycle of the therapy. Interestingly, higher eradication rate was observed in the subjects who received the combination of pantoprazole, amoxicillin and metronidazole compared to omeprazole, amoxicillin and metronidazole, supporting to the fact that pantoprazole has lowest potential for metabolic interaction among other proton pump inhibitors [310].

On the other hand, Kodaira et al [311] reported that in 15 healthy Japanese volunteers who received oral lansoprazole 30 mg for 15 days, the drug plasma concentrations were higher in subjects with the *3435TT* genotype compared to *3435CC* and *3435CT*, respectively. The impact of *MDR1 C3435T* polymorphism on the lansoprazole pharmacokinetics was observed to be smaller after subjects received repeated doses; implying that in long term therapy such as that used for gastroesophageal reflux disease, lansoprazole pharmacokinetic could be less affected by this polymorphism.

Gawronska-Szklarz et al [312] reported that there was no significant association between *MDR1 C3435T* and the *H. pylori* eradication rate among Polish Caucasian peptic ulcer subjects whom received combination of pantoprazole, amoxicillin and metronidazole. This finding was different to what had been observed in other studies [313-314] where *MDR1 C3435T* polymorphism was significantly associated with successful eradication in clarithromycin-resistance *H. pylori*- positive subjects.

Oh et al [315] also failed to demonstrate an association between *MDR1 C3435T* polymorphism and the outcome of *H. pylori* eradication therapy amongst a group of

Korean subjects treated with the combination of pantoprazole 40 mg, clarithromycin 500 mg and amoxicillin 1000 mg twice daily for 7 days. The dissimilarities reported regarding *MDR1 C3435T* polymorphism and *H. pylori* treatment outcomes or infection risk may be contributed to by differences in protein analysis techniques, the various antibodies used and/or patient demographics.

2.3 *Helicobacter pylori* adherence in disease development

2.3.1 Principles of bacteria pathogenesis

A bacterial pathogen must first enter a host, find a unique niche, avoid other competing microbes and host defence barriers before multiplying sufficiently to establish itself or to be transmitted to a new susceptible host [316]. The bacterial pathogenesis comprises two major mechanisms, first is the invasiveness which include the ability to adhere and colonize the tissue; production of extracellular substances to facilitate the invasion and ability to overcome any host defence mechanism. The second mechanism is the ability of the bacteria to produce toxins, either exotoxins or endotoxins.

An important step in the host-pathogen interaction is adherence of the pathogen to host surfaces [317]. Adherence also involves internalization, deeper tissue penetration and possible systemic distribution. Once pathogens adhere to a specific host cell surface such as mucous membranes or endothelial tissues, they initiate specific biochemical pathways including proliferation, toxin secretion, host cell invasion and activation of host cell signaling cascades resulting in disease development [318]. Bacterial invasion can be divided into two different types, namely extracellular and intracellular. Extracellular invasion is when the pathogens break down the tissue barrier to disseminate in the host while they remain outside the host cells.

For example, *Pseudomonas aeruginosa* is an opportunistic pathogen that exploits any compromised host defences to initiate an infection [319]. This pathogen secretes an enzyme to aid the lung tissue invasion in individuals with cystic fibrosis. *P.*

aeruginosa then has access to the tissues and can proliferate before disseminating to other sites in the body to express toxins and later initiate inflammatory responses. On the other hand, a number of Gram negative and positive bacteria demonstrate the ability to cause the invasion intracellularly [318]. *H. pylori* exhibits this type of invasion and is able to form large vacuolar compartments for the bacteria to persist for long periods and evade the host immunological defenses [320].

Fundamentally, the ability of pathogenic bacteria to cause disease in a susceptible host is determined by multiple virulence factors acting individually or together at different stages of the infection [321]. Virulence factors are the molecules that are produced by a microorganism to evoke disease [322]. Bacterial virulence factors may be encoded on chromosomal, plasmid, or bacteriophage DNA. They can be divided into several groups based on their mechanism of virulence and function. For example, some bacteria such as *Salmonella* spp. and *Escherichia coli* will produce pili (fimbriae), cell wall adhesion proteins or biofilm-producing capsules in order to adhere to host cells and avoid any physical removal.

Colonization, with or without subsequent invasion, is the consequence of bacterial adhesion [323]. Bacteria will later produce endotoxins or exotoxins which are vital to their ongoing viability. For example, *Listeria monocytogenes* avoids phagosomal killing by producing a toxin known as lysteriolysin that allows them to escape into the cytosol before further replicating and spreading through other cells through their actin-tail-based motility [324]. Many bacterial pathogens also produce a specialized secretory system that resembles a syringe and use it as effectors to inject toxins into the host cell [325]. These effectors will mimic the function of host proteins and substantially alter mammalian signaling pathways, subsequently contributing to the disease process. Additionally, cells of the innate immune system such as dendritic cells and macrophages also provide an innate protection against microorganisms that are newly encountered by the host [326].

The ability to avoid detection by either the innate or acquired host immune system is important for bacterial pathogens to survive [327]. One strategy is to disguise the surface of the microbe or the infected cell such that it is not recognized by host surveillance systems. Since the host's immune system rapidly develops specific

responses to an invading pathogen, microbial mechanisms for phase and antigenic variation can reduce the restrictive effect of host immune responses [328]. This is often accomplished by bacterial genetic mechanisms which involve DNA rearrangement. In order to promote their life cycle, bacterial pathogens may also use host cells to aid their own adherence, replication or dissemination [329]. After entry to host cells, invasive pathogens are either localized in the cytosol or sequestered in vesicular structures. Bacterial pathogens could further manipulate the cytoskeleton by interacting with actin filaments and modulating G proteins to help invade a host cell or to gain motility in the cell. Many bacterial pathogens also manipulate actin-filament dynamics so that they can move within the infected host cell after the invasion and escape from membrane-enclosed vesicles into the cytosol.

Another crucial step in the development of infection and disease progression is the ability of intracellular pathogens to exit the host cell once replication has ceased, thereby allowing infection of new host cells [330]. *Listeria* for example, promote their escape from phagosomes through the action of pore-forming cytolysins [331]. Cellular release then occurs as these bacteria use actin polymerization to protrude out of the cell. Meanwhile, intracellular bacteria that reside within a vacuole have the additional challenge of needing to traverse two membranes to successfully escape the host cell. One example is the merozoite-stage rupture of erythrocytes by the malaria parasite *Plasmodium falciparum* where cysteine proteases have been shown to be essential for sequential rupture of the vacuole and cell.

A comprehensive understanding of the mechanisms underlying the bacteria pathogenesis is a must in order to design an effective drug therapy while avoiding any risk of bacterial resistance. The increasing prevalence of bacterial strains that are resistant to available antibiotics demands the discovery of new therapeutic approaches [332]. As some elements of virulence are fundamental for many pathogens, the new combination therapy may be designed so that bacterial clearance is mediated by standard antibiotics and the symptoms of virulence are suppressed by the anti-virulence drugs. The principle behind this combination therapy is to disarm the pathogens rather than kill it and further alleviate the problem of resistance [333].

2.3.2 *Helicobacter pylori* pathogenesis

H. pylori is mainly found attached to the gastric mucous layer [334]. While in a small number of the population, *H. pylori* has been observed to invade the lamina propria of antrum, causing more severe mucosa inflammation [335]. *H. pylori*-induced gastric epithelial damage allows the bacteria to translocate to the gastric lymph nodes and chronically stimulate the host immune system. *H. pylori* adherence to gastric epithelial cells facilitating access to nutrients and delivery of effector molecules has been considered essential for development of disease [336].

Peristalsis and gastric acidity are the fundamental barriers to successful colonization of *H. pylori* in the stomach, hence the bacteria has to establish several mechanisms to escape from those primary host defences [337]. Upon colonization, *H. pylori* penetrates the mucus layer to multiply near to the surface epithelium. *H. pylori* uses their flagella in order to facilitate their motility within gastric mucus and to overcome peristalsis [338]. Each flagellum is about 3 μm long and exhibits a typical bulb-like structure at its distal end that represents a dilation of the flagellar sheath. The flagellar sheath is an extension of the bacterial outer membrane that is important in protecting the acid-labile flagellar structure from the attack of the stomach acid. Interestingly, *H. pylori* also uses the flagella to mediate chemotaxis to promote infection [339].

H. pylori uses a variety of strategies to undermine the innate and adaptive immune systems of the host, therefore represents a remarkable medical challenge [340]. *H. pylori* biofilm formation has been reported to be involved in the colonization of the human stomach [341]. The biofilm is likely to provide greater protection for *H. pylori* under stressful conditions. In antral biopsies from patients with peptic ulcer disease and urease positive, almost the entire mucosal surface was covered with mature biofilms, while coverage was less than 2% in urease-negative patients [342]. To adhere to gastric epithelial cells, *H. pylori* also can assume either a spiral or coccoid form, passing through an intermediate U-shape during the conversion from one form to another [343]. *H. pylori* is known to mainly present in a spiral shape but it converts into a coccoid shape when exposed to detrimental environmental circumstances [344]. Coccoid *H. pylori* is found attached to severely damaged gastric

epithelial cells and can be identified in up to 93% of biopsy specimens from patients with *H. pylori*-associated adenocarcinoma [345].

H. pylori not only survives the low pH of the stomach, but thrives in it [346]. To overcome acid stress, *H. pylori* produces urease, which catalyses the hydrolysis of urea to carbon dioxide and ammonia. Urease also helps the bacterium to maintain a proton motive force that is essential for their metabolism and survival. Meanwhile, ammonia molecule helps to buffer the *H. pylori* cytoplasm and the microenvironment as it is secreted from the bacterial cell. Urease is important to the pathogenesis of *H. pylori* infection and urease-negative mutants have been demonstrated to fail in colonizing various animal models [347]. Urease protein activity is elevated under acidic condition. *H. pylori* may then modulate the urease activity *in vivo* through activation of nickel transporters such as NixA.

During colonization, the gastric epithelium lining will respond to the infection by degenerative changes such as mucin depletion, cellular exfoliation and compensatory regenerative changes and production of cytokines such as interleukins and tumor necrosis factor alpha [348]. Host innate immunity functions as a pathogen sensor also begin to induce the pathogen eradication and establish the adaptive immunity [349]. The innate toll-like receptor (TLR) system is usually recognizes bacterial lipopolysaccharides before triggering the pro-inflammatory response. There is evidence showing that *H. pylori* is able to bypass the TLR as the latter does not react towards lipopolysaccharides from the bacteria cell wall. *H. pylori* flagellum function was also shown to not signal through TLR and stimulate any innate immune response. In addition, *H. pylori* also modulates the adaptive immune system by blocking the antigen dependent proliferation of T cells partially by delivery of the vacuolating cytotoxin, *VacA*, which blocks T-cell receptor signaling events that normally lead to the production of cytokines, important mediators of the immune response [350]. The risk of ulceration is higher with more virulent strains which express active forms of *VacA* and posses the cytotoxin associated gene (*cagA*) that stimulates the host inflammatory response [351].

These virulence factors of *H. pylori* play important roles in gastric mucosal injury, such as gastric inflammation, peptic ulceration, atrophy, intestinal metaplasia,

dysplasia, and malignancy [352]. The *VacA* gene is present in all *H. pylori* strains where the subtype *s1, m1* genotype produces higher levels of cytotoxin activity than other genotypes. *CagA* is a marker for a pathogenicity island which is not present in every *H. pylori* strain but is associated with more severe clinical outcomes [353]. The *cagA* gene which encodes a family of high-molecular-mass protein (120 to 140 kDa) is present in approximately 60% of *H. pylori* isolates. The *cagA* is directly secreted from bacterial cells into epithelial cells via the *cag*-encoded type IV translocation apparatus and disturbs the physiological signal transduction [354]. This causes pathological cellular responses such as increased cell proliferation, motility, apoptosis and morphological changes.

While *H. pylori* has also been shown to produce reactive oxygen species (ROS) in infected gastric mucosa, *cagA* itself can induce ROS production in gastric epithelial cells [355]. Serologic response to *cagA* has been detected in *H. pylori*-infected patients with peptic ulcer disease more frequently than in the infected patients with gastritis alone. Serum IgG *cagA* antibodies were present in 44.1% of healthy *H. pylori* infected volunteers, 58.8% of those peptic ulcer disease, 57.7% of those with duodenal ulcer and in 63.3% of those with gastric ulcer [295]. Infection with a *cagA*-positive *H. pylori* strain increases the risk of development of gastric ulcer and presence of *cagA*-positive isolates has been proven to contribute to difficulties in bacterial eradication [356].

One final mechanism that may be crucial for *H. pylori* to escape from the host immune response is the ability to undergo genetic rearrangement that either eliminates particular immunostimulatory gene products or causes variation in potentially immunostimulatory molecules [357]. Additionally, *H. pylori* may also hide from the cell host defences through intracellular invasion of the epithelial cell lines [358]. *H. pylori* needs to establish adherence with the epithelial cells before gain the entry into a cell. *H. pylori* attachment to cell lines is associated with protein phosphorylation and cytoskeleton rearrangement leading to formation of pseudopodia [359]. *H. pylori* adhesins may be involved in this process, for instance *SabA* is involved by mediating the binding to gangliosides and stimulation of G-proteins, and the activation of phagocytosis and oxidative burst reactions of neutrophils [360]. Additionally, the *cagA* mediated reorganization of the host cell

membrane and signaling is probably fundamental in bacterial adhesin accumulation in the bacterial–host cell interface.

H. pylori may colonize the human gastric mucosa for long periods, rather than just days or weeks as occurs with other gastrointestinal pathogens [361]. Gastric inflammation always leads the development of peptic ulceration and is a critical component in initiating the progression towards gastric carcinogenesis [362]. Although all *H. pylori* strains induce gastritis, strains that contain the *cag* PAI (*cag*+) enhance the severity of gastritis and atrophic gastritis and distal gastric cancer risks compared to those with strains which lack the *cag* island (*cag*-deficient mutants) [363]. Chronic gastritis induced by *H. pylori* further increases the risk for various gastrointestinal diseases including peptic ulcer disease, distal gastric adenocarcinoma and gastric mucosal lymphoproliferative diseases such as non-Hodgkin's lymphoma [364].

2.3.3 *Helicobacter pylori* adherence to gastric tissues

H. pylori internalization starts from cell surface through the gastric epithelial cells during the early phase of the infectious process [365]. The adherence and adhesion of the bacteria to the gastric mucosa are key steps in the establishment of a successful infection [366]. Bacterial adhesion to a material surface can be described as a two-phase process; Phase 1 which is an initial, instantaneous and physically reversible and Phase 2 which is a time-dependent and irreversible molecular and cellular phase [367]. On the other hand, adherence is a general description of bacterial adhesion which is the initial process of bacteria directly binding to the surface. *H. pylori* adhere specifically to epithelial cells in the antral and fundal mucosa of human stomach [368]. The close association of *H. pylori* with gastric epithelial cells stimulates not only morphological but also physiological changes within gastric epithelial cells [369]. This *in vitro* study has confirmed the specificity of *H. pylori* for human gastric epithelial cells and its lack of binding to human esophageal epithelial cells and human fibroblasts.

H. pylori adherence has been demonstrated *in vitro* using human tissue culture lines such as KATO-III and AGS that are derived from gastric cancer patients [370]. In

their study, AGS human gastric carcinoma tissue culture cell monolayers were infected with *H. pylori*. The authors found that only 0.6-2.0% of the inoculums were actually internalized by the host cells. *H. pylori* were also seen within coated pits and appeared to be taken into the host cell by endocytosis. Once taken into the cell, bacteria were seen within vacuoles in the epithelial cells. Some of the *H. pylori* that were not internalized made their way into the tight junctions and were seen between cells. Earlier, Hemalatha and colleagues [371] examined the adherence of *H. pylori* to KATO-III cells, an epithelial cell line derived from a human gastric adenocarcinoma. The author demonstrated *in vitro* that adherence of *H. pylori* to gastric epithelial cells involved attachment and effacement mechanisms. Some *H. pylori* were shown to adhere to the intact microvillus, while others closely adhere to the plasma membrane in regions where microvilli were effaced. Interestingly, their adherence assays also showed that binding of *H. pylori* was temperature- and gastric pH-dependent.

In another study, Nilius et al [372] compared attachment of five *H. pylori* strains taken from patients with gastritis (4 strains) and duodenal ulcer (1 strain) to three different gastric cell lines namely human surface mucous cells (HSMC), tumour surface mucous cells (TSMS) and bovine surface mucous cells (BSMC). All strains consistently showed adhesion to human epithelial mucous cells (HSMC and TSMS) with one strain additionally adhering to BSMC. The single *H. pylori* strain which adhered to bovine stomach had been isolated from a patient with duodenal ulcer, whereas the remaining four strains came from patients with gastritis. As the adhesion of this *H. pylori* strain to BSMC is apparently in contradiction to earlier reports of a selective affinity of *H. pylori* for human gastric surface mucous cells *in vivo*, the authors suggested that some bacteria may adhere because of different adherence factors, possibly related to their virulence potential.

There was an assumption that infection can be prevented by inhibiting the pathogens adherence to a cells surface [373]. For instance, the pathogens adherence inhibition could be achieved by using commensal bacteria to interfere with microbial pathogenesis at certain mucosal sites to compete for nutrients and host-cell-binding sites. If bacteria cannot adhere to the mucosal layer, they will be removed rapidly by the local, nonspecific host-defence mechanisms such as peristalsis, ciliary activity

and turnover of the epithelial cell populations and mucous layer [374]. The carbohydrate compositions of natural glycoconjugates that are genetically regulated in individuals may also act as natural antimicrobial clearance factors. It is an interesting concept that the initial adherence of bacteria to cell surface receptors might play such an important role in pathogenesis and any changes to the adherence process could subsequently affect the infectious diseases predisposition.

In a preliminary study, Crowe and Bebawy [375] proposed that P-glycoprotein expression in the gastrointestinal tract may prevent the bacterial toxicity by inhibiting the bacterial adherence to the human enterocytes cells. Three human cell lines (Caco-2, RKO and MCF7) and six species of bacteria (*Staphylococcus aureus*, *Salmonella typhimurium*, *Klebsiella pneumoniae*, *Clostridium sporogenes* and *Pseudomonas aeruginosa*) were used. These authors found that the P-glycoprotein inhibition did lead to a significant increase in the adherence of all bacteria species studied to the Caco-2 cells but this was not the case with RKO and MCF7 cells. As Caco-2 cells that originated from human colon adenocarcinoma is known to express P-glycoprotein and has been extensively used as a model for the intestinal barrier, the study by Crowe and Bebawy demonstrated the role of P-glycoprotein in preventing the bacterial adherence and its potential in protecting gastrointestinal mucosa tissue. Their study may be expanded to determine the influence P-glycoprotein on the adherence of other bacteria species, such as *H. pylori* adherence and to determine whether manipulating its expression can influence to the occurrence and severity of bacteria-related gastrointestinal diseases. P-glycoprotein has been reported to increase stem cell proliferation and regulate apoptosis and whilst its absence results in decreased repair of intestinal epithelial cells after chemical injury [376]. Microbial and host factors that determine the outcome of *H. pylori* colonization have been difficult to define, in part because both *H. pylori* and humans are genetically diverse [377]. *H. pylori* ability to establish physical contact with the gastric epithelium is an important factor for their pathogenesis, therefore affecting its ability to influence mucosal epithelial cells may be a way to modulate its carcinogenicity.

CHAPTER 3

MATERIALS AND METHODS

CHAPTER 3 MATERIALS AND METHODS

3 Overview

This study was divided into two – Phase 1 (*Helicobacter pylori* and P-glycoprotein expression) and Phase 2 (*Helicobacter pylori* attachment to gastrointestinal cell lines).

3.1 Phase 1 : *Helicobacter pylori* and P-glycoprotein expression

3.1.1 Patient recruitment

All patients who attended the Endoscopy Unit, Department of Gastroenterology/Hepatology, Sir Charles Gairdner Hospital, Nedlands, Western Australia from October 2010 to July 2011 for an upper gastroendoscopy were invited to participate in the study. On the day of the procedure, the doctor obtaining consent for the upper gastrointestinal endoscopy had assessed the patient eligibility for the study. Patients who were unable to converse in English, were unwilling to participate or deemed too unwell by the doctor had been considered ineligible for study. Patients who were on any antibiotics upon the endoscopy also had been excluded from this study.

When the patient was deemed eligible, the study had been explained and the signed consent form was obtained from patients who agree to participate. Participants also required to fill-in a set of questionnaire (comprehended of four questions) attached with the informed consent. The information regarding participant`s medical and medication history also had been taken before proceeded to the endoscopy procedure. On the day of recruitment, all the participants underwent an upper gastroendoscopy as scheduled by their doctor where four additional biopsies were collected for the research purpose. Whole blood samples (10 mL) also were collected by jugular venipuncture into vacutainer tubes with anticoagulant, Ethylene diamine Tetra-acetic Acid (EDTA).

3.1.2 Sample size calculation

It is reported that 48% of patients diagnosed with peptic ulcer disease are caused by *Helicobacter pylori* [245]. Previous study had shown that about 15 to 20% of patients diagnosed with *H. pylori* were expected to have an increased in P-glycoprotein levels [305]. While the majority (80% to 85%) of patients having normal or lower P-glycoprotein levels.

Based on the number of endoscopy procedure performed which was 1818 for the year 2009 at Department of Gastroenterology/Hepatology at Sir Charles Gairdner Hospital, Nedlands, the prevalence of P-glycoprotein expression in *H. pylori*–positive patients had been estimated along with its 95% confidence interval (CI). The sample size has been selected to give adequate precision to this prevalence estimate. Allowing for failure to obtain some data, about 200 participants was expected to be included in this study to ensure a 95% CI of approximately ± 6% as described in **Table 3-1**.

Table 3-1: Estimation of sample size (N) for Phase 1 recruitment

Prevalence of P-glycoprotein	**Precision**	
	95% Confidence interval [CI]	**N**
20%	± 8%	96
	± 7%	125
	± 6%	170
15%	± 8%	76
	± 7%	100
	±6%	136

3.1.3 Ethics approval

This study was approved by ethics committee at Sir Charles Gairdner Hospital, Nedlands which conforms to the provisions of the World Medical Association's Declaration of Helsinki in 1995 (as revised in Tokyo 2004) followed by human ethics clearance granted by Curtin University Ethics Committee in September 2010. The copy of ethic approval is attached in **Appendix II** and **Appendix III** along with participant information sheet and consent form **(Appendix IV).**

3.1.4 Questionnaire

All the participants were required to fill in the questionnaire before the endoscopy procedure. Participants also were required to submit additional information in the set of questions regarding their past medical and medication history. The questionnaire contained the following four questions:

A. Have you ever been tested for bacteria named *Helicobacter pylori*?

- Yes
- No
- Don`t know

B. If yes, do you know the result of the test?

- Positive
- Negative
- Don`t know

C. Have you ever been treated for *Helicobacter pylori* infection?

- Yes
- No
- Don`t know

D. If yes, was the therapy successful in eliminating the infection?

- Yes
- No
- Don`t know

3.1.5 Endoscopy procedure

All the participants underwent upper gastroendoscopy as planned by their doctors. The participants were required to be in fasting condition and some pre-medications were prescribed to assist the procedure such as intravenous Fentanyl 100 mcg and Midazolam 5 mg. The gastroscopy video system (GIF-H180) had been used to assist endoscopist in obtaining best upper gastrointestinal image and collecting the biopsies. All the endoscopy procedures were conducted in sterile environment.

All our participants have been informed that antibiotic therapy, bismuth or proton pump inhibitors should be ceased a few weeks before biopsies needed to be taken. Four additional biopsies have been collected for the purpose of this study (two samples from antrum and two samples from duodenum). All biopsies had been labelled with participant`s identification number, date, time and site of biopsy. Upon the upper gastroendoscopy procedure, one antral biopsy that would be used to determine *H. pylori* status was immediately placed in transport media (brain heart infusion broth 2% with 20% glycerol, PathWest Media, Western Australia). For other antral and duodenal biopsies, there were immediately kept on dry ice before being transferred to the laboratory for storage.

In addition, a 10 mL blood was collected by jugular venipuncture into vacutainer tubes with anticoagulant, Ethylene diamine tetra-acetic acid (EDTA) and placed on the dry ice together with the biopsies in a closed container. All the specimens obtained on that particular day were then transported back to the laboratory within four hours and kept in the freezer for -80 °C storage before any further analysis.

3.1.6 *Helicobacter pylori* infection status

The *H. pylori* infection status was determined based on culture or polymerase chain reaction (PCR) results using antral biopsies. Samples were considered positive for *H. pylori* if one of the methods (culture or PCR) yielded positive results. The rapid urease test (CLOtest) and antibiotic sensitivity test was only performed on

participants that had been diagnosed with failure towards *H. pylori* eradication therapy upon recruitment.

3.1.6.1 Culture

In this current study, all bacterial culture work has been conducted in a special laboratory that only dedicated for Helicobacter work. The antral biopsy was removed from the transport media and crushed using a sterile blade before being transferred to two different blood agar bases (Columbia Blood Agar Base, Oxoid, Australia) with and without *H. pylori*-selective supplement. Supplemental antibiotic (*H. pylori*-selective supplement, Dent, Oxoid, Australia) was introduced to our Columbia blood agar base to inhibit growth of other organisms, maintain the *H. pylori* viability and optimize the survival conditions. The supplement consists of vancomycin, trimethoprim, cefsulodin and amphotericin B. Another blood agar base without antibiotic supplement was also used to culture the *H. pylori* from each sample.

All the blood agar plates were incubated at 37 °C under microaerobic conditions (90% CO_2, 4% N_2, 2% H_2) for five to seven days. After the first 48 hours, each plate was examined and any colony that resembled *H. pylori* was transferred to a new blood agar for further incubation periods. About 48 hours later, the colonies were re-suspended with a cotton swab in 1 mL of sterile saline. A 90 µL of bacteria suspension then was added to the surface of a new blood agar and incubated for another 48 hours. As expected, the conventional media without the antibiotic supplement did allow growth of few other bacteria; however *H. pylori* could be differentiated based on its shape under microscope, pattern of colonization on the blood agar as well as urease and catalase tests.

Isolates were identified based on colony morphology, gram staining, oxidase, catalase and urease tests [378]. For Gram staining, crystal violet was used to flood the glass slide for about one minute. The stain was washed off with deionised water before been flooded again with Gram iodine solution for another minute before been removed again by deionised water. Alcohol 95% was used for 10 seconds to cover the glass slide and drained. The glass slide was then flooded with carbol fushin for two to three times before been drained and blot dried with clean paper towel. The

glass slide was then immersed with oil immersion lens before been examined under the microscope.

To confirm the *H. pylori* infection status, sterile inoculating loops aseptically transferred colonies of pure *H. pylori* to two separate curvetttes containing catalase and urease reagents. After a few minutes, a positive urease test of *H. pylori* resulted in changes of orange to pink solution. Meanwhile, a positive catalase test was indicated by rapid bubbling of the peroxide solution. On the other hand, an oxidase strip was directly placed on the bacteria colony on a blood agar where positive result for *H. pylori* was shown by rapid development of blue colour.

3.1.6.2 Polymerase chain reaction

Polymerase chain reaction (PCR) was used to determine presence of *H. pylori* in antral biopsies. Earlier, the boiling method was used to obtain bacteria DNA from the biopsies as adapted from Holmes & Quigley [379]. The specimen was placed in 50 µL of Tris buffer and boiled in a dry bath at 90 °C for about 15 minutes. This was followed by vortexing and centrifugation at 12,000rpm for 5 minutes. A supernatant containing the DNA was transferred to a new tube and kept at -20°C storage. The PCR analysis was performed in a 50 µL of reaction volume that consisted of 1 µL of genomic DNA, 25 µL of MasterMix, 2µL of each oligonucleotide primers and 22 µL of pure water. The PCR cycle was performed using PTC-100™ Programmable Thermal Controller (MJ Research, Minnesota) for 95 ^{o}C, 30 seconds; 95 ^{o}C, 18 seconds ; 55 ^{o}C, 30 seconds (30 cycles); 68 ^{o}C ,90 seconds; 68 ^{o}C, 5 min. About 5 µL of amplified samples with 1 µL of loading dye were electrophoresed on 1.5% agarose gel in tris-borate-EDTA (TBE) buffer for 85 minute at 85 volt and the amplified bands were visualised under ultraviolet light. All the primer sequences and PCR conditions used were listed in **Table 3-2.**

With availability of commercial bacteria DNA purification kits, our specimen DNA was then purified using EDNA Hispex™ Kit (Saturn Biotech, Western Australia) and quantified using Biospec-Nano (Shimadzu Biotech, New South Wales). The PCR analysis was carried out in a 25 µL of reaction volume consists of 1 µL of

Table 3-2: Conditions for two different polymerase chain reaction methods to detect *H. pylori*

Target Genes	Fragment length	Primer sequences	PCR conditions	Reference
16S rRNA	109 bp	5'-CTGGAGAGACTAAGCCCTCC-3' 5'-ATTACTGACGCTGATTGTGC-3'	95 ^{o}C, 3 min; 95 ^{o}C, 20 s; 60 ^{o}C, 20 s (35 cycles) ; 72 ^{o}C , 1 min ; 72 ^{o}C , 5 min.	[380]
*Vac*A	700 bp	5`-ACAACCGTGATCATTCCAGC-3' 5`-ATACGCTCCCACGTATTGC-3`	95 ^{o}C, 30 s; 95 ^{o}C, 18 s ; 55 ^{o}C, 30 s (30 cycles); 68 ^{o}C ,90 s; 68 ^{o}C, 5 min.	[381]

- The sensitivities of the vacA and 16S rRNA assays were 89.5 and 85.0%, respectively, and their specificities were 99% and 98.1%, respectively [380].

genomic DNA and 24 µL of reaction mixture containing 10x PCR buffer, 25mM $MgCl_2$, 5mM dNTPs, 25 pmol/µL of each oligonucleotide primers and thermo-stat Taq DNA polymerase in PTC-100™ Programmable Thermal Controller (MJ Research, Minnesota).As such DNA sequences encoding rRNA are highly ubiquitous, there is a possible risk of nonspecific products, particularly when analyzing DNA extracted from mammalian tissue (380). Therefore, *vacA* gene, a species-specific and highly conserved locus in *H. pylori* was also targeted in our PCR. *H. pylori 26695* was used as positive control and sterile water was used as negative control. About 10 µL of amplified samples were electrophoresed on 2% agarose gel in tris-acetate-EDTA (TAE) buffer for 60 minute at 80 volts. The gel was stained with 30 µL gel red in 100 µL of TAE buffer and the amplified bands were visualised under ultraviolet light

3.1.6.2 Rapid urease test (CLOtest)

For a few participants, their *H. pylori* infection status also was confirmed by incubating the antrum biopsy in CLOtest (Kimberly Clark, Sydney) at the time of gastroendoscopy. A biopsy of gastric mucosa was taken from the antrum and placed into a medium containing urea while phenol red acted as indicator. *H. pylori* then produced urease which transformed the urea to ammonia and the increased pH of the medium would change the colour from yellow (negative) to red (positive).

3.1.6.3 Antibiotic sensitivity test

Antibiotic sensitivity test was performed in the resistance group by E-test (Biodisk, BioMérieux France) for amoxicillin (AC), clarithromycin (CH), metronidazole (MZ), tetracycline (TC), rifampicin (RI) and ciprofloxacin (CI). A strip that had been impregnated with the respective antibiotic was laid on a blood agar plate where the *H. pylori* had already been spread.

After 24 hours of incubation, an elliptical zone of inhibition (if any) would be seen and reading for minimal inhibitory concentration was obtained. Resistance was

defined according to National Committee for Clinical Laboratory Standards [382] for antibiotic minimal inhibitory concentration (MIC) : AC, MIC ≥ 2 μg/ml ; CH, MIC ≥ 1 μg/ml ; MZ, MIC ≥ 8 μg/ml ; TC, MIC ≥ 1 μg/ml ; RI, MIC ≥ 4 μg/ml and CI, MIC ≥ 1 μg/ml.

3.1.7 Protein analysis

The protein analysis involved protein concentration determination by the Micro-Lowry Method for total protein and Western Blot Analysis for P-glycoprotein expression.

3.1.7.1 Sample preparation

In sample preparation for Western Blot Analysis, proteolysis degradation is the main concern during fractionation of cell extracts [383]. SIGMAFAST™ protease inhibitor tablet (Sigma-Aldrich, St Louis MO) and Phenylmethylsulfonyl fluoride (Sigma-Aldrich, St Louis MO) were added to tissue lysis buffer to ensure stable protein collection. Tris was added to permeabilize the cell membrane by releasing the lipopolysaccharides of the lipid bilayer. In this study, biopsies prepared for protein analysis were placed in 300 μL of an ice-cold tissue lysis buffer (0.05 M Tris Hydrochloride, 20% glycerol, 2 mM Ethylenediaminetetraacetic acid, 0.2 mM Phenylmethanesulfonyl and one protease inhibitor tablet) and homogenized.

3.1.7.2 Protein concentration

Protein concentration were estimated using a Micro-Lowry Method [384] adapted for use with multi well plates on a TECAN Sunrise™ (TECAN Group Ltd, Switzerland) 96- well plate spectrophotometer with a 750 nm filter using Magellan 3 software for Windows 2000. For about 1 to 30 μg protein in reaction, a CTC reagent was prepared consisting $CuSO_4.5[H_2O]$, Na-K-Tartrate and Na_2CO_3 that had been dissolved in nanopure water to make 100 mL of total solution. A solution of 0.8M NaOH was then prepared by dissolving the NaOH pellets in nanopure water to make 100 mL of total solution. A Lowry reagent was prepared by adding one part of CTC

reagent to 2 parts of nanopure water and 1 part of 0.8M NaOH solution. To prepare a Folin reagent, 2N Folin was added to nanopure water.

A bovine serum albumin (BSA) stock solution of 2 mg/mL was prepared from analytical grade BSA (Sigma-Aldrich, St Louis MO) and a concentration range of 10 to 1500 µg/mL was used as our protein standard. All the protein samples, standard and stock solution were transferred by pipettes into a 96- well plate accordingly. Lowry reagent was added to each well using a multi-channel pipettor and incubated for 15 minutes at room temperature. At the end of 15 minute-incubation period diluted Folin reagent (1:4) was added into each well. The 96- well plate was then incubated again for another 30 minutes.

Using a spectrophotometer set to 750 nm, the absorbance value was measured by Magellan 3 software. A standard curve was generated by plotting the average blank-corrected 750 nm values for each BSA standard versus its concentration in µg/mL. The standard curve was used to determine the protein concentration of each sample.

3.1.7.3 Western Blot Analysis

Western Blot Analysis was used to quantify expression of P-glycoprotein from biopsies with B-actin as a loading control in a NuPage® Novex 3-8 % Tris-Acetate gels with fifteen 1.5 mm x 25 uL wells (Invitrogen, Victoria). The X-cell apparatus (Xcell *Sure Lock*™, Invitrogen, Victoria) were set up and 20 µL of solution prepared previously were loaded to each well. The Novex Sharp® Standard was loaded to the last well of the gel as molecular weight marker proteins. The X-cell apparatus was run using 150 V for 67 minute.

A Immun-Blot® PVDF cut 8 x 9 cm membrane was used to electrophoretically transfer proteins from polyacrylamide gel using the Xcell II™ Blot Module. The Xcell II blot module was run for 105 minute and 33 V. By using a clean forceps, the Immun-Blot® PVDF membranes were collected and washed with Tris-Buffered Saline with 0.5% Tween-20 (TBST) twice. SimplyBlue™ SafeStain (Invitrogen, Victoria) was used to detect protein transfer by visualizing protein bands on polyacrylamide gels. The mini gels were rinsed with double deionised water three

times for five minute each before the stain solution was poured to each gel and left for about one hour. The gels were washed again with double deionised water and were rocked for one hour to remove the blue stain. Any visible band on the gel was then examined.

Meanwhile, Ponceau S method was used to determine protein transfer on the PVDF membranes using the commercially available Ponceau S solution (0.1% in 5% acetic acid, Sigma-Aldrich, St Louis MO). The blots were removed from the transferring sandwich in Xcell II™ Blot Module and rinsed three times with distilled water. 100% methanol was used to saturate the PVDF membrane for a minute before the staining solution was poured to the blots for another minute. Any visible band on the gel was then examined and the stain could be removed by rinsing it in 0.1 M NaOH for a few minutes. Both membranes were washed for once in TBST and left sitting the TBST for another 10 minute before being rinsed off. Blocking agents from Western Breeze®, (Invitrogen, Victoria) consisting of 20 % blocker A, 30% blocker B and 50% double deionised water were added and membranes were left blocked overnight at 4 °C.

On day 2, the membranes were washed twice in TBST for 5 minute each on a rocking platform to remove blocking agents. TBST was rinsed off and 10 mL antibody solution from Western Breeze® (Invitrogen, Victoria) containing 40 µL of PgP Sc13131 Mdr [G-1] mouse monoclonal IgG2b (200 µg/ml*;* Santa Cruz Biotechnology) and 2 µL of mouse anti-B-actin (42kDa protein; Sigma-Aldrich, St Louis MO) were added to each membranes. B-actin, a cytoskeletal protein is very abundant in mammalian and act as a housekeeping protein on the same blot. Membranes were left on rocking tray at room temperature for two hours. After 2 hours, primary antibodies were removed by washing the membranes four times in antibody wash solution with 5 minute for each wash. 10 mL diluted secondary antibody (Goat anti mouse IgG AP Pre-diluted, Western Breeze®, Invitrogen, Victoria) was added to each membrane and incubated for 1.5 hour on room temperature. Both membranes were washed four times for five minute each in antibody wash solution (Western Breeze, Invitrogen, Victoria) and rinsed once with double deionised water. A 2 ml of the Chemiluminescent substrate were applied evenly to the membrane surface and left for about 5 minute to develop the reaction.

An AlphaInnotech-ChemiImager 4400 (Alpha Innotech, San Leandro, CA) with a CoolSnap HQ charge-coupled device camera (Roper Scientific, Trenton, NJ) was used to capture chemiluminescence of any p-glycoprotein expression and recorded as a image after 10 to 15 minutes of exposure. The intensity of P-glycoprotein band was quantified based on the corresponding pixel intensity values designated as IDV (Integrated Density Value) in Spot Density Tools from the AlphaImager 4400 which represent p-glycoprotein expression level. With the chemiluminescence intensity mentioned in its raw form, simple intensity numbers that relevance is in its comparative nature to other bands measured in the same image and other plots using the same exposure time and equipment i.e. what is important is how many fold higher or lower these photon intensity plots are to each other.

3.1.8 *MDR1 (C3435T)* polymorphisms

The genotyping for *MDR1 (C3435T)* involved blood DNA preparation from frozen sample, PCR for determining P-glycoprotein band, followed by enzyme digestion with Mbo-1 to obtain particular band for *3435CC,3435 TT* and *3435CT* genotype.

3.1.8.1 Blood DNA preparation

DNA could be extracted from whole EDTA-treated blood, buffy coat or lymphocyte preparations using a commercial kit or home-made lysis buffer (red cell and nuclei) followed by precipitation with ammonium acetate and ethanol. In this study, genomic DNA was extracted from venous blood using home-made lysis buffer. The frozen blood had been thawed in the room temperature upon the laboratory work. The thawed blood then was decanted into a sterile conical centrifuge tube and red cell lysis buffer was added. The mixture was mixed gently by inversion for several times at room temperature before being centrifuged at 2000 x g for 10 minutes. The supernatant was discarded and a fresh red cell lysis buffer was added again to the conical centrifuge tube followed by inversion and centrifuging. That step was repeated three to four times. Following the final centrifugation step, the supernatant was discarded and remaining pellet was vortexed. White cell lysis buffer was added

to the dispersed pellet. The lysis buffer and pellet were mixed gently by repeated aspiration with a pipette, before being transferred to a fresh sterile conical centrifuge tube and incubated for 10 minutes at 40 ^{o}C. A Proteinase K 10 mg/ml was added to each conical centrifuge tube and the incubation continued at 55 ^{o}C for 60 minutes. A 10M ammonium acetate solution was added to each conical centrifuge tube, followed by vortex then centrifuging process at 2000 x g for 10 minutes. The supernatant was then decanted into an amount of absolute ethanol in a fresh small conical tube. The solution was mixed by gentle inversion until the DNA becomes visible as white strands or clumps. The tubes were left for several hours or overnight at -20 ^{o}C.

All the conical tubes containing the DNA strands were then centrifuged at 2000 x g for 10 minutes. The supernatant was discarded and the pellet was washed with small amount of 70% ethanol before being transferred to an eppendorf tube. The eppendorf tube was then centrifuged at 12 000 RPM for 10 minutes. The 70% ethanol was discarded carefully by inverting the eppendorf tubes on an absorbent paper and each sample was rehydrated with Tris-EDTA buffer. The DNA quantification was read by using Biospec-Nano (Shimadzu Biotech, New South Wales) with Tris-EDTA buffer as blank solution. All the DNA samples were stored at -20 ^{0}C before further analysis.

3.1.8.2 Polymerase chain reaction

The PCR analysis for determining P-glycoprotein band was carried out in 25 uL of reaction volume consisting of 1 µL of genomic DNA and 24 µL of reaction mixture containing 10× PCR buffer, 25mM $MgCl_2$, 5mM dNTPs and 25 pmol/µL of each oligonucleotide primers (forward 5`-TCTTTTCAGCTGCTTGATGG-3` and reverse 5`-AAGGCATGTATGTTGGCCTC-3`) in PTC-100™ Programmable Thermal Controller (MJ Research, Minnesota) [385]. Thermo-stat Taq DNA polymerase was added to each reaction volume. The Caco-2 cell line was used as a positive control and sterile water was used as negative control. The amplification cycles consisted of 95 ^{o}C for 3 minutes, 35 cycles of 95 ^{o}C for 20 seconds, 60 ^{o}C for 20 seconds and 72 ^{o}C for 1 minute with final extension at 72 ^{o}C for 5 minute. To determine P-glycoprotein band, about 10 µL of amplified samples were electrophoresed on 2% agarose gel in TAE buffer for 60 minute at 80 volt. The gel was stained with 30 µL

gel red in 100 µL of TAE buffer and the amplified bands were visualised under ultraviolet light.

3.1.8.3 Enzymatic digestion for *C3435T* polymorphisms

A 20 µL of reaction consisted of 10x buffer, 2 units of Mbo-1 enzyme (New England BioLabs Inc, Ipswich MA), pure water and 10 µL of PCR product obtained from previous step was set on ice. After incubation overnight at 37^{o}C, the amplification of DNA was analysed by agarose gel electrophoresis using standard procedures where 10 µL of amplified samples were electrophoresed on 2.5% agarose gel in TAE buffer for about 60 minute at 80 volt. The gel was stained with gel red (30 µL in 100 µL TAE buffer) and the amplified bands were visualised under ultraviolet light. The final genotype bands were examined by two independent investigators. Enzymatic digestion with 2 units of Mbo-1 that had been performed to analyse *C3435T* polymorphism would be yielded products of one band (197 bp) for homozygous *3435TT*, 158 bp (*3435CC*) and two bands (197 and 158 bp) for heterozygous *3435CT* genotype [386].

3.1.9 CYP2C19 polymorphisms

An additional work of *CYP2C19* genotyping was undertaken for a group of participants who were referred as "failure towards *H. pylori* eradication therapy". Basically, a 25 µL of reaction volume consists of 1 µL of genomic DNA and 24 µL of reaction mixture containing Taq 2x MasterMix (New England BioLabs Inc, Ipswich MA), PCR grade water and oligonucleotide primers (**Table 3-3)** was prepared for *CYP2C19*2* or *CYP2C19*3* analysis and run in PTC-100™ Programmable Thermal Controller (MJ Research, Minnesota). Similar to the PCR work in determining *MDR1 C3435T* band, the amplification cycles consisted of 95 ^{o}C for 3 minutes, 35 cycles of 95 ^{o}C for 20 seconds, 60 ^{o}C for 20 seconds and 72 ^{o}C for 1 minute with final extension at 72 ^{o}C for 5 minute. About 10 µL of amplified samples were electrophoresed on 2.5% agarose gel in TAE buffer for 60 minute at 80 volt. The gel was stained with 30 µL gel red in 100 µL of TAE buffer and the

Table 3-3: List of primers for *CYP2C19*2* and *CYP2C19*3* analyses

Allele	Fragment length	Primers
*CYP2C19*2*	300 bp	Forward primer 5'-CAACCAGACTTGGCATATTG-3' Reverse primer 5'-CACAAATACGCAAGCAGTCAC-3'
*CYP2C19*3*	247 bp	Forward primer 5'-CACCCTGTGATCCCACTTTC-3' Reverse primer 5'-ACTTCAGGGCTTGGTCAATA-3'

Reference : [387]

amplified *CYP2C19*2* (300 bp) or *CYP2C19*3* (247 bp) bands were visualised under ultraviolet light. To determine whether the *CYP2C19*2* band was present, a 20 µL of reaction consisted of 10x buffer, 2 units of restriction enzymes (SmaI, New England BioLabs Inc, Ipswich MA), pure water and 10 µL of the amplified PCR product of 300 bp was prepared and left for incubation overnight at 25^{o}C. For *CYP2C19*3*, the enzymatic digestion was performed with 2 units of BamHI (New England BioLabs Inc, Ipswich MA) and incubated overnight at 37 ^{o}C. The reaction was electrophoresed on 2.5% agarose gel in TAE buffer for about 60 minute at 80 volt, followed by the gel red staining before the amplified bands were visualised under ultraviolet light. As the restriction site is absent in the mutant alleles for both *CYP2C19*2* and *CYP2C19*3*, the 300 or 247 bp of PCR product would not digested by the restriction enzymes [387]. The subjects were then classified into three different genotypes groups as follows: homozygous extensive metabolizer (*CYP2C19*1/*1*), heterozygous extensive metabolizer (*CYP2C19*1/*2* or *CYP2C19*1/*3*) and passive metabolizes (*CYP2C19*2/*2* or *CYP2C19*3/*3*).

3.1.10 Statistical analysis

Standard descriptive statistics (frequencies and percentages for categorical variables, median, means, standard deviations and ranges for variables measured on a continuous scale) were used to summarise the demographic data for the study participants. SAS® software version 9.2 (SAS Institute Inc. Carry North Carolina USA) was used in this study and in all analyses, p-value less than 0.05 was taken to indicate a statistically significant association. Differences in these variables between subjects who were *H. pylori* – positive and *H. pylori* – negative were examined and p-values calculated. Similarly, differences in distribution of demographic variables across *MDR1 C3435T* polymorphisms were compared using Chi-square statistic. P-glycoprotein expressions according to the *MDR1 C3435T* polymorphisms were compared between antrum and duodenum using a paired t-test. A regression model was used to identify any association of antrum and duodenum P-glycoprotein values with Helicobacter infection status and *MDR1* genotype. The tables quoted raw medians of the P-glycoprotein expression but p-values (including the paired t-test) were based on the log-transformed data.

3.2 Phase 2: *Helicobacter pylori* attachment to gastrointestinal cell lines

3.2.1 Caco-2 cell culture

Originally, the human adenocarcinoma cell line (Caco-2) is supplied by American Type Culture Collection (ATCC; Manassas, VA, USA) in liquid nitrogen. Caco-*2* cell cultures are routinely maintained in Nunc 25 cm^2 tissue culture flasks in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich New South Wales) supplemented with 10% foetal calf serum (FCS), 2 mM $_L$-glutamine, 1 mM non-essential amino acids and 100 U/mL penicillin/streptomycin antibiotic solution. The cell cultures were maintained in CO_2 incubator at 37°C (5% CO_2, 95% air atmosphere) with the culture media replaced every 2 days.

Once the cell cultures reached around 90% confluence, the old culture medium was removed and cell lines were washed with PBS or saline. The solution then was aspirated out and trypsin-EDTA was added to detach cells from flask surface. The cell flask was then returned to the CO_2 incubator for about eight minutes. Any floating cells that lifted from the bottom of the flask were viewed under the microscope. When most of the cells had lifted and were seen floating, a serological pipette was used to mix the solution in the flask. The clumping of any remaining cells was reduced by forcefully pipetting the mixture against side of flask for few times. After the final mixing of saline, trypsin and cells, the solution was transferred to a fresh conical tube containing of fresh DMEM growth medium to inactivate the trypsin. The conical tube was capped tightly and centrifuged at 1600 RPM for seven minutes. The supernatant was aspirated out after the centrifuging and a new DMEM growth medium was added to the remaining pellet. The pellet and growth medium were mixed using a sterile transfer pipette a number of times to attempt to dissociate clumping cells prior to seeding in few plates and flasks. To determining the number of cells in the suspension, a haemocytometer was used. A mixture consisting 25 µL of saline, 25 µL of 0.4% trypan blue and 25 µL of cell suspension was prepared. A heavy coverslip was placed over the counting surface and the hemocytometer

counting chamber was then placed on the microscope stage and viewed under 100 x magnifications for the total cells calculation. After cell counting by haemocytometer, about three to four drops of cells suspension was transferred into a new 25 cm^2 tissue culture flask for passage maintenance. A 3.5 mL DMEM growth medium was added and the flask was returned to the CO_2 incubator. Cells in the flask were fed again by changing growth media approximately once every two days and passaged once it reaches the 90% confluence. For the purpose of this study, a 96-well plate (black) was fed with 2000 cells per well. The 96-well plate was feed for every other day for 19 to 21 days before it was ready for the bacterial adherence experiment.

3.2.2 LS174T cell culture

LS174T cells are also human adenocarcinoma cells and are used as another human gut cell line for our bacterial attachment study. LS174T cells were acquired from ATCC and grown in 25 cm^2 flasks in growth media containing 10% foetal calf serum (FCS), 2 mM $_L$-glutamine, 1 mM non-essential amino acids and 100 U/mL penicillin/streptomycin antibiotic solution. LS174T cells also were seeded into 96-well plates at 2000 cells per well and incubated for 9 days. All cell cultures were maintained in CO_2 incubator at 37°C (5% CO_2, 95% air atmosphere) with the growth media replaced every 2 days. A 96- well plate and 25 cm^2 flasks were seeded with LS174T cells.

3.2.3 Bacterial cultures

Two strains of *H. pylori* causing gastric cancer, namely G27 (NCBI 563041) and J99 (ATCC 700824) were donated by the *Helicobacter pylori* Research Laboratory, University of Western Australia. *H. pylori* strain G27 has been used extensively in *H. pylori* research [388]. Each strain was grown on a blood agar plate enriched with *H. pylori*-selective supplement (Dent, Oxoid, Australia). All the blood agar plates were incubated at 37 °C under microaerobic conditions (90% CO_2, 4% N_2, 2% H_2) for five to seven days upon the experiment. The aerobic bacteria (*Escherichia coli* W (ATCC 9637) and *Staphylococcus aureus* (ATCC 6538) were stored in nutrient broth at 4 °C. Upon the experiment, all aerobic bacteria were inoculated onto nutrient agar plates

and incubated for 24 to 48 hours at 37 °C. All nutrient agar and nutrient broth used in this study were obtained from Oxoid (Adelaide).

3.2.4 *Bac*Light green preparation

All bacteria colonies were scraped from their agar plates and suspended in 10 mL of cold sterile phosphate buffered saline (PBS, Sigma-Aldrich, New South Wales). The bacteria suspension was then centrifuged at 4000g for 10 minutes. Each bacterial pellet was re-suspended in 1000 µl cold PBS and pipette tips were used to resuspend the pellet before being transferred to microcentrifuge tubes. A 9 µl of a 100 µM *Bac*Light Green solution in DMSO Molecular Probes (Eudene, Oregon USA) was added to each of the bacterial suspensions in each centrifuge tube to give a final dye concentration of 900 nM. The *Bac*Light Green bacterial stain is a fluorescent labeling reagent for detecting and monitoring live bacteria. Bacteria stained with the *Bac*Light Green will exhibit bright green fluorescence (absorption/emission about 480/516 nm). These tubes were incubated for 1 hour at 37 °C in a rotating incubator at 650 RPM. The samples were subsequently centrifuged at 11 000g for 5 minutes, aspirated and washed with multiple times of cold PBS. After the final wash, the pellets were re-suspended in PBS to the final volume of 1 mL.

For the bacterial attachment study with the presence of P-glycoprotein inhibitor, Hepes Balanced Salt Solution (HBSS) was prepared. A tube containing 30 mL of HBSS and 10% foetal calf serum (FCS) and a combination of HBSS, FCS and 4 µM PSC-833 (valspodar) were also prepared. To make up bacterial solution, two eppendorf tubes were prepared for each bacteria strain. One eppendorf tube contained HBSS, FCS and bacterial solution while the other eppendorf tube contained HBSS, FCS, bacterial solution and PSC-833.

3.2.5 Rapid bacteria count

A new 96- well plate was used for rapid bacteria count. 100 µL of cold PBS was put to the beginning of each row followed by 100 µL of each bacterial solution. Doubling dilution was conducted along each well of the row where the bacteria

solution was added. The absorbance measurement was read at 415 nm wave length using a TECAN Sunrise™ (TECAN Group Ltd, Switzerland) 96-well plate reader using the Magellan 3 software for Windows 2000. Bacterial cell count was determined from the absorbance of 400 nm.

3.2.6 Bacterial attachment study

3.2.6.1 Caco-2 cells and P-glycoprotein expression inhibition

A potent P-glycoprotein inhibitor, PSC-833 (valspodar) was used in this study. 21 day old Caco-2 cells grown in black 96-well plates were used for bacterial attachment study. Medium was replaced with HEPES Balanced Salt Solution at pH 7.4. The P-glycoprotein inhibitor, PSC833 was incubated with Caco2 cells for only 30 minutes prior to the first bacterial incubation period to allow P-glycoprotein transport sites to be blocked prior to exposure to bacteria [389]. *S. aureus* and *E.coli* W were used as the positive controls as both bacteria may also colonize the gastrointestinal tract.

One 96- well plate was used for one bacterial strain. The bacterial solutions were loaded into the black 96-well plates accordingly. Quadruplicate wells were each incubated with bacteria in growth medium for 30, 60, 90, 120, 180 and 240 minutes and incubated at 37 °C during this time period. Concurrently with bacterial attachment studies done at these time points, additional wells of the plate were incubated with PSC-833 to block P-glycoprotein function (**Figure 3-1)**. Bacteria were also incubated at 30, 60, 90, 120, 180 and 240 minutes in here. Once the addition of bacterial solution to the cell lines at the required times had been completed, loosely associated bacteria were washed off from the well with at least three washes of cold PBS. Finally, a 96-well fluorescence plate reader (Fluostar Optima) from BMG LabTech (Mornington, Victoria) was used to detect fluorescent bacteria in these black 96-well plates. A 485 nm excitation filter and 520 nm emission filter were used for detection using top excitation and top emission.

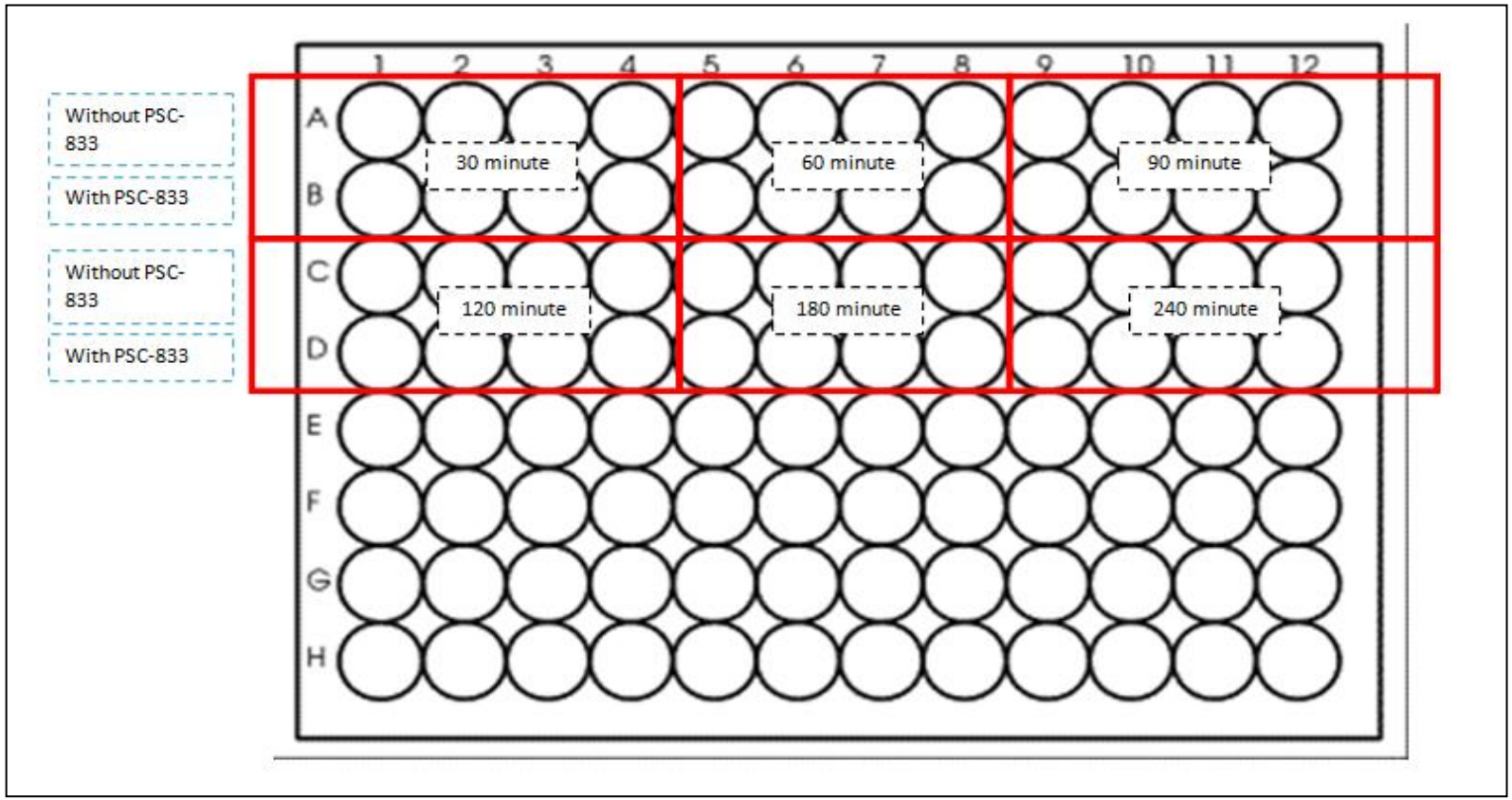

Figure 3-1: The arrangement of bacteria solution in a 96- well plate seeded with Caco-2 cells with incubation period of 30, 60, 90, 120, 180 and 240 minutes. PSC-833 solution was added only to the lane B and D. (Original by M.Omar)

To check the bacteria stickiness to the well, another black 96-well plates was filled with each bacteria solution (with and without PSC-833) and incubated at 37 °C for about 60 minutes. After end of incubation period, the plate had its fluorescence measured using the Fluostar Optima before and after aspiration of the fluorescence bacteria.

3.2.6.2 LS174T cells and P-glycoprotein expression inhibition

Black 96- well plate was seeded with LS174T cells and grown up to ten days upon the experiment in a CO_2 incubator at 37°C (5% CO_2, 95% air atmosphere) with the growth media replaced every two days [375]. Similar method as in Chapter 3.2.6.1 was utilized for bacterial attachment study in LS174T cells with the presence of P-glycoprotein inhibitor, PSC-833.

3.2.6.3 LS174T and P-glycoprotein expression induction

In this current study, P-glycoprotein expression of LST174T cells was induced with rifampicin. After four days of seeding, growth media containing 10 µM rifampicin was given to the LST174T cells in black 96- well plate and parallel 25 cm^2 flasks that had been used for collecting protein for Western Blot Analysis. The growth media containing rifampicin was replaced every 2 days. The LST174T cells were used after 6 days of exposure to rifampicin. For bacterial attachment study in LS174T cells with the presence of P-glycoprotein inhibitor (PSC-833), the method used was similar to Chapter 3.2.6.1. One 96-well plates was used for each bacteria strain and quadruplicate wells were used for each incubation period as the arrangement illustrated in **Figure 3-2**.

In order to determine the influence of P-glycoprotein induction by rifampicin, P-glycoprotein expression in 10 days old LS174T was measured by Western Blot Analysis. Each 25 cm^2 flasks containing LS147T was feed either with rifampicin enriched growth media or normal growth medium. For protein collection, the growth medium were removed from each flask and rinsed with cold PBS. About 1 mL of

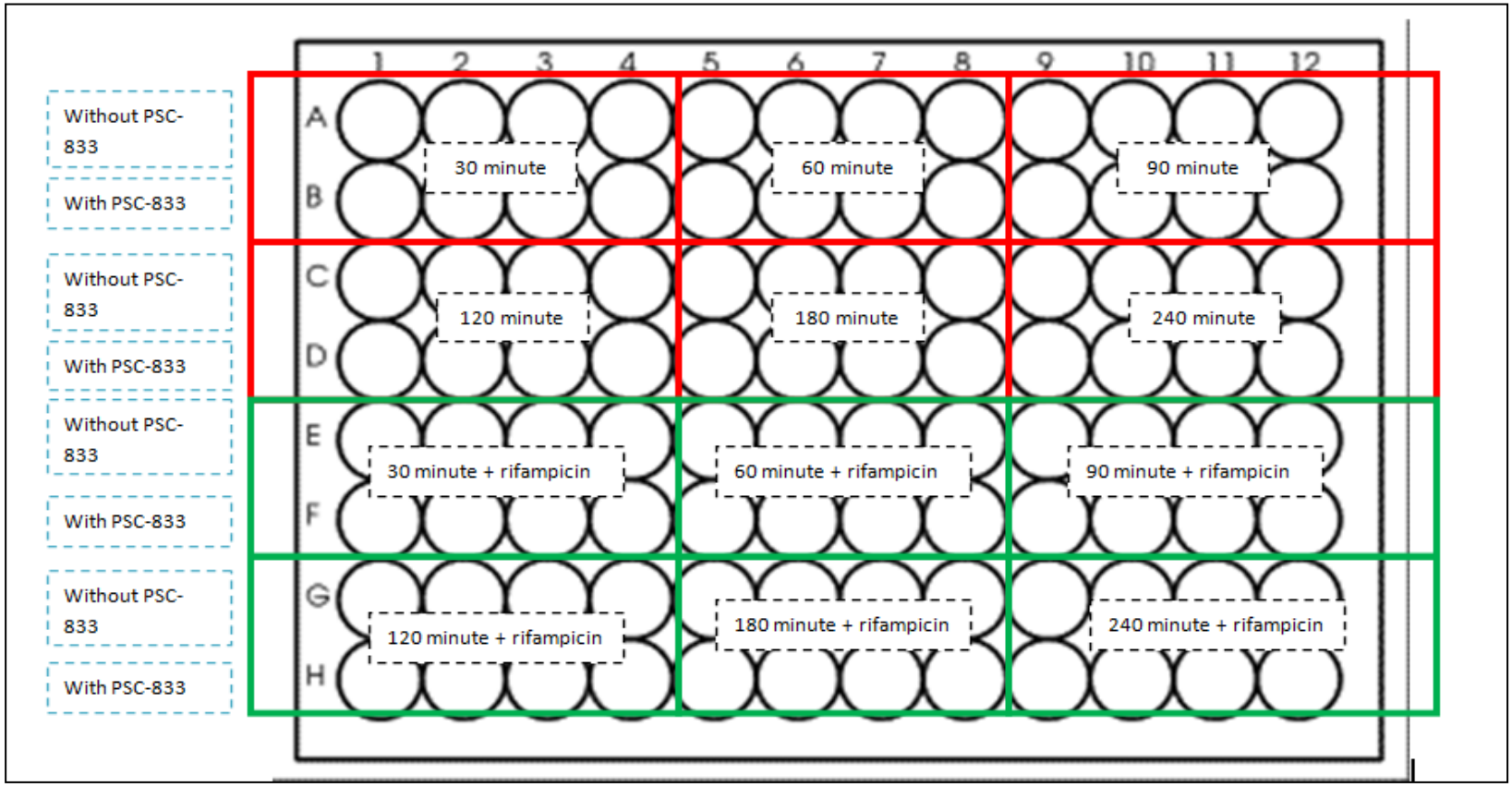

Figure 3-2: The arrangement of bacteria solution in a 96- well plate seeded with LS174T cells with incubation period of 30, 60, 90, 120, 180 and 240 minute. Rifampicin was added into second half of the 96-well plates to induce P-glycoprotein expression of LS174T cells. PSC-833 solution was added only to the lane B, D, F and G. (Original by M.Omar)

tissue cell lysis buffer was added into each flask to over the cells surfaces. After 10 minutes, the cells were extracted out from the flasks and transferred into new centrifuge tubes. The tubes were sonicated for 10 minutes in sonicating water. After final centrifugation, the supernatant were collected from each tube and kept at -20 °C until the protein determination and Western Blot Analysis could be done. The Western Blot Analysis was performed based on the method outlined in Chapter 3.1.7.3. ChemiDoc™ MP programme was used to determine the P-glycoprotein expression from Western Blotting

3.2.7 Statistical analysis

For bacterial attachment studies using Caco-2 cells, the fluorescence intensity for cells that has been incubated with 4 µM PSC-833 were compared to its control cells using student t-test. Results were presented as the mean ± standard deviation of quadruplicate well at each incubation period. SAS® software (SAS Institute Inc.) was used in this study and in all analyses, p-values less than 0.05 were taken to indicate a statistically significant difference.

For bacterial attachment studies using LS174T cells, the fluorescence intensity for cells that has been incubated with 4 µM PSC-833, 10 µM rifampicin or 4 µM PSC-833 plus 10 µM rifampicin were compared to its control cells using student t-test. Results were presented as the mean ± standard deviation of quadruplicate well at each incubation period. SAS® software (SAS Institute Inc.) was used in this study and in all analyses, p-values less than 0.05 were taken to indicate a statistically significant difference.

CHAPTER 4

RESULTS

CHAPTER 4 RESULTS

4 Overview

This study was divided into two phases: Phase 1 (*Helicobacter pylori* and P-glycoprotein expressions) and Phase 2 (*Helicobacter pylori* attachment to gastrointestinal cell lines).

4.1 Phase 1: *Helicobacter pylori* and P-glycoprotein expression

4.1.1 Participant`s demographic

In this phase of study, a total of 91 participants had been recruited. A total of 33 participants have been classified as *H. pylori* –positive including 11 whom had been diagnosed with failure towards *H. pylori* eradication therapy. Another 58 participants were *H. pylori* – negative. Participant`s demographic based on their Helicobacter infection status has been summarized in **Table 4-1. Table 4-2** demonstrates the indication for endoscopy.

The distribution of endoscopy findings is illustrated in **Figure 4-1**. Most of study subjects demonstrated spectrum of normal upper gastrointestinal tract anatomy during their endoscopy (normal endoscopy, n = 34, 37%). In relation to the age group, most subjects with normal endoscopy came from 40-49 years old (n = 9, 26.5%) followed by 60-69 years old (n = 8, 23.5%) and 50-59 years old (n = 7, 20.6%) as opposed to younger subjects (20-39, n = 4, 11.8%) and older age group (70-99, n = 6, 17.6%).

Table 4-1: Demographic characteristic of study subjects according to *H. pylori* infection status

		***Helicobacter pylori* infection status**		**p-value**
		***H. pylori*-positive**	***H. pylori*-negative**	**(Chi-**
		n = 33 (36.3%)	**n = 58 (63.7%)**	**square)**
Gender	Male	14 (42.4%)	28 (48.28%)	0.48
	Female	19 (57.6%)	30 (51.72%)	
Age	20-29	3 (9.09%)	4 (6.89%)	0.78
	30-39	4 (12.12%)	5 (8.62%)	
	40-49	8 (24.24%)	13 (22.41%)	
	50-59	4 (12.12%)	12 (17.24%)	
	60-69	6 (18.18%)	14 (24.14%)	
	70-79	5 (15.15%)	6 (10.34%)	
	80-99	3 (9.09%)	4 (6.89%)	
Endoscopy finding	Peptic ulcer disease	9 (27.27%)	6 (10.34%)	0.025
	Others	24 (72.73%)	52 (89.65%)	
MDR1	*CC*	9 (27.27%)	11 (18.96%)	0.334
C3435T	*CT*	12 (36.36%)	31 (53.45%)	
genotype	*TT*	12 (36.36%)	16 (27.58%)	
Use of proton pump inhibitor	Yes	10 (30.30%)	27 (46.55%)	0.034
	No	23 (69.69%)	31 (53.45%)	

Table 4-2: Primary indication for endoscopy of study subjects

Indication		Age (average, year)	Number of participant (percentage)
Abdominal pain		66	2 (2.19%)
Epigastric pain		56.5	8 (8.79%)
Anaemia		58.7	10 (10%)
Dyspepsia		50.2	13 (14.28%)
Dysphagia		58.3	7 (7.69%)
Varices		58.9	9 (9.89%)
Bleeding		51.7	3 (3.3%)
Malignancy		45.1	11 (12.09%)
Ulcer		63	5 (5.49%)
Abdominal imaging		68.6	7 (7.69%)
Others	Anorexia	53.7	1 (1.1%)
	Atypical chest pain		1 (1.1%)
	Barret`s syndrome		1 (1.1%)
	Cough		1 (1.1%)
	Diarrhoea		3 (3.3%)
	Duodenal biopsy		1 (1.1%)
	Gastroparesis		1 (1.1%)
	Heartburn		1 (1.1%)
	Reflux syndrome		1 (1.1%)
	Stricture		1 (1.1%)
	Vomiting		1 (1.1%)
	Antral biopsy		3 (3.3%)

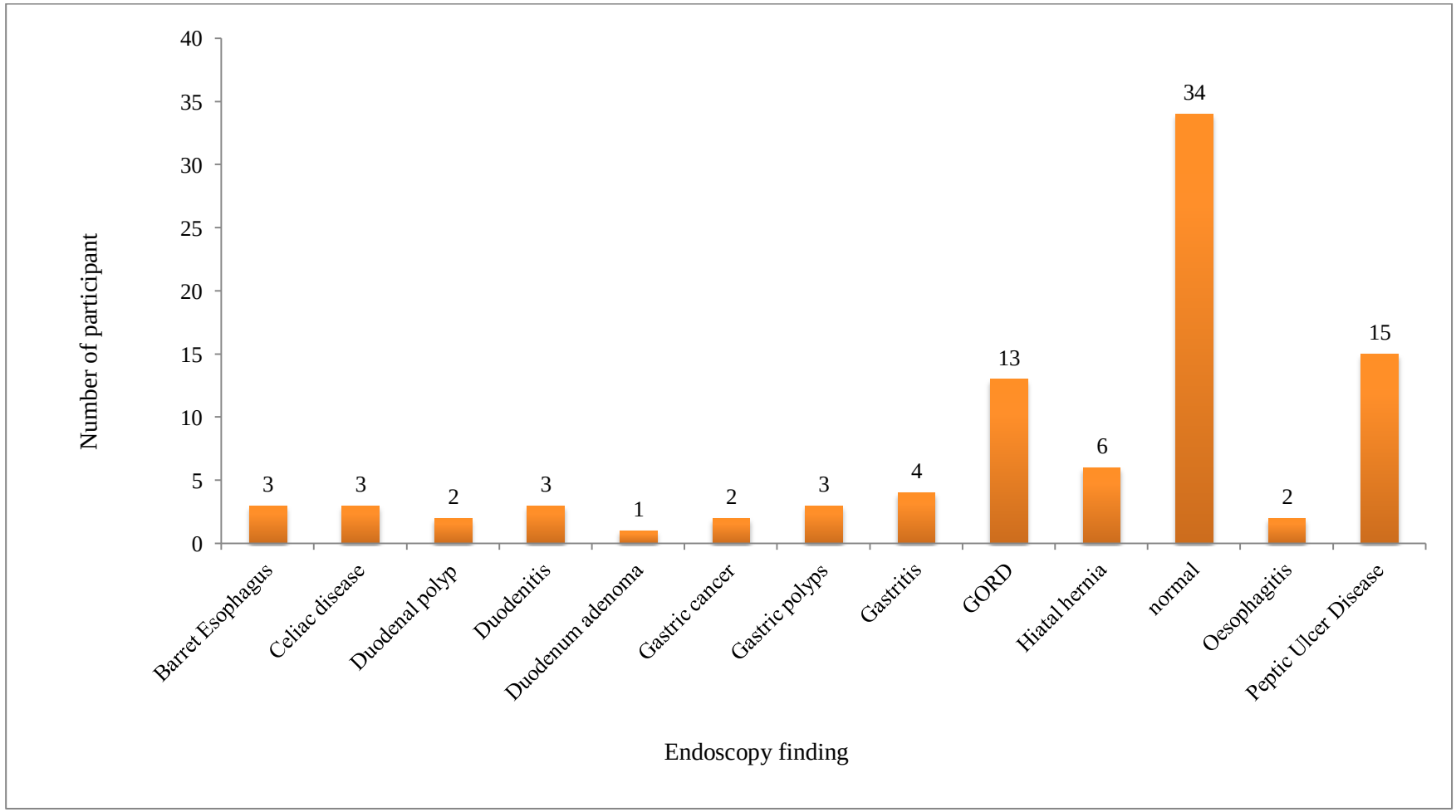

Figure 4-1: The distribution of endoscopy findings of study subjects

4.1.2 Questionnaire

Participants were required to answer two questions upon the endoscopy procedure. The answer for the questionnaire was populated in **Table 4-3**. Overall, most of the participants did not know whether they were ever tested for *H. pylori* (41.76%, n = 38). Only 32.97% of the participants (n = 30) stated that they were tested for the bacterial infection while the remaining 25.27% of the participants (n = 23) have not received any test for *H. pylori*. On the other hand, nearly quarter of the participants (24.17%, n = 22) acknowledged to receive treatment for *H. pylori* infection. About 34.1% of the participants (n = 23) stated that have not received any treatment for *H. pylori* before the recruitment. Again, most of the participants did not know whether they were ever treated for *H. pylori* (41.76%, n = 38).

Table 4-3: Distribution of answer for all questionnaires

Question 1	**Choice of answer**	***H. pylori* positive (n = 33)**	***H. pylori* negative (n = 58)**	**p-value (Chi square]**
Have you been tested for bacteria named *Helicobacter pylori?*	Yes	13 (39.39)]	17 (29.31%)	0.4474
	No	9 (27.27%)	14 (24.14%)	
	I don`t know	11 (33.33%)	27 (46.55%)	

Question 2	**Choice of answer**	***H. pylori* positive (n = 33)**	***H. pylori* negative (n = 58)**	**p-value (Chi square]**
Have you been treated for bacteria named *Helicobacter pylori?*	Yes	12 (36.36%)	10 (17.24%)	0.0757
	No	10 (30.30%)	21 (36.21%)	
	I don`t know	11 (33.33%)	27 (46.55%)	

4.1.3 *Helicobacter pylori* infection status

All the antral biopsies collected upon the endoscopy were sent to the laboratory to determine *H. pylori* infection status based on culture and polymerase chain reaction (PCR). In addition, few antral biopsies have been sent off to the pathology laboratory for histology and the result was obtained from participant`s medical record. For those *H. pylori* - resistance subjects, the gastroenterologist also had performed additional test (CLOtest) in the endoscopy suite, however their antral biopsies still went to the laboratory for culture and PCR.

Figure 4-2 shows the positive growth of *H. pylori* on blood agar after been cultured for four days. The example of PCR positive results using two different primers could be seen in **Figure 4-3** and **Figure 4-4**. Overall, 33 of our subjects were diagnosed as *H. pylori*-positive. The total positive cases came from the combination of various tests conducted here in our laboratory as well as in the recruitment site. In our laboratory, 23% (n = 21) of our subjects were detected positive based on bacteria culture. By using PCR, the percentage of positive subjects was increased to 30.76% (n = 28). In addition, 10 antral biopsies were sent to the hospital laboratory for histology and only one was confirmed positive for *H. pylori* infection. A number of subjects (n = 14) also underwent CLOtest during their endoscopy procedure and all came back positive for *H. pylori* infection.

Figure 4-2: Example of positive growths of *Helicobacter pylori* on blood agar after been cultured for four days at 37 °C under microaerobic conditions (90% CO_2, 4% N_2, 2% H_2). (Picture courtesy of Chin Yen Tay, University of Western Australia, published with permission)

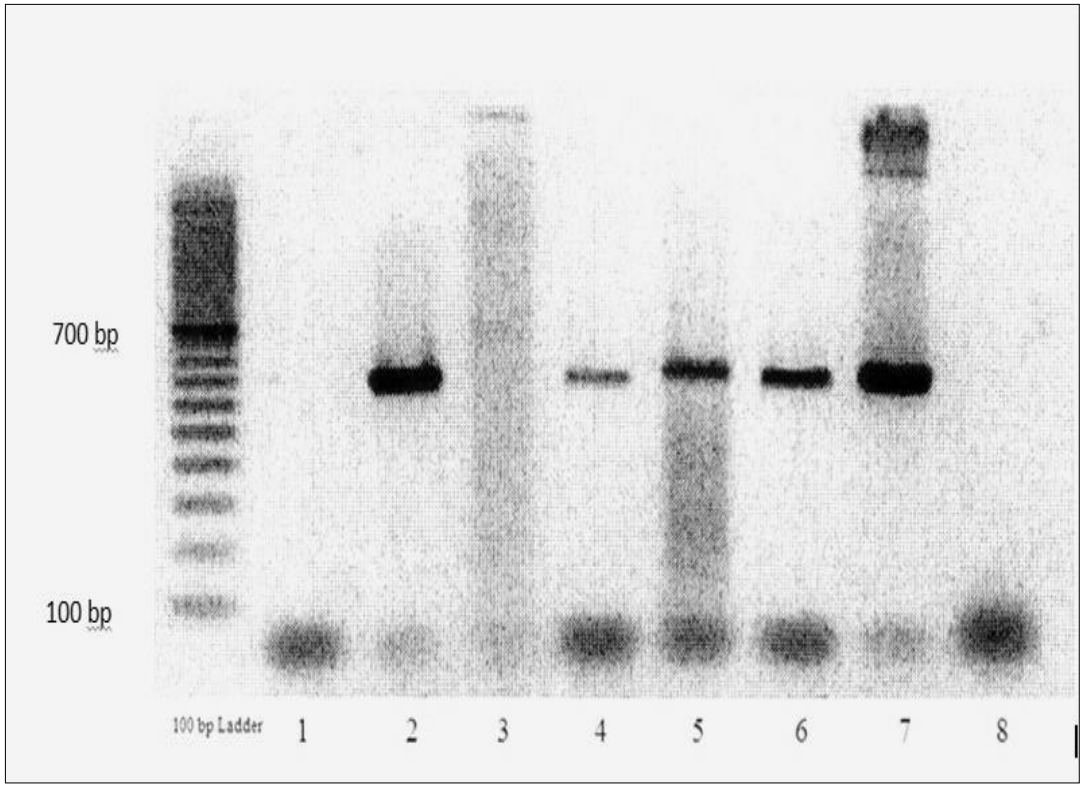

Figure 4-3: Example of primary amplication of *vacA* gene of *Helicobacter pylori* specific sequences using *vacA 1* and *vacA 2* primers in antral biopsies with 100 bp ladder as molecular marker. Lane 1, 3 were negative; lane 2, 4, 5, 6 were positive. *H. pylori* 26695 was used as positive control (lane 7) and lane 8 was reserved for the negative control.

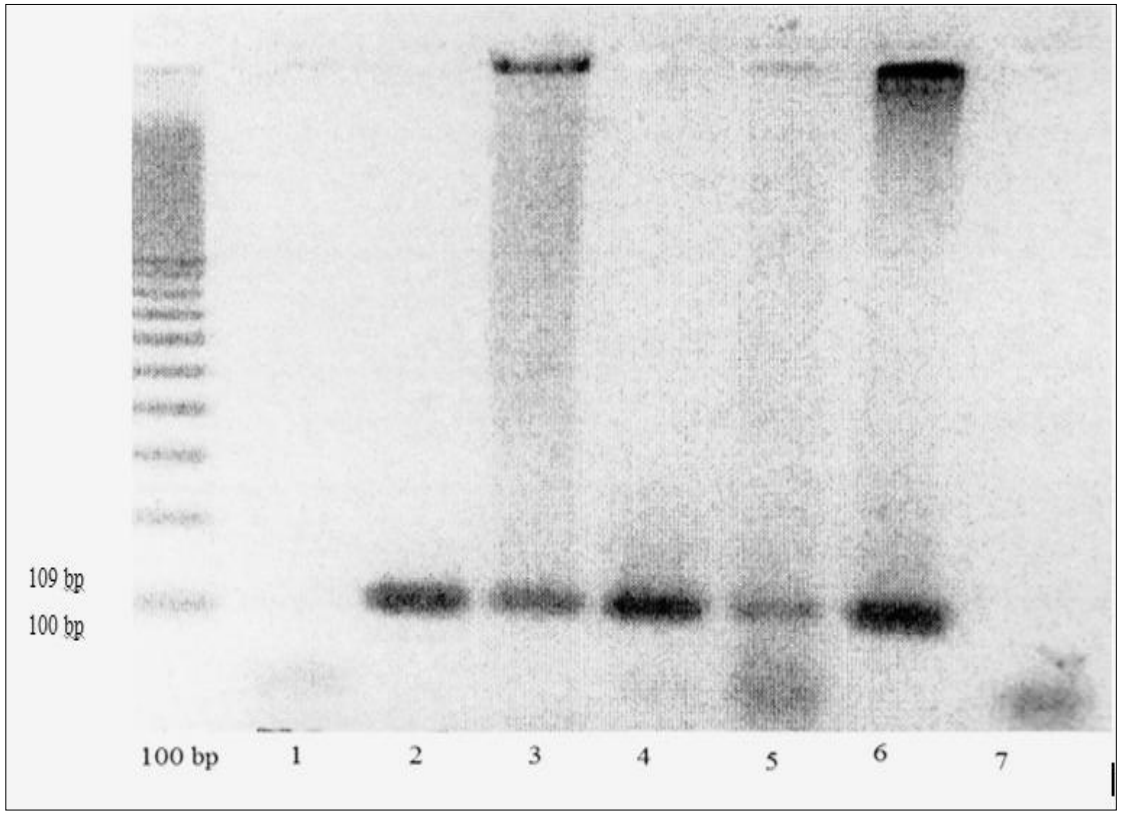

Figure 4-4: Example of primary amplication of 16S rRNA gene of *H. pylori* specific sequences using 16S rRNA primers in antral biopsy specimens with 100 bp ladder as molecular marker. Lane 1 was negative and lane 2, 3, 4 and 5 were positive. *H. pylori* 26695 was used as positive control (lane 6) and lane 7 was reserved for the negative control.

4.1.4 Protein analysis

The protein concentration was determined using a Micro-Lowry Method using TECAN Sunrise 96 well plate spectrophotometer and Magellan 3 software before been used running protein analysis on Western Blot. The example of Western Blot gel and its P-glycoprotein expression is illustrated in **Figure 4.5**.

The statistical analysis was divided into two parts; the first one was for the first 76 participants recruited in this study. While the second part of the data analysis only concentrated on the group of participants (n = 15) who were recruited from the referral list due to resistance to *H. pylori* eradication therapy.

4.1.4.1 P-glycoprotein expression in antrum and duodenum

In the 76 subjects recruited in the first part of this study, there was a significant difference between P-glycoprotein expression in antrum and duodenum (medians 218 *vs.* 797, $p < 0.0001$) as illustrated in **Figure 4.6**. The P-glycoprotein expression in the duodenum was found to be higher than that in the antrum.

4.1.4.2 P-glycoprotein expression and *Helicobacter pylori* infection status

In 76 subjects, a significant difference in P-glycoprotein expression between *H. pylori* positive and *H. pylori* negative subjects (means ratio of antrum to duodenum: 0.38 ± 0.19 *vs.* 0.29 ± 0.15, $p = 0.028$) was detected [**Figure 4.7**]. The P-glycoprotein expression in *H. pylori* positive was found to be higher than that in the *H. pylori* negative subjects. That ratio of P-glycoprotein expression was not influenced by the proton pump inhibitor taken by the subjects prior to the endoscopy ($p = 0.171$). Gender or age also had no influence on P-glycoprotein expression in the antrum or duodenum ($p > 0.05$).

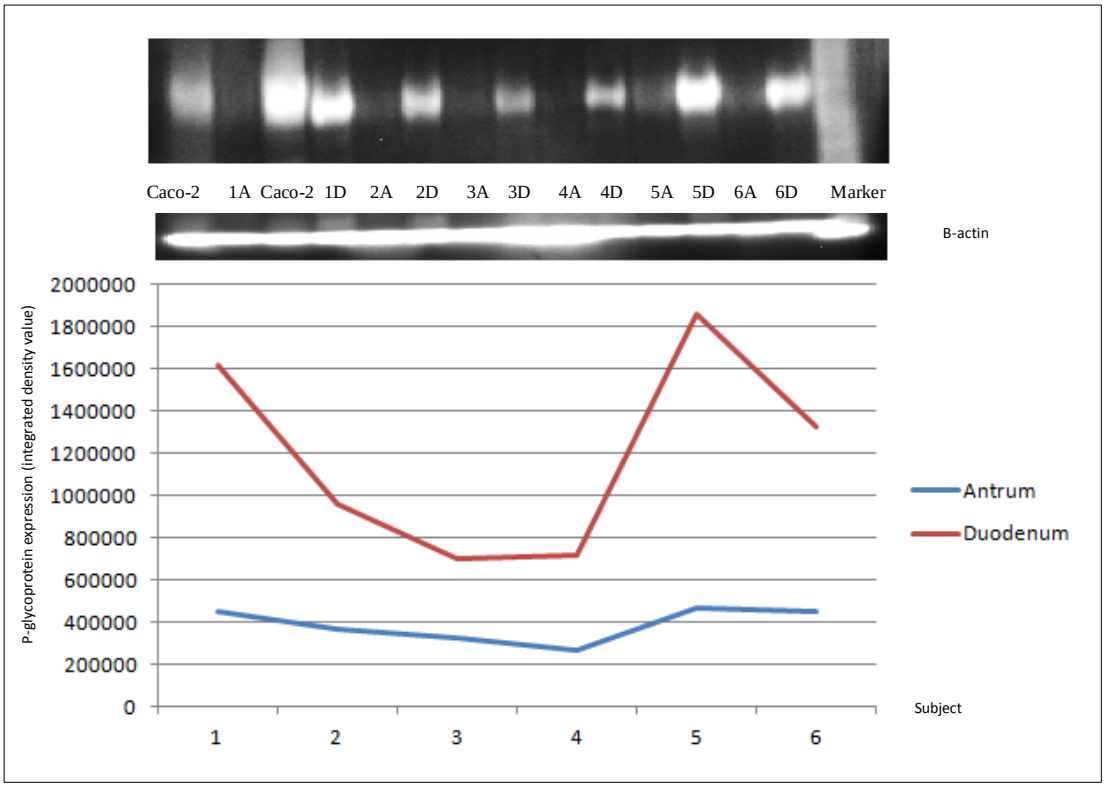

Figure 4-5: Expression of P-glycoprotein in subject 1 to subject 6. Representative western blot gel showing P-glycoprotein expression in antral (A) and duodenal (D) biopsies with two different Caco-2 passages as positive controls (upper figure). The expression of P-glycoprotein was detected by incubating the blot membranes with PgP Sc13131 Mdr [G-1] mouse monoclonal IgG2b antibodies. The mouse anti-B-actin was used as control to normalize the gel loading and western blot transfer (middle figure). Relative P-glycoprotein expression level in antrum and duodenum according to the each subject (1 to 6) assessed by densitometry (lower figure).

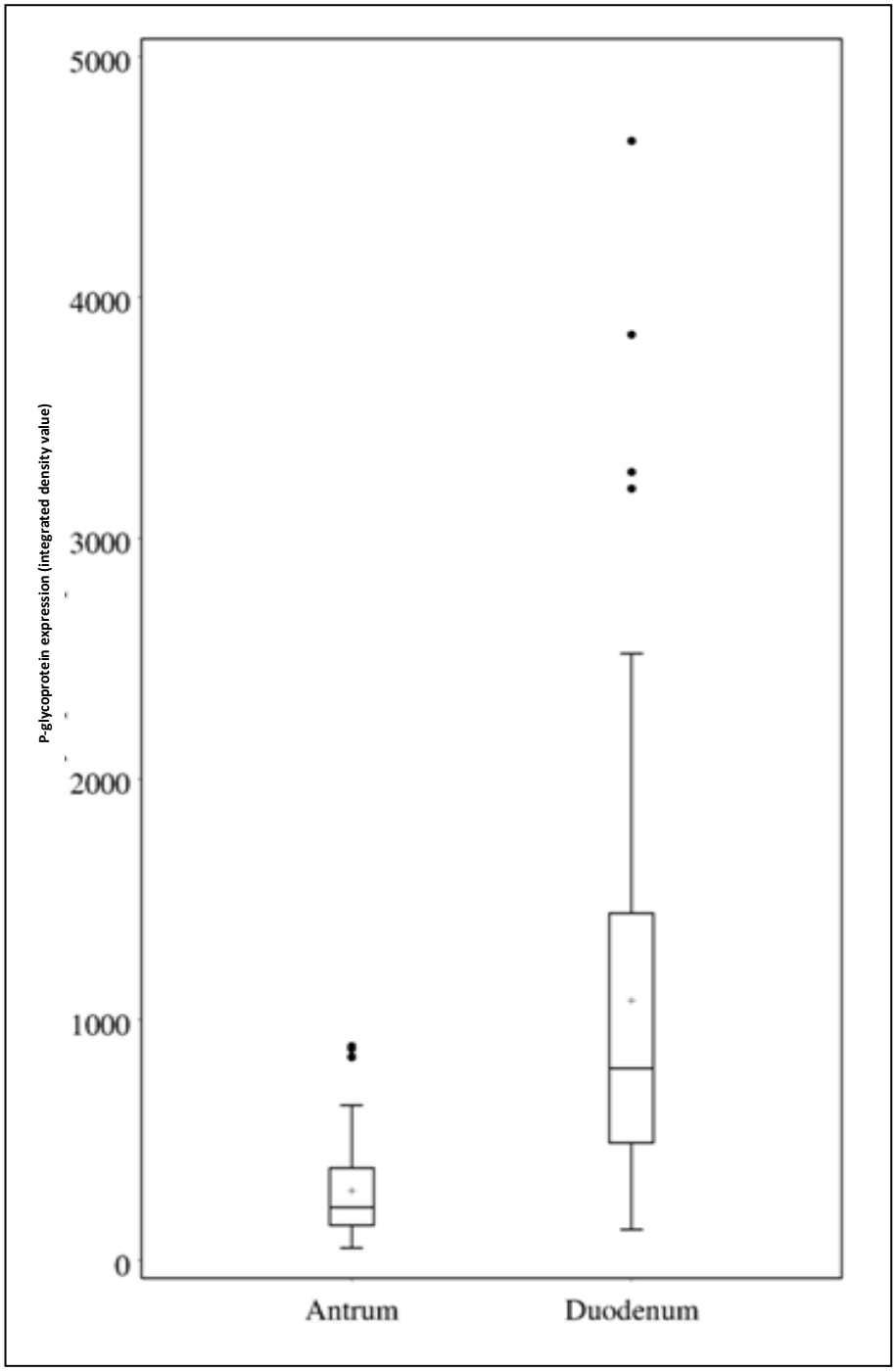

Figure 4-6: The distribution of P-glycoprotein expression in antrum and duodenum from overall subjects (n = 76). Antrum: medians (218), mean (452), standard deviation (936). Duodenum: medians (797), means (1798), standard deviations (3714).

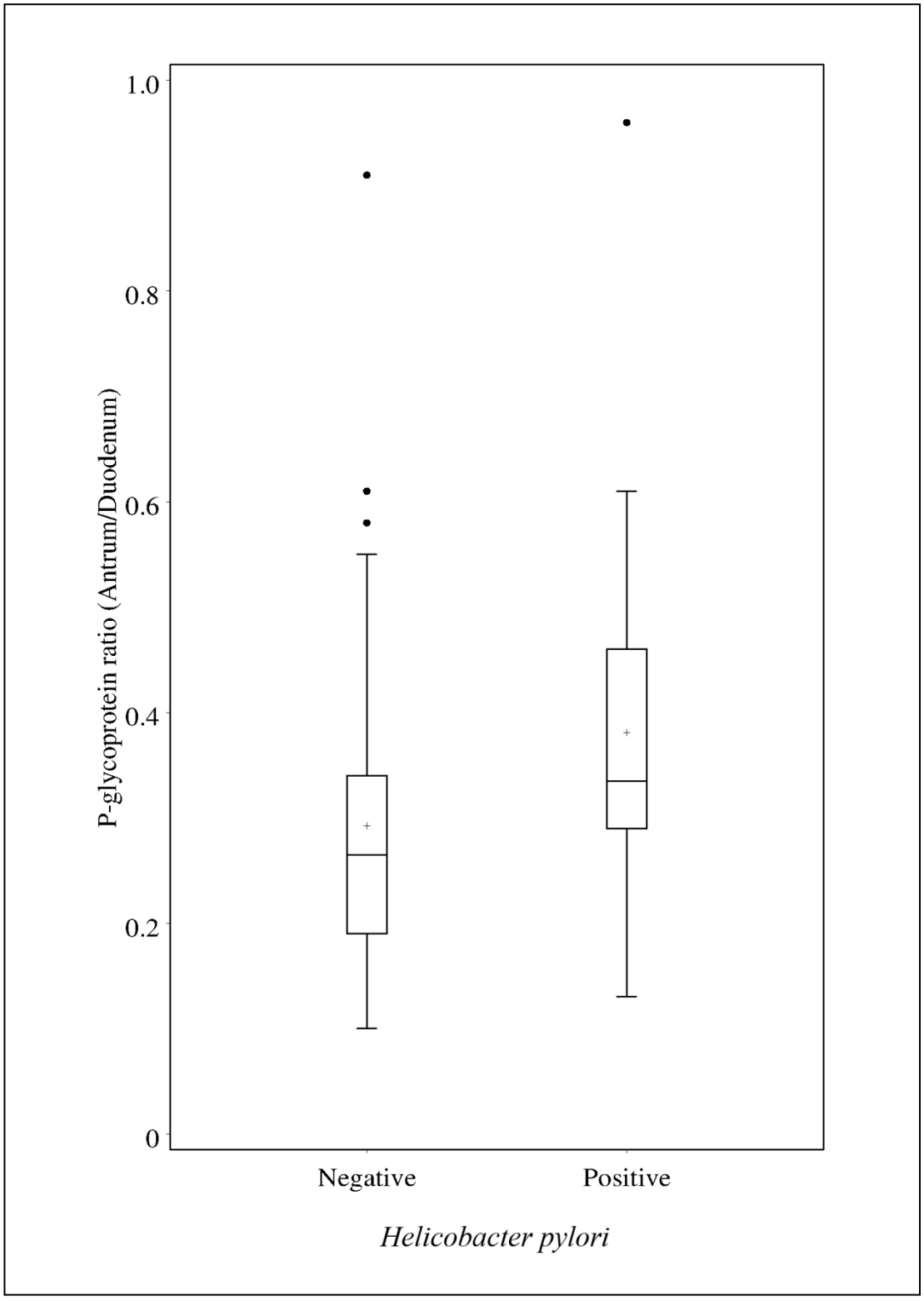

Figure 4-7: Comparison of P-glycoprotein expression (log antrum / duodenum) in *Helicobacter pylori* – positive and –negative subjects

4.1.5 Influence of *MDR1 C3435T* polymorphisms

The blood DNA quantification was obtained using a Biospec-Nano spectrophotometer (Shimadzu Biotech New South Wales) at 712 nm with TE buffer as blank solution for all samples. Using the blood DNA, the C3435T polymorphism band was yielded followed by the enzymatic digestion with 2 units of *Mbo-1* enzyme to produce one band (197 bp) for homozygous TT, 158 bp for homozygous CC and two bands (197 and 158 bp) for heterozygous CT genotype (**Figure 4-8)**.

A regression model was used to identify if there was any difference between MDR1 genotype and P-glycoprotein levels from antrum **(Table 4-4)**. The heterozygous subjects demonstrated to have an intermediate level of P-glycoprotein expression when compared to the homozygous (*CC* and *TT*) although the different was not statistically significant ($p = 0.1049$). However, based on the paired t-test, the *3435TT* genotype appeared to have a significantly lower P-glycoprotein expression than *3435CC* subjects ($p = 0.041$).

Figure 4-9 illustrates the distribution of homozygous (*CC* and *TT*) and heterozygous (CT) subjects with *H. pylori*-positive and *H. pylori*-negative in relation to P-glycoprotein expression (antrum to duodenum ratio). There was an increasing trend in P-glycoprotein expression linked to the presence of *H. pylori* for all the genotypes, however only the homozygous *TT* subjects demonstrated a statistically significant difference in their P-glycoprotein when compared between *H. pylori* –positive and –negative groups (means 1.03 *vs.* 1.47, $p=0.0293$).

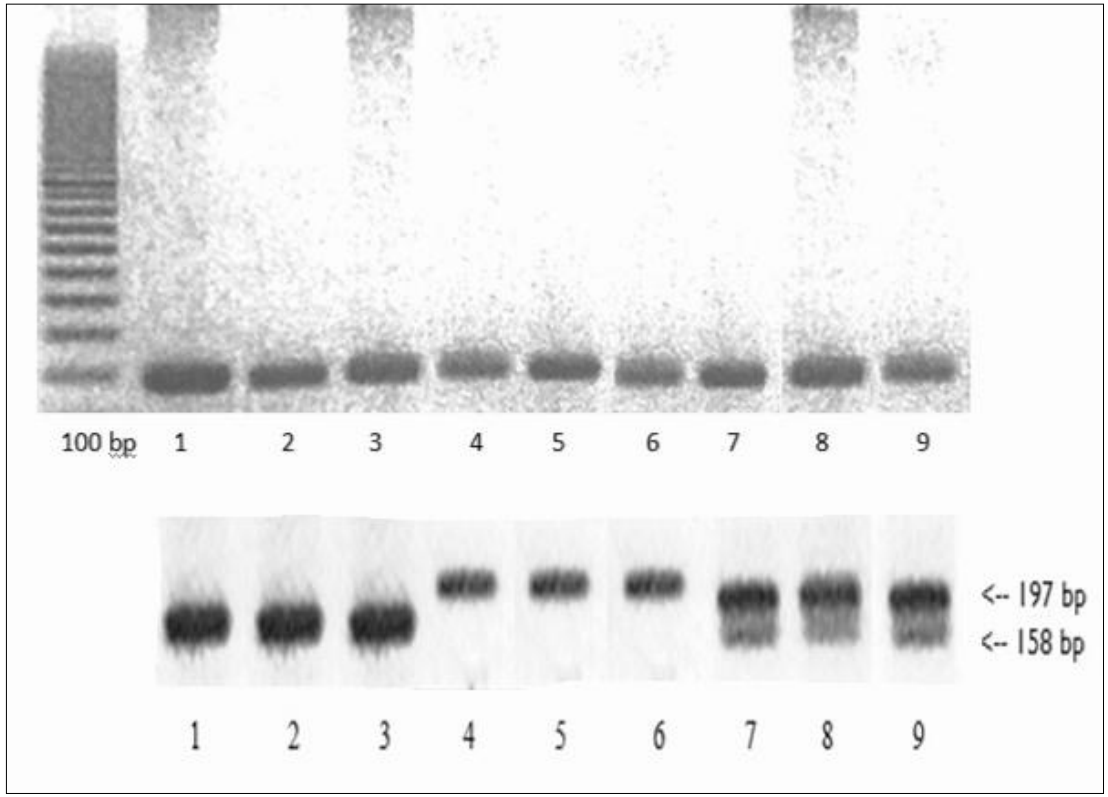

Figure 4-8: *MDR1 C3435T* bands determined by PCR-RFLP using blood DNA of subjects 1 to 9 (upper figure). *MDR1 C3435T* SNPs after Mbo-1 incubation as determined by PCR-RFLP using blood DNA of subjects 1 to 9 (lower figure) where two bands indicated heterozygosity (lane 7, 8, 9); one lower band indicated the *C/C* allele (lane 1,2,3) and one upper band indicate the *T/T* alleles (lane 4,5, 6).

Table 4-4: P-glycoprotein expression in antrum and *MDR1 C3435T* genotype

Site	*MDR1*	N	Mean (SE) (P-glycoprotein)	Overall p-value	Pair wise p-values	
					CT	*TT*
Antrum	*CC*	15	383.4 (61.4)	0.1049	0.075	
	CT	38	274.3 (31.6)			
	TT	23	246.7 (36.7)			

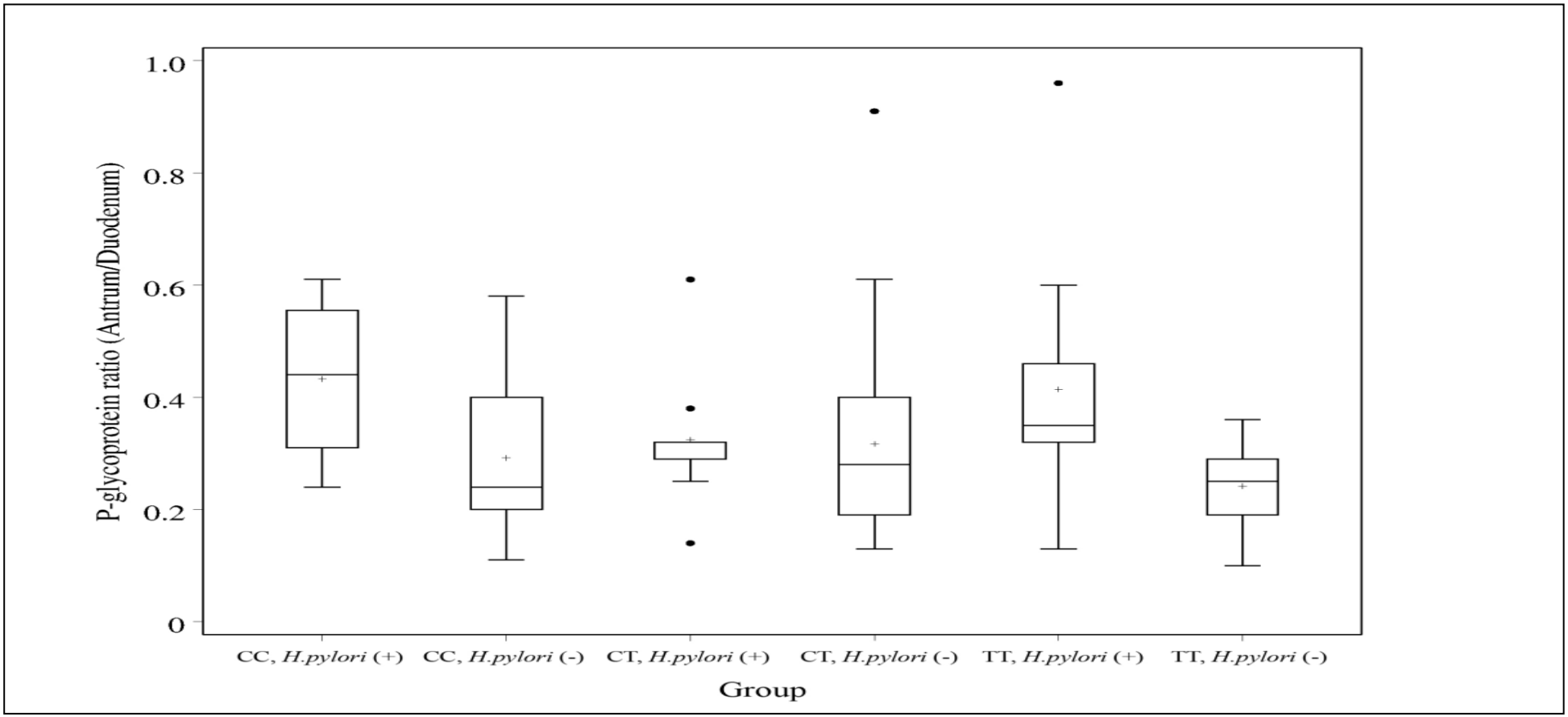

Figure 4-9: Comparison of P-glycoprotein ratio (antrum/ duodenum) according to *MDR1* genotype (*CC, CT* and *TT*) and Helicobacter status (*H. pylori*- positive and – negative)

4.1.6 *Helicobacter pylori* treatment resistance and CYP2C19 polymorphisms

Out of 91 participants that had been recruited, 15 of them had been referred as "failure towards *H. pylori* eradication therapy". Those 15 patients were treated with either Nexium HP7 or Klacid HP7 regimen previously. Among them, only 11 were tested positive for *H. pylori* upon referral based on CLOtest and culture.

Table 4-5 shows the demographic characteristic of resistance subjects in comparison to the *H. pylori*-negative subjects. Antibiotic sensitivity profile for 11 participants with *H. pylori* positive and had failed their eradication therapy is given in **Table 4-6**. Details of their medication regimens taken pre- and post-referral are listed in **Table 4-7**. Their P-glycoprotein levels were determined using Western Blot Analysis (**Figure 4-10)**. Most of the subjects demonstrated resistance to clarithromycin (72%) and metronidazole (63.6%). No resistance to amoxicillin, tetracycline or ciprofloxacin was observed in any of our subjects and rifampicin resistance was limited to one subject. Six of our subjects (54.5%) demonstrated resistance to both metronidazole and clarithromycin. Interestingly, two subjects demonstrated no antimicrobial resistance despite a history of repeated treatment failure.

Based on the enzymatic digestion with SmaHI (**Figure 4-11)** or BamHI (**Figure 4-12**), the study subjects were classified into five different allelic patterns as follows: homozygous for wild type alleles (*1) in exons 4 and 5 (*1/*1), heterozygous extensive metabolizers for the CYP2C19*2 polymorphism without the CYP2C19*3 polymorphism (*1/*2), heterozygous extensive metabolizers the CYP2C19*3 polymorphism without the CYP2C19*2 polymorphism (*1/*3), homozygous passive metabolizers for the CYP2C19*2 polymorphism without the CYP2C19*3 polymorphism (*2/*2) and homozygous passive metabolizers for the CYP2C19*3 polymorphism without the CYP2C19*2 polymorphism (*3/*3) as illustrated in **Figure 4-13**.

Table 4-5: Demographic characteristic of *H. pylori* treatment resistant and *H.pylori*-negative groups

Variable		*Helicobacter pylori* status		p-value
		***H. pylori*-treatment resistant (n = 11)**	***H. pylori* – negative (n = 54)**	
Gender	Male (%)	3 (27 %)	25 (46%)	0.44
	Female (%)	8 (73%)	29 (54%)	
Age	Mean (SD)	46.8 (10.4)	55.7 (16.1)	0.09
	Range	35-68	23-88	

Table 4-6: Resistance profile for participants with *H. pylori* - positive whom failed their eradication therapy

Participant	CLOtest	Culture	Antibiotic minimal inhibitory concentration					
			MZ	AC	TC	CI	RI	CH
1	Positive	Positive	R	S	S	S	R	R
2	Positive	Positive	S	S	S	S	S	S
3	Positive	Positive	R	S	S	S	S	R
4	Positive	Positive	R	S	S	S	S	S
5	Positive	Positive	R	S	S	S	S	R
6	Positive	Positive	R	S	S	S	S	R
7	Positive	Positive	S	S	S	S	S	S
8	Positive	Positive	R	S	S	S	S	R
9	Positive	Positive	R	S	S	S	S	R
10	Positive	Positive	S	S	S	S	S	R
11	Positive	Positive	S	S	S	S	S	R

- Antibiotic sensitivity testing was performed by E-test (Biodisk) for amoxicillin (AC), clarithromycin (CH), metronidazole (MZ), tetracycline (TC), rifampicin (RI) and ciprofloxacin (CI).
- R for resistance and S for sensitive strains
- Resistance was defined according to National Committee for Clinical Laboratory Standards for antibiotic minimal inhibitory concentration (MIC) : AC, MIC $\geq$ 2 μg/ml ; CH, MIC $\geq$ 1 μg/ml ; MZ, MIC $\geq$ 8 μg/ml ; TC, MIC $\geq$ 1 μg/ml ; RI, MIC $\geq$ 4 μg/ml and CI, MIC $\geq$ 1 μg/ml.

Table 4-7: Medication regimens taken pre- and post-referral for participants with *H. pylori* positive whom failed their eradication therapy

Subject	Medication	
	Pre-referral	**Post-referral**
1	Nexium HP7 [+]	Regimen A[++]
2	Nexium HP7 [+]	Regimen A[++]
3	Nexium HP7 [+]	Regimen A[++]
4	Nexium HP7 [+] + Metronidazole	Regimen A[++]
5	Metronidazole	Regimen B[+++]
6	Nexium HP7 [+]	Regimen A[++]
7	Nexium HP7 [+]	Regimen B[+++]
8	Nexium HP7 [+]	Regimen A[++]
9	Nexium HP7 [+]	Regimen A[++]
10	Nexium HP7 [+]	Regimen A[++]
11	Nexium HP7 [+]	Regimen C[++++]

[+] Nexium HP7 consists of esomeprazole, amoxicillin and clarithromycin, Klacid HP7 consists of clarithromycin, amoxicillin and omeprazole

[++] Regimen A consists of rabeprazole 20 mg (3 times daily for 10 days), amoxicillin 1000 mg (3 times daily for 10 days), ciprofloxacin 500 mg (starting from day 6, 2 times daily for 5 days) and rifabutin 150 mg (starting from day 6, 2 times daily for 5 days)

[+++] Regimen B consists of rabeprazole 20 mg (3 times daily for 10 days), bismuth subcitrate 240 mg (4 times daily for 10 days), ciprofloxacin 500 mg (2 times daily for 10 days) and rifabutin 150 mg (2 times daily for 10 days)

[++++] Regimen C are consists of rabeprazole 20 mg (3 times daily for 10 days), amoxicillin 1000 mg (3 times daily for 10 days) and metronidazole 400 mg (10 days)

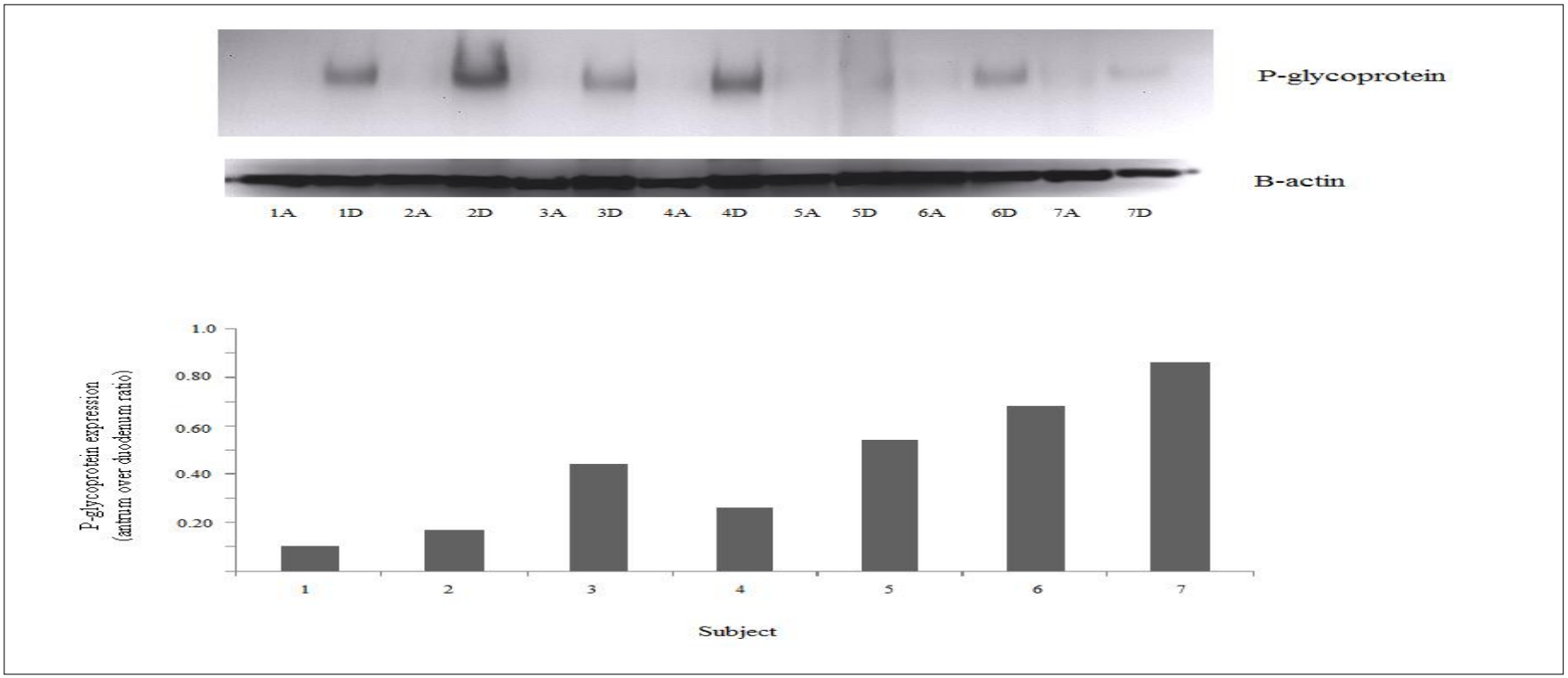

Figure 4-10: Western blot showing P-glycoprotein expression from antrum (A) and duodenum (D) of study subject 1 to 6 (upper figure) with B-actin as a loading control. The relative quantities was determined using densitometry from CoolSnap HQ charge-coupled device camera and presented as P-glycoprotein expression (antrum over duodenum ratio) (bottom figure). Subject 1-4 were *H. pylori* - negative and Subject 5-7 were *H. pylori* - treatment resistant.

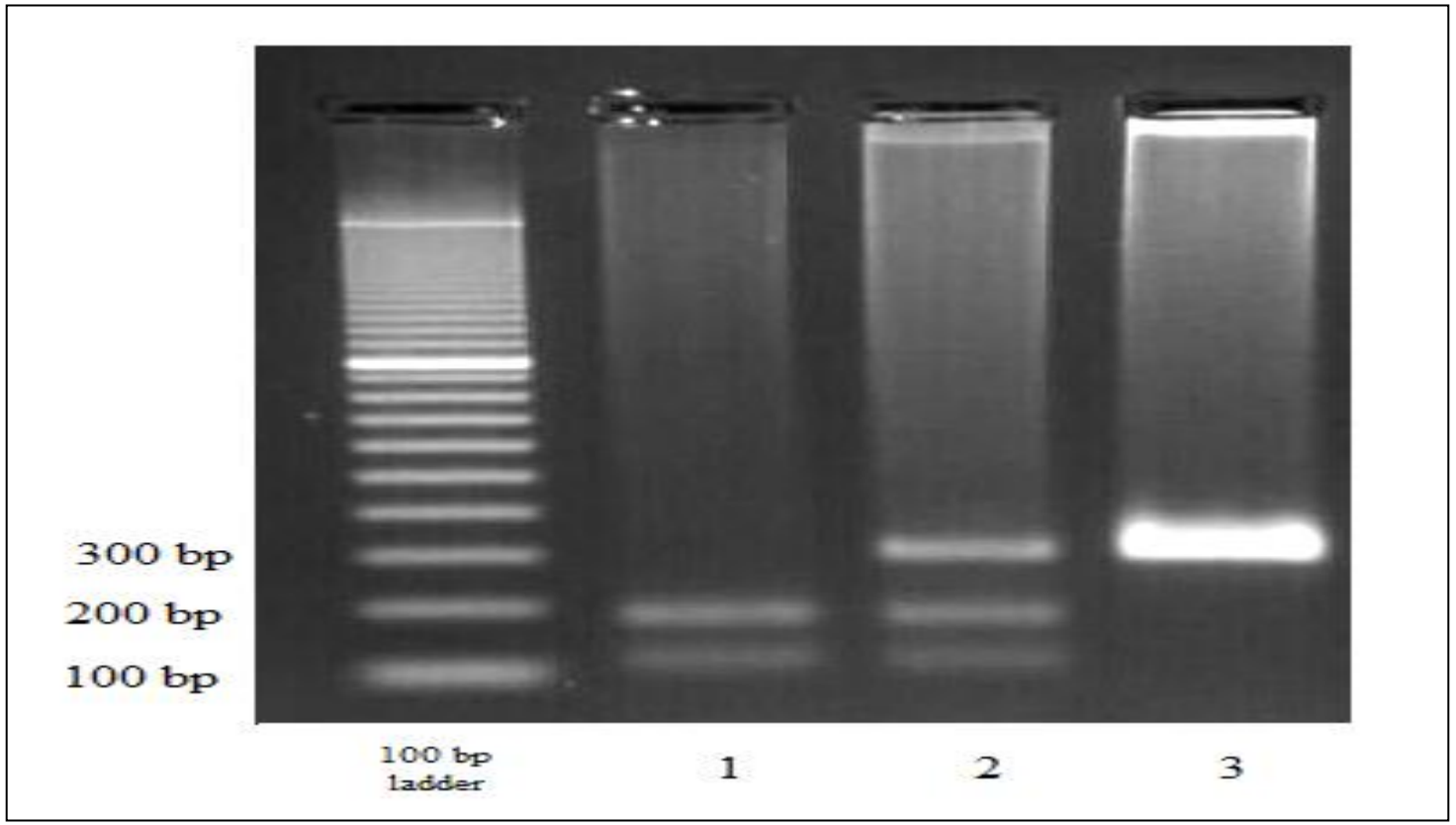

Figure 4-11: Gel electrophoresis for the *CYP2C19*2* analysis. The PCR-RFLP of exon 5 digested overnight with SmaI for *CYP2C19*2* genotypes (M: 100 bp ladder marker, lane 1: 1*/1*, lane 2: 1*/2*, lane 3: 2*/2*)

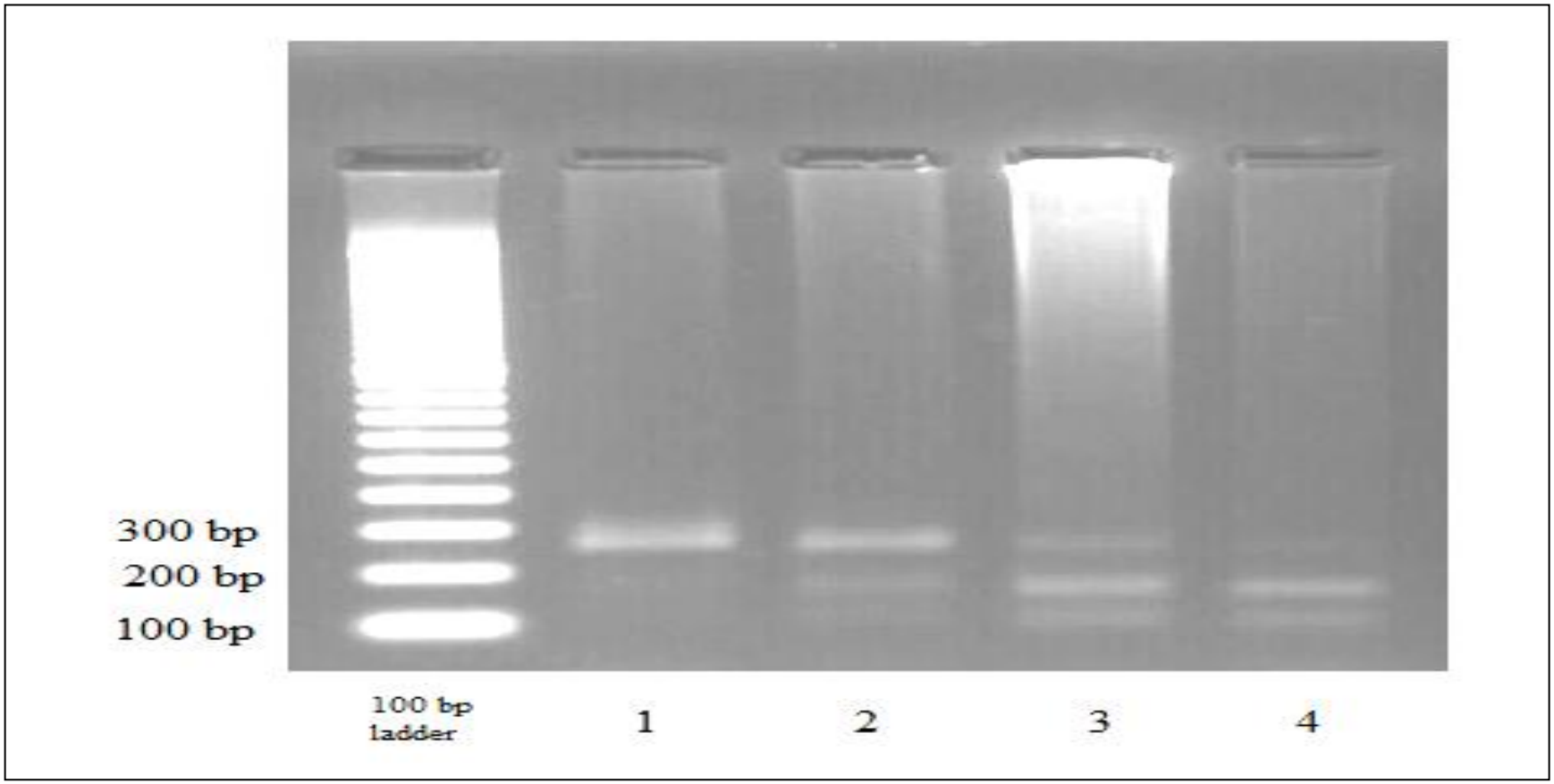

Figure 4-12: Gel electrophoresis for the *CYP2C19*3* analysis. The PCR-RFLP of exon 4 digested overnight with BamHI for *CYP2C19*3* genotypes (M: 100 bp ladder marker, lane 1: 3*/3*, lane 2 and 3: 1*/3*, lane 4: 1*/1*)

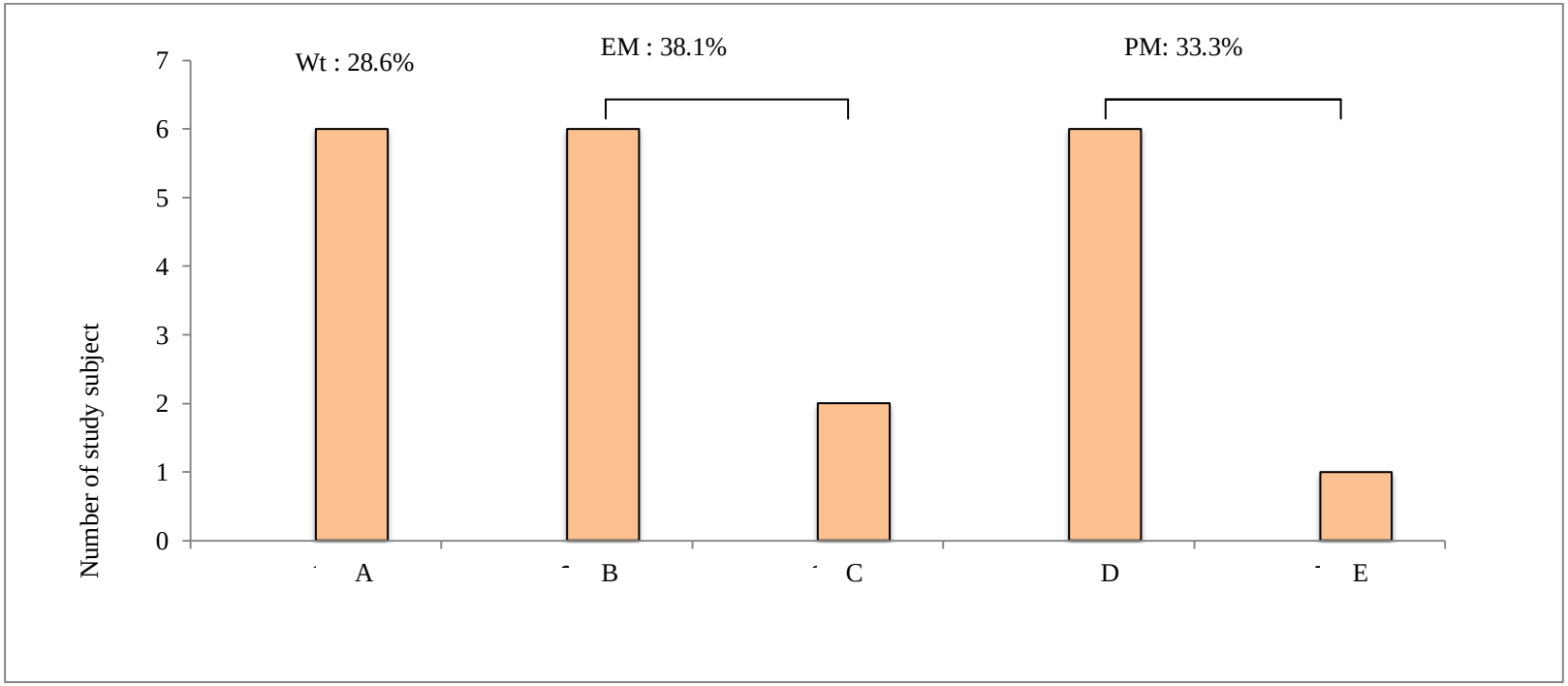

Figure 4-13: The distribution of resistant subjects according to five different allelic patterns of CYP2C19 genotypes. (A) Homozygous for wild type (Wt) alleles (*1) in exons 4 and 5 (*1/*1). (B) Heterozygous extensive metabolizers (EM) for the *CYP2C19*2* polymorphism without the *CYP2C19*3* polymorphism (*1/*2). (C) Heterozygous extensive metabolizers the *CYP2C19*3* polymorphism without the *CYP2C19*2* polymorphism (*1/*3). (D) Homozygous passive metabolizers (PM) for the *CYP2C19*2* polymorphism without the *CYP2C19*3* polymorphism (*2/*2).(E) Homozygous passive metabolizers for the *CYP2C19*3* polymorphism without the *CYP2C19*2* polymorphism (*3/*3)

4.1.7 *Helicobacter pylori* treatment resistance: P-glycoprotein expression and *MDR1 C3435T* polymorphisms

A total number of 15 subjects were referred due to *H. pylori* eradication therapy failure. Only 11 participants were confirmed positive for *H. pylori* and subsequently resistant towards *H. pylori* therapy (*H. pylori*- treatment resistant). **Table 4-8** and **Figure 4-14** show the demographic characteristic and P-glycoprotein expression levels in antrum of resistant and *H. pylori*-negative groups, respectively. For both *H. pylori*- treatment resistant and *H. pylori*-negative groups, the P-glycoprotein expression was higher in the duodenum rather than in antrum ($p = 0.027$).

The mean of P-glycoprotein expression (antrum over duodenum) among *H. pylori*-treatment resistant subjects was found higher when compared to the *H. pylori*-negative group (mean P-glycoprotein expression 0.37 *vs.* 0.29, $p = 0.0361$) suggesting that the P-glycoprotein increases with presence of *H. pylori*. The P-glycoprotein expression in *H. pylori*- treatment resistant subjects was compared to the subjects that were also *H. pylori*- positive but yet to receive any eradication therapy (*H. pylori* – positive - treatment naïve, $n = 22$). There was no difference of P-glycoprotein expression observed between the *H. pylori*-positive- treatment naïve ($n = 22$) and *H. pylori*- treatment resistant ($n = 11$) groups ($p = 0.319$).

In the *H. pylori*- treatment resistant group, all the *MDR1 C3435T* genotypes showed an increasing trend of P-glycoprotein expression with the presence of *H. pylori* though these did not reach statistically significance ($p = 0.056$) as illustrated in **Figure 4-15**.

Table 4-8: Demographic characteristic of *H. pylori*-treatment resistant and *H. pylori*-negative groups

Variable		*Helicobacter pylori* status		p-value
		***H. pylori*- treatment resistant (n = 11)**	***H. pylori* – negative (n = 54)**	
Gender	Male (%)	3 (27 %)	25 (46%)	0.44
	Female (%)	8 (73%)	29 (54%)	
Age	Mean (SD)	46.8 (10.4)	55.7 (16.1%)	0.088
	Range	35-68	23-88	
MDR1	*3435CC*	5 (45.45%)	11 (20.37%)	0.138
3435 C>T genotype	*3435CT*	3 (27.27%)	29 (53.7%)	
	3435TT	3 (27.27%)	14 (25.93%)	

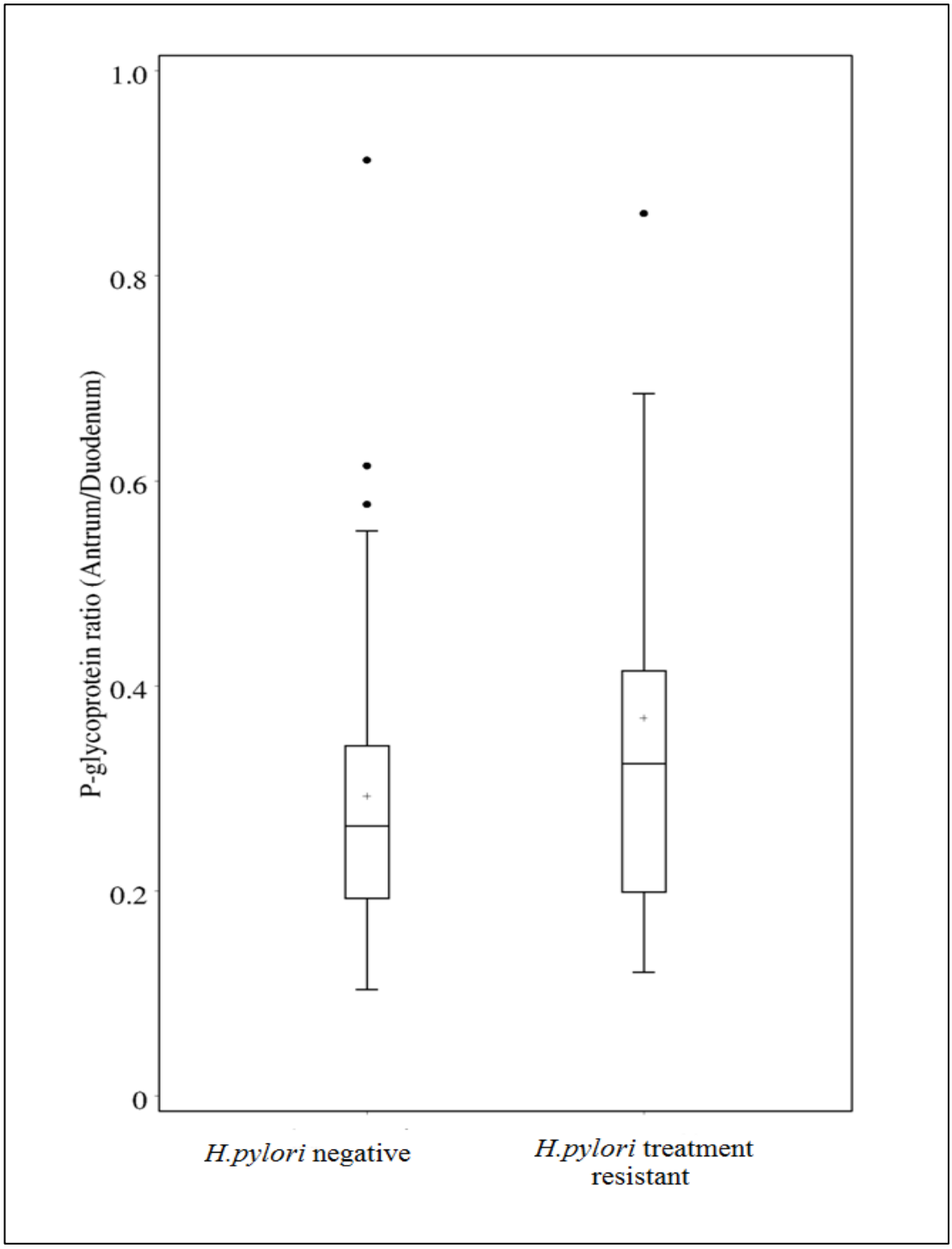

Figure 4-14: The differences in P-glycoprotein expression (antrum over duodenum) between the *H. pylori*- treatment resistant subjects (n = 11, mean P-glycoprotein = 0.37) and *H. pylori* - negative subjects (n = 54, mean P-glycoprotein = 0.29), p = 0.0361

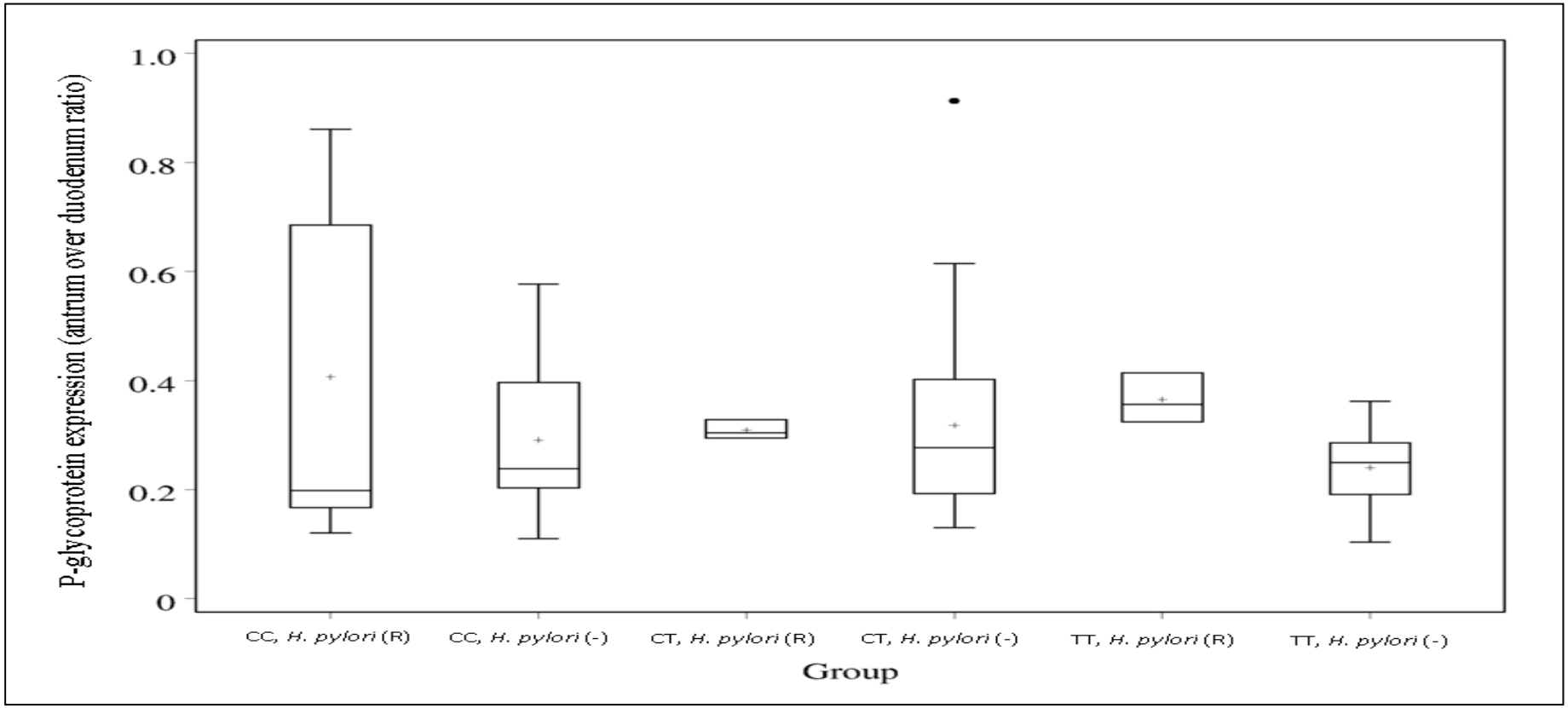

Figure 4-15: The differences in P-glycoprotein expression (antrum over duodenum) according *MDR1 C3435T* genotypes (*CC, CT* and *TT*) between the *H. pylori* - treatment resistant (*H. pylori*, R) and *H. pylori* - negative (*H. pylori* -) groups

4.2 Phase 2: *Helicobacter pylori* attachment to gastrointestinal cell lines

4.2.1 Caco-2 cells and bacterial attachment study

All the Caco-2 cells used in this study were grown in a 25 cm^2 flask and fed every other day with DMEM + 10% FCS. Once the cells achieved 90% confluence, the cells were split and seeded onto 96-well plates and fed with growth medium up to 21 days. In this study, potent P-glycoprotein inhibitor (PSC-833) was used to assess the influence of P-glycoprotein expression towards attachment of two different strains of *H. pylori*. The intensity of bacterial fluorescence represented the bacteria remaining associated with the human cell monolayers after the washing steps.

Overall, both strains of *H. pylori* showed an increasing bacterial attachment to Caco-2 cells over the 4 hour study period (**Figure 4-16** and **Figure 4-17)**. *H. pylori G27* demonstrated significantly higher bacterial attachment to Caco-2 cells at 30, 90, 180 and 240 minute when PSC-833 was introduced compared to the control cells ($p < 0.05$). While *H. pylori J99* was also observed to have higher bacterial attachment to Caco-2 cells, significantly at 90 and 180 minute in the presence of P-glycoprotein inhibitor compared to the control Caco-2 cells ($p < 0.05$). Increase bacterial attachment to Caco-2 cells over 4 hour study period was also observed with *E. coli* and *S. aureus* (**Figure 4-18** and **Figure 4-19)**. For *E. coli*, the differences between bacterial attachment to Caco-2 cells with and without PSC-833 were significant at 60, 90, 180 and 240 minute ($p < 0.05$). Whilst *S. aureus* demonstrated marked high bacterial attachment to Caco-2 cells in the presence of PSC-833 at 30 and 90 minute ($p < 0.05$).

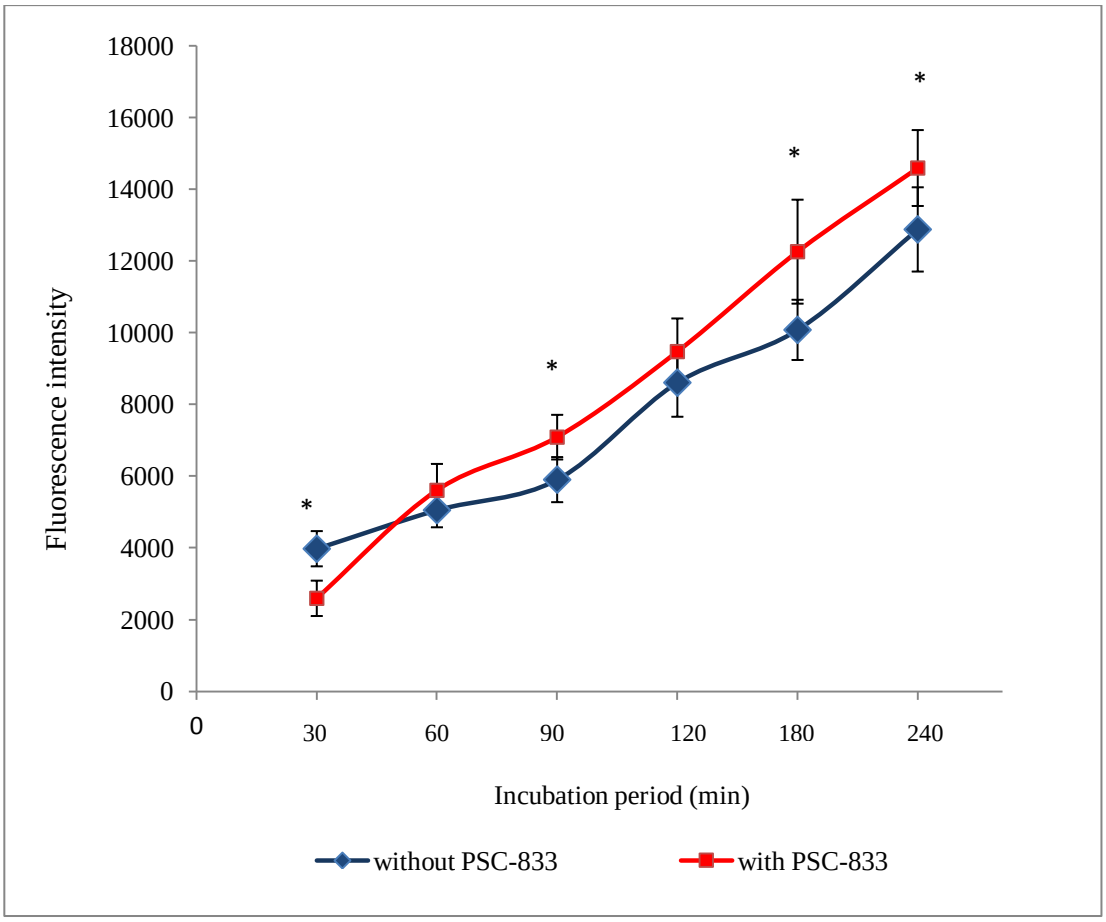

Figure 4-16: 4 hour study of *Bac*Light Green labelled *H. pylori* G27 attachment to the Caco-2 cells grown for 21 days in a 96-well plate. Control Caco-2 cells (filled diamond) was compared to the Caco-2 cells that was incubated with 4 µM of PSC-833 (filled square) the 30, 60, 90, 120, 180 and 240 minute incubation period. Results shown are the mean ± standard deviation of quadruplicate wells (minus blank) at each time point. * $p < 0.05$ illustrates significantly higher fluorescence for the study cells compared to the control cells.

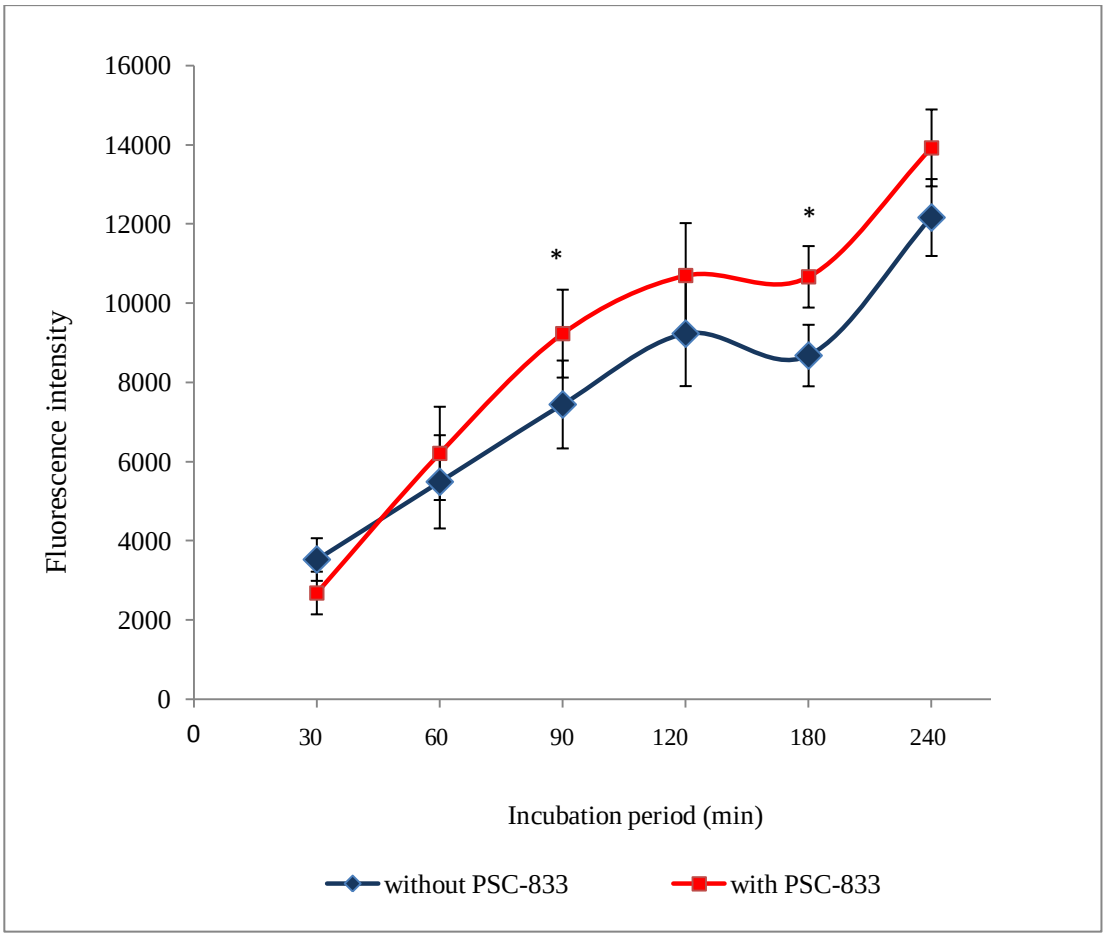

Figure 4-17: 4 hour study of *Bac*Light Green labelled *H. pylori* J99 attachment to the Caco-2 cells grown for 21 days in a 96-well plates. Control Caco-2 cells (filled diamond) was compared to the Caco-2 cells that was incubated with 4 µM of PSC-833 (filled square) the 30, 60, 90, 120, 180 and 240 minute incubation period. Results shown are the mean ± standard deviation of quadruplicate wells (minus blank) at each time point. $p < 0.05$ illustrates significantly higher fluorescence for the study cells compared to the control cells.

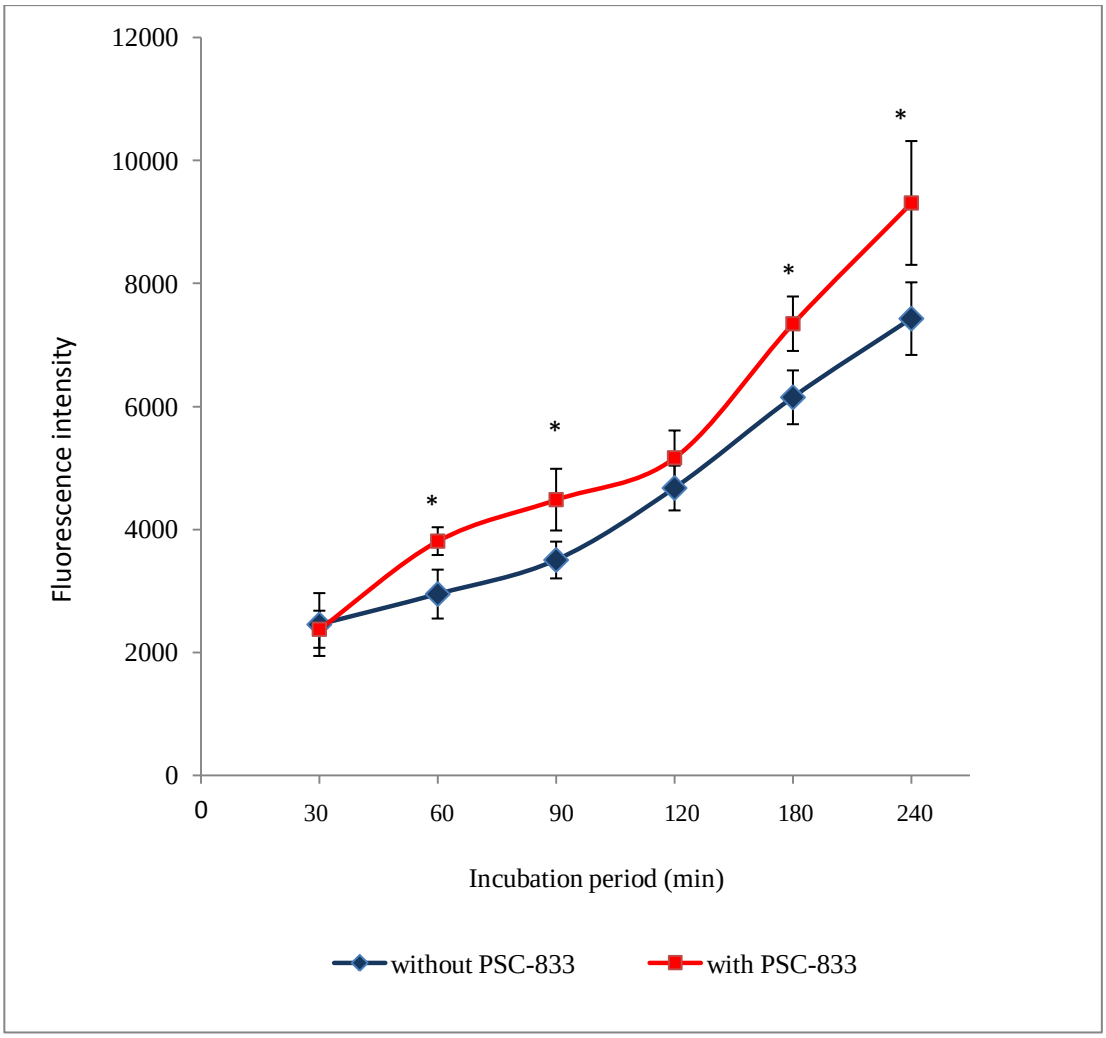

Figure 4-18: 4 hour study of *Bac*Light Green labelled *E .coli* W attachment to the Caco-2 cells grown for 21 days in a 96-well plates. Control Caco-2 cells (filled diamond) was compared to the Caco-2 cells that was incubated with 4 µM of PSC-833 (filled square) the 30, 60, 90, 120, 180 and 240 minute incubation period. Results shown are the mean ± standard deviation of quadruplicate wells (minus blank) at each time point. $p < 0.05$ illustrates significantly higher fluorescence for the study cells compared to the control cell.

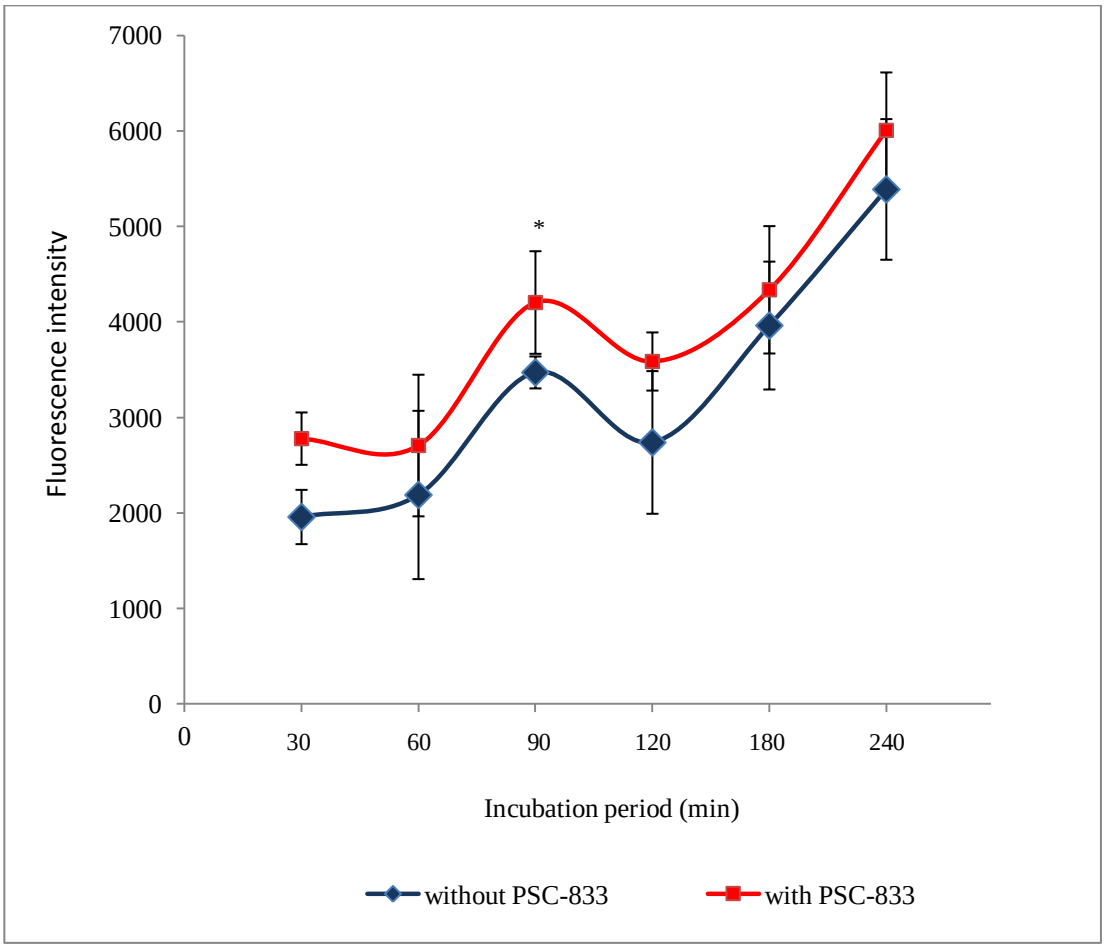

Figure 4-19: 4 hour study of *Bac*Light Green labelled *S. aureus* attachment to the Caco-2 cells grown for 21 days in a 96-well plate. Control Caco-2 cells (filled diamond) was compared to the Caco-2 cells that had been incubated with 4 µM of PSC-833 (filled square) during the 30, 60, 90, 120, 180 and 240 minute incubation period. Results shown are the mean ± standard deviation of quadruplicate wells (minus blank) at each time point. * $p < 0.05$ illustrates significantly higher fluorescence for the study cells compared to the control cells.

4.2.2 LS174T cells and bacterial attachment study

All the LS174T cells used in this study were grown in a 25 cm^2 flask and fed every other day with DMEM + 10% FCS. Once the cells achieved the 90% confluence, the cells were split and seeded onto 96-well plates and fed with growth medium up to 10 days. 10 µM rifampicin was given to half of LS174T cell lines in the 96-well plates to induce P-glycoprotein expression. Similar to the Caco-2 cells and bacterial attachment study, potent P-glycoprotein inhibitor (PSC-833) was also used to assess the influence of P-glycoprotein expression towards attachment of two different strains of *H. pylori*. *S. aureus* and *E. coli* W were used as positive controls in this study.

The bacterial attachment of both *H. pylori G27* and *J99* strains to LS174T cell lines were found to be unaffected by the levels of P-glycoprotein expression (**Figure 4-20** and **Figure 4-21**). There was no difference found between three treatment conditions and the control cells for *H. pylori G27* and *J99* strains. Initially, binding of *H. pylori G27* and *H. pylori J99* to the control LS174T cell lines were reduced at the 60 minute of incubation period. After that, both *H. pylori G27* and *J99* strains managed to overcome the P-glycoprotein barrier formed in LS174T cells. In contrast, *E. coli* W demonstrated significantly more bacterial attachment to the LS174T cells that have been pre-incubated with PSC-833 at 90, 120, 180 and 240 minutes than the control cell lines ($p < 0.001$). There was no significant difference found between bacterial attachment to the LS174T cells that have been pre-incubated with rifampicin and the control cells at all time points except for 180 minutes as shown in **Figure 4-22.** Additionally, the pre-incubated rifampicin treated LS174T cells with PSC-833 showed significant difference of the *E. coli* W attachment when compared to the control cells at all time points except for 30 and 60 minutes.

In this study, *S. aureus* showed significantly less bacterial attachment to the LS174T cells that have been pre-incubated with rifampicin than the control cell lines at 90, 120, 180 and 240 minutes ($p < 0.001$). *S. aureus* also demonstrated significantly more bacterial attachment to the LS174T cells that have been pre-incubated with PSC-833 at

than the control cell lines at all time points ($p < 0.001$) as shown in **Figure 4-23**. Further, the pre-incubated rifampicin treated LS174T cells with PSC-833 showed significant difference of the *S. aureus* attachment when compared to the control cells at all time points except for 30 minutes.

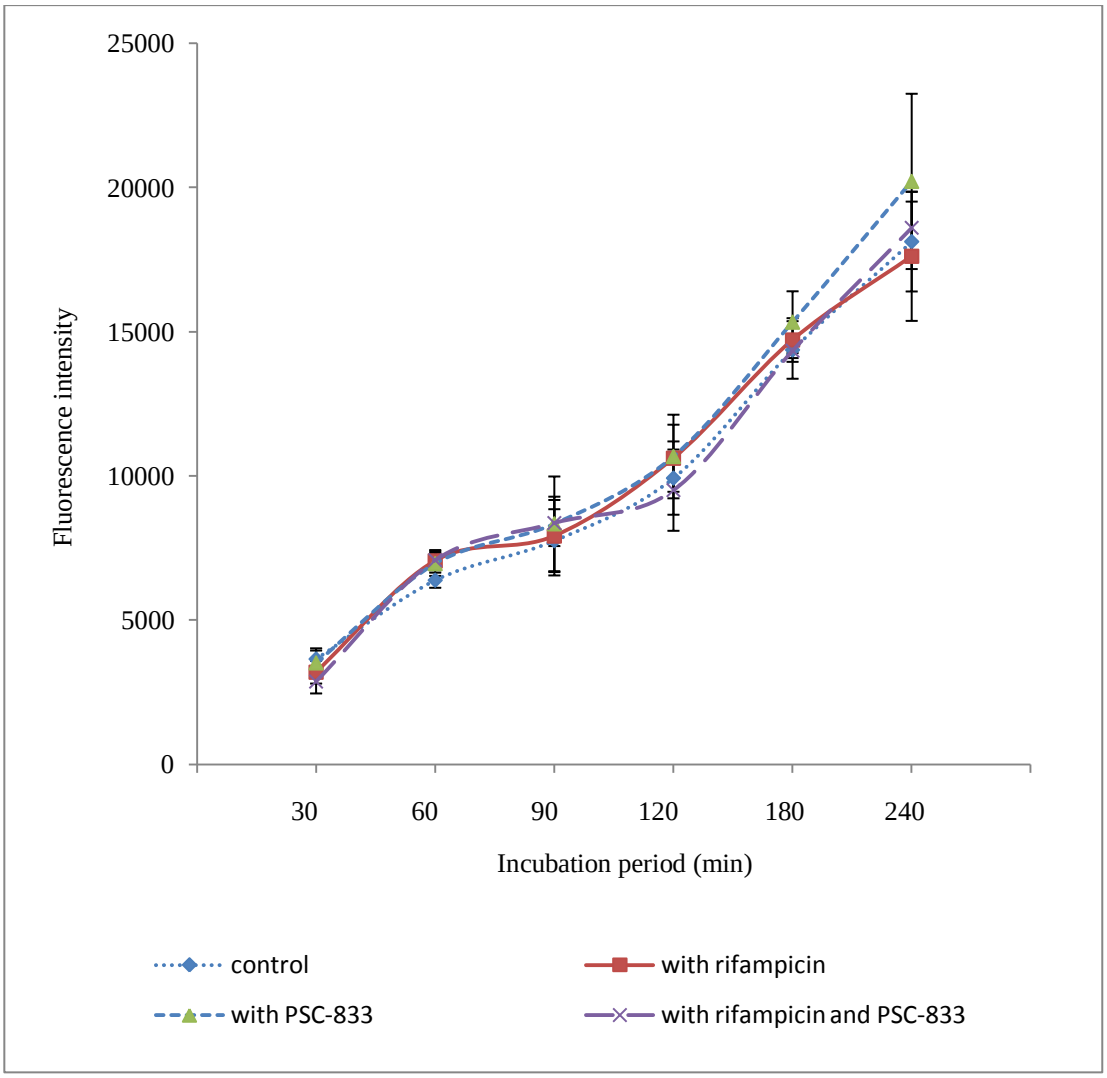

Figure 4-20: 4 hour study of *Bac*Light Green labelled *H. pylori* G27 attachment to the LS174T cells grown up to 10 days in a 96-well plate. Control LS174T cells (filled diamond) was compared to the LS174T cells that was incubated with 4 µM of PSC-833 only (filled triangle), LS174T cells that was incubated with 10 µM of rifampicin only (filled square) and similar pre-incubated rifampicin treated LS174T cells with 4 µM PSC-833 (cross) during the 30, 60, 90, 120, 180 and 240 minute incubation period. Results shown are the mean ± standard deviation of quadruplicate wells (minus blank) at each time point.

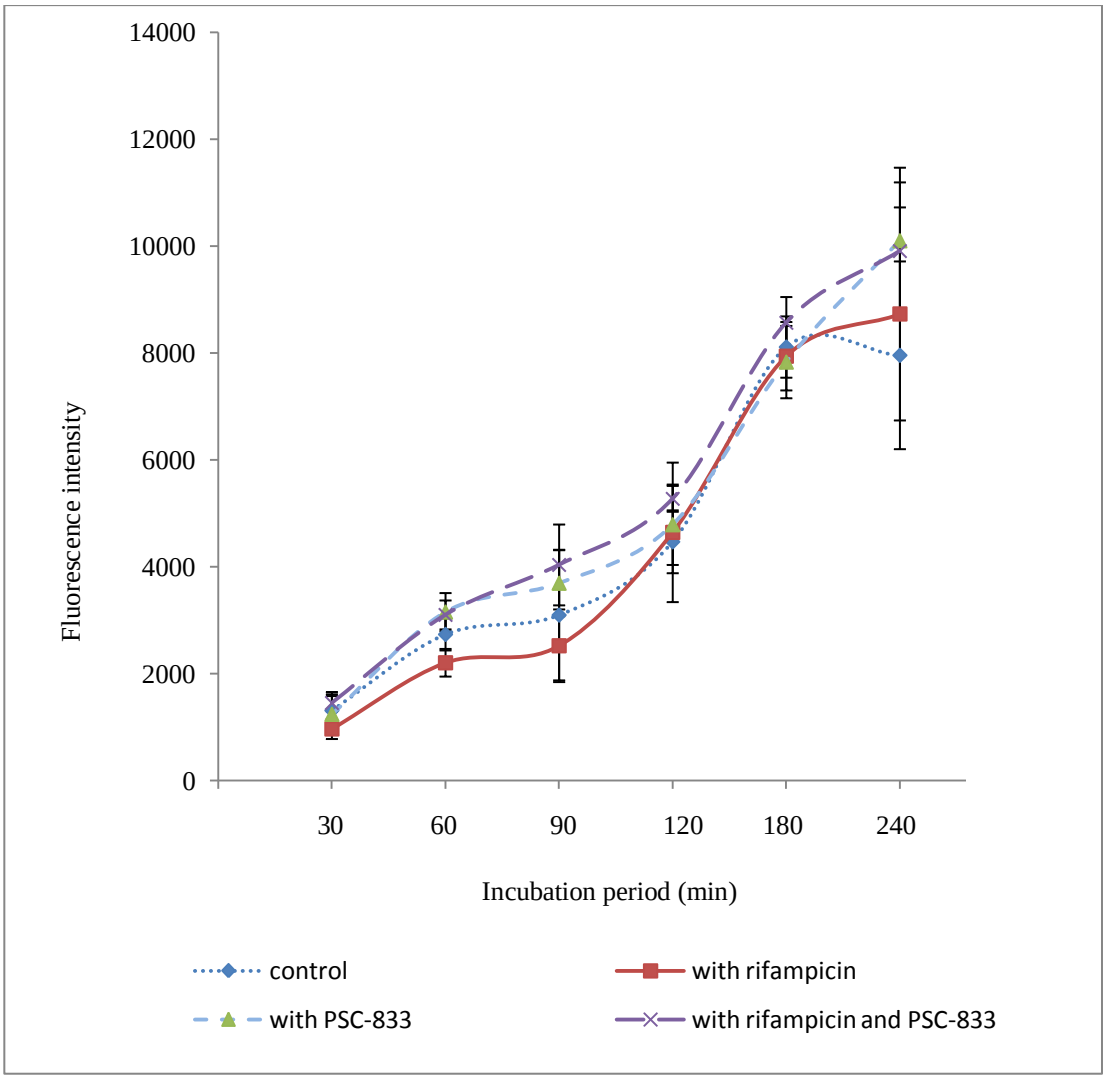

Figure 4-21: 4 hour study of *Bac*Light Green labelled *H. pylori* J99 attachment to the LS174T cells grown up to 10 days in a 96-well plate. Control LS174T cells (filled diamond) was compared to the LS174T cells that was incubated with 4 µM of PSC-833 only (filled triangle), LS174T cells that was incubated with 10 µM of rifampicin only (filled square) and similar pre-incubated rifampicin treated LS174T cells with 4 µM of PSC-833 (cross) during the 30, 60, 90, 120, 180 and 240 minute incubation period. Results shown are the mean ± standard deviation of quadruplicate wells (minus blank) at each time point.

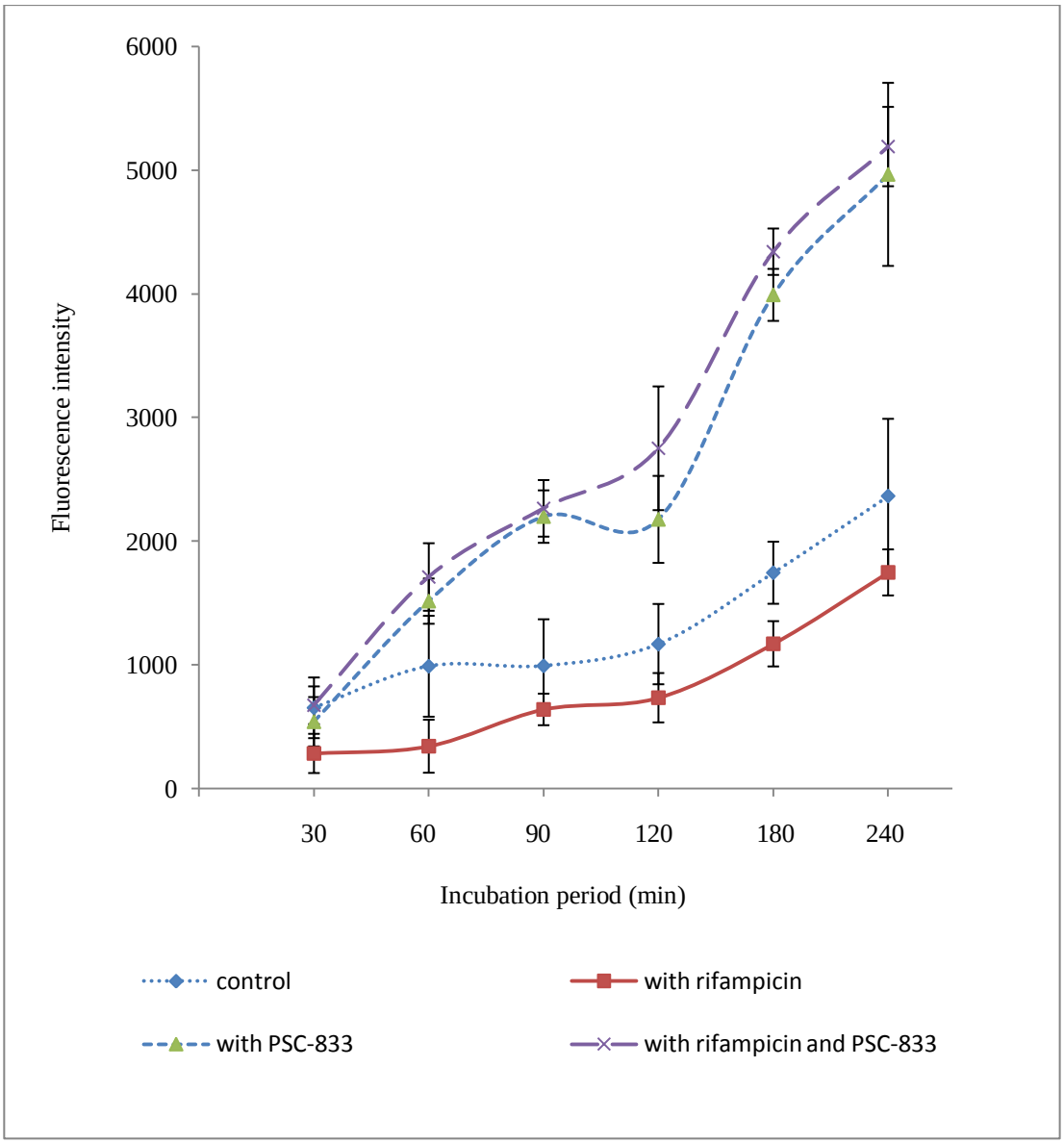

Figure 4-22: 4 hour study of *Bac*Light Green labelled *E. coli* W attachment to the LS174T cells grown up to 10 days in a 96-well plate. Control LS174T cells (filled diamond) was compared to the LS174T cells that was incubated with 4 µM of PSC-833 only (filled triangle), LS174T cells that was incubated with 10 µM of rifampicin only (filled square) and similar pre-incubated rifampicin treated LS174T cells with 4 µM of PSC-833 (cross) during the 30, 60, 90, 120, 180 and 240 minute incubation period. Results shown are the mean ± standard deviation of quadruplicate wells (minus blank) at each time point.

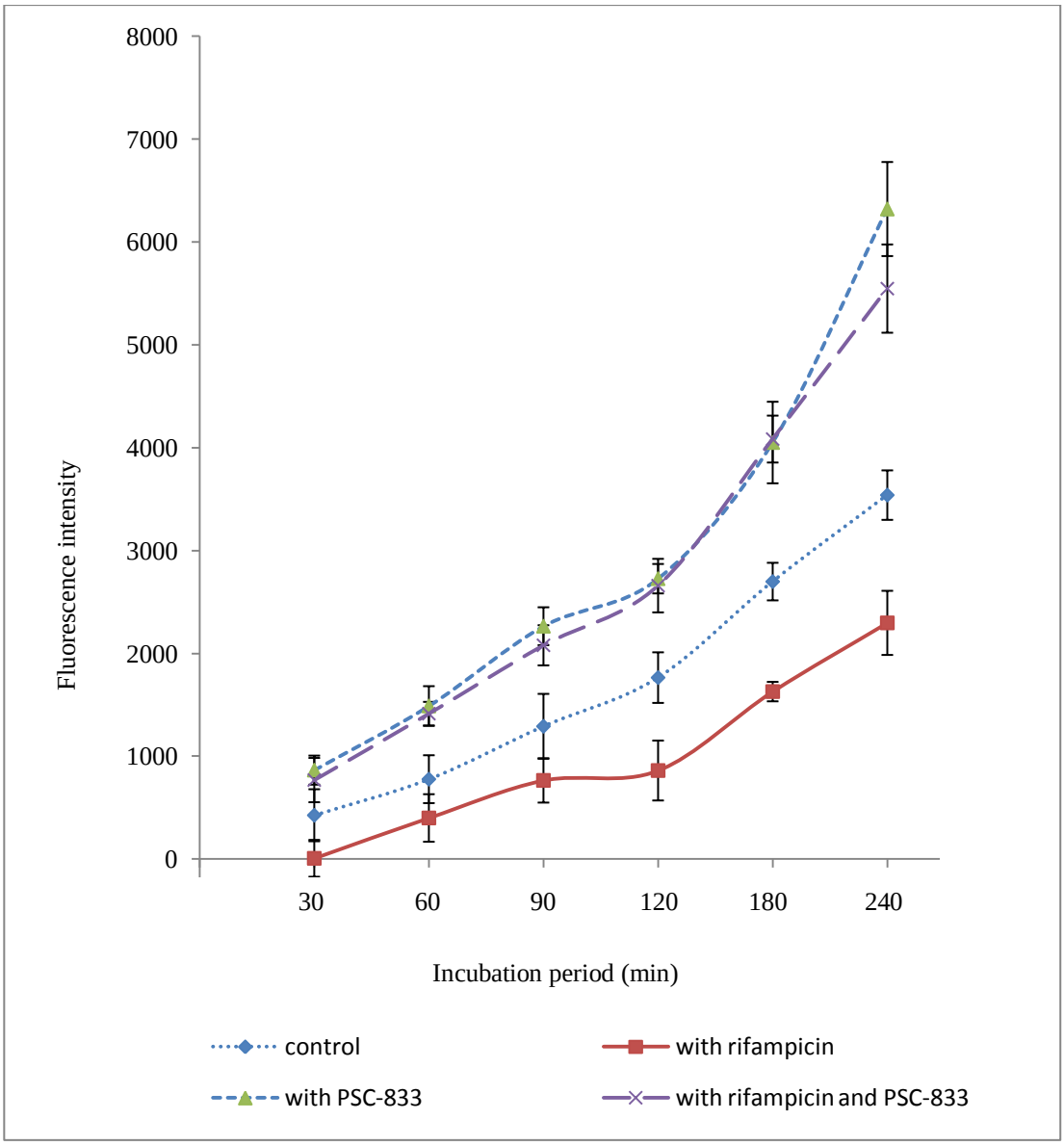

Figure 4-23: 4 hour study of *Bac*Light Green labelled *S. aureus* attachment to the LS174T cells grown up to 10 days in a 96-well plate. Control LS174T cells (filled diamond) was compared to the LS174T cells that was incubated with 4 µM of PSC-833 only (filled triangle), LS174T cells that was incubated with 10 µM of rifampicin only (filled square) and similar pre-incubated rifampicin treated LS174T cells with 4 µM of PSC-833 (cross) during the 30, 60, 90, 120, 180 and 240 minute incubation period. Results shown are the mean ± standard deviation of quadruplicate wells (minus blank) at each time point.

4.2.3 Induction of P-glycoprotein expression in LS174T cell lines

Although it has been confirmed in two separate publications [389, 582] that rifampicin at 10 µM for a week exposure can vastly increase P-glycoprotein expression, still it was considered important to confirm P-glycoprotein expression in paired control cells and parallel to the bacterial attachment study. P-glycoprotein over-expression test would prove that the cells in the bacterial attachment experiment did indeed have high P-glycoprotein levels. Thus to confirm the induction of *MDR1* gene by rifampicin, LS174T cells were treated with rifampicin in this study. The P-glycoprotein expression was determined by western blotting. The relative expression of P-glycoprotein in 10 days old LS174T cells and in 10 days old LS174T cells following 6 days exposure to 10 µM rifampicin were measured.

The data from both LS174T cell lines were compared to 21 days old Caco-2 cells as illustrated in **Figure 4-24**. It was clear that based on the densitometry, the 10 days old LS174T cells (sample 1) have shown the lowest P-glycoprotein level as compared to the 10 days old LS174T cells following 6 days exposure to 10 µM rifampicin (sample 2) and Caco-2 cells (sample 3). The control LS174T cells had a relatively low expression of P-glycoprotein when grown without any P-glycoprotein inducer. It was found that the P-glycoprotein expression in LS174T cells that was induced by rifampicin became nearly 3.7- fold higher than the control LS174T cells. Nevertheless, pre-incubation of LS174T cells with rifampicin did not increase P-glycoprotein levels beyond that of the Caco-2 cells. Caco-2 cells proved to have significant quantities of P-glycoprotein expression, about 20-30% higher than LS174T cells induced by rifampicin and 5–fold higher than the control LS174T cells.

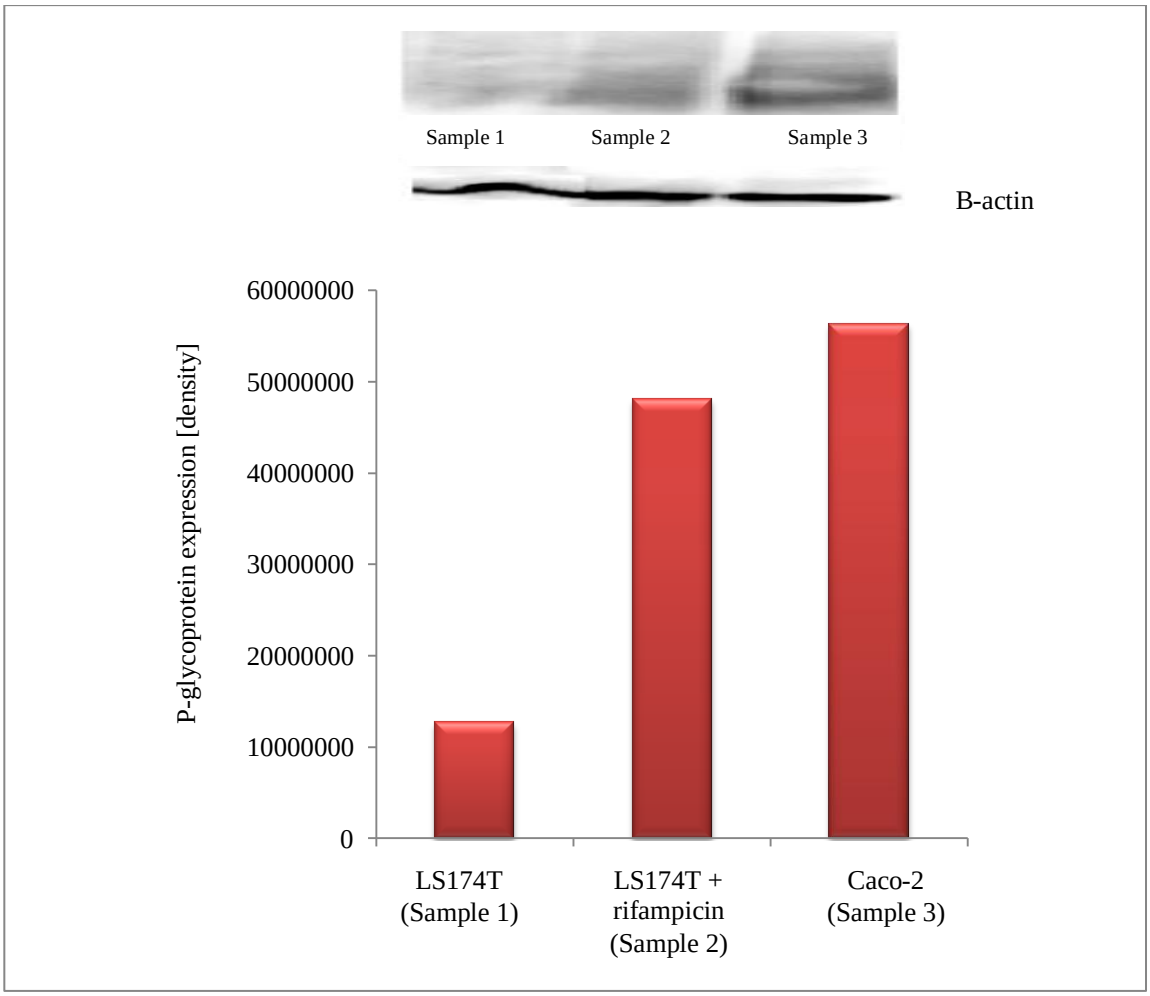

Figure 4-24: Expression of P-glycoprotein in 10 days old LS174T cells (sample 1), in 10 days old LS174T cells following 6 days exposure to 10 µM rifampicin (sample 2) and 21 days old Caco-2 cells (sample 3). The expression of P-glycoprotein was detected by incubating the blot membranes with PgP Sc13131 Mdr [G-1] mouse monoclonal IgG2b antibodies. The mouse anti-B-actin was used as control to normalize the gel loading and western blot transfer. Graph shows relative P-glycoprotein expression level (integrated density value) in each sample assessed by densitometry.

CHAPTER 5

DISCUSSION

CHAPTER 5 DISCUSSION

5 Overview

This study was divided into two phases: Phase 1 (*Helicobacter pylori* and P-glycoprotein expression) and Phase 2 (*Helicobacter pylori* attachment to gastrointestinal cell lines).

5.1 Phase 1: *Helicobacter pylori* and P-glycoprotein expression

5.1.1 Participant demographics

It was initially anticipated that it would be possible to recruit 200 participants in one year for our research based on the number of patients referred for upper gastroendoscopy at Sir Charles Gairdner Hospital in 2009. However, the number of patients reduced in 2010 and 2011 as more patients were referred for the lower gastroendoscopy rather than upper endoscopy. Despite this 91 subjects were recruited which provided an adequate sample size to give good precision to the prevalence estimate of P-glycoprotein expression in *H. pylori*–positive patients.

Dyspepsia was the most common indication for endoscopy in our study followed by anaemia and epigastric pain. Keren et al [390] reported that dyspepsia and anaemia remain the commonest reasons of endoscopy referral, especially in patients aged more than 45 years old. Dyspepsia refers to chronic or recurrent pain centered in the upper abdomen [391]. The prevalence of dyspepsia is around 20-40% of the population and majority of them presented with functional dyspepsia [392]. As one of the criteria for functional dyspepsia is epigastric pain or epigastric burning [393], this may explain of why patients with epigastric pain were commonly referred for upper gastroendoscopy. Further, epigastric pain is a common organic symptom of gastro-oesophageal reflux disease (GORD) and peptic ulcer disease that should not be ignored [394]. Meanwhile, chronic occult blood loss from the gastrointestinal tract is widely accepted as a major

cause of anaemia with or without iron deficiency [395]. Iron deficiency anaemia occurs in less than 5% of adults but increases to 7% in geriatric patients [396]. Iron deficiency anaemia due to chronic blood loss is usually undetectable and becomes pronounce only when patients become symptomatic [397]. Endoscopy is a sensitive test for investigating the source of gastrointestinal bleeding; it can determine the cause of anaemia in between 30 and 50% of patients [398].

In this study, the 40 to 49 years age group has the highest rate of participation followed closely by the 60 to 69 and 50 to 59 years age group. It was expected to see this age pattern as endoscopy is recommended for dyspeptic patients over the age of 45 or those with certain alarm symptoms such as weight loss, recurrent vomiting, dysphagia, bleeding or anaemia [399]. Patients with dyspepsia who are younger than age 50 and without alarm features are commonly evaluated by one of three methods namely noninvasive testing for *H. pylori*, a trial of acid suppression or an initial endoscopy [400]. Although an age of 45 years appears to be a reasonable threshold for endoscopy, physicians should remain aware of the possibility of malignancy in younger patients and provide an early endoscopy if required [401]. Even if it is still unclear whether early endoscopy is necessary in cases of undiagnosed dyspepsia, one study did recommend endoscopy be undertaken in dyspeptic patients as young as 40 years of age to prevent undiagnosed gastric cancer cases in these younger patients [402]. Another study also suggested that the age threshold for endoscopy should be lowered in men in view of higher risk of cancer as opposed to women [403].

Normal upper gastrointestinal tract anatomy (normal endoscopy) was the most common finding in our study, followed by peptic ulcer disease. The higher percentage normal endoscopies among the younger participants compared to the older group was similar to the findings of Aduful et al [404]. It has been shown that dyspepsia is strongly associated with normal endoscopy [405]. Hence, with an open-access system and high number of referrals for dyspepsia, it is not surprising a normal endoscopy finding was common. Kerrigan et al [406] reported that as many as 40% of upper gastrointestinal endoscopies performed had normal findings. Nevertheless, a normal endoscopy should

not be dismissed as it may rule out serious upper gastrointestinal disease diagnosis. It also provides strong reassurance of good health for both patients and medical practitioners leading to less empirical therapy and reduced medication costs.

Endoscopies have been reported to detect peptic ulcer disease in about 5.3% of dyspeptic Canadians and 15.7% of Danes [407]. Peptic ulcer disease is commonly associated with *H. pylori* infection and the increased use of non-steroidal anti-inflammatory drugs (NSAIDs) since the 1950`s [408]. The American Society for Gastrointestinal Endoscopy (ASGE) recommends for upper gastroendoscopy to investigate peptic ulcer disease in patients aged more than 50 with new onset of dyspepsia, as well as in patients of any age with alarm symptoms including a family history of gastrointestinal malignancy, unexplained weight loss and anaemia [400].Over the past 15 years the incidence of peptic ulcer disease, duodenal ulcer and duodenitis cases world-wide have been reported to have significantly decreased following the introduction of proton pump inhibitors and better endoscopic techniques for the early disease detection [409]. The decrease in peptic ulcer disease cases has also been attributed to better standards of living affecting *H. pylori* transmission and wider use of empiric antibiotics [410].

About 40% of our participants were using proton pump inhibitors pre-endoscopy. Pantoprazole remains the most common proton pump inhibitor used among our participants as opposed to esomeprazole and omeprazole. Proton pump inhibitors are normally prescribed in acid-related gastrointestinal disorders such as GORD, peptic ulcer disease and Zollinger-Ellison syndrome [411]. They are also indicated for the treatment of or prophylaxis against ulcers related to NSAIDs and as part of *H. pylori* eradication regimens. Pantoprazole was listed among 100 most costly drugs to Australian Government [412]. Both 20 mg and 40 mg pantoprazole have been demonstrated to be safe and effective in managing GORD [413]. It has been reported that pantoprozole provided prompt symptomatic relief in most patients with upper gastrointestinal acid-related problem [414], as do all of the other proton pump inhibitors.

5.1.2 *Helicobacter pylori*: participant awareness and acceptance of screening or treatment (Questionnaire)

The prevalence of *H. pylori* infection in Australian adults is lower than other developed countries [415]. This bacterial infection is most common amongst adults living with low socio-economic disadvantage or who were born overseas. Public awareness and acceptance towards screening of *H. pylori* are still limited although people have received information about the bacterium through the media [416]. Nevertheless, despite the perceived lack knowledge of *H. pylori*, it has been reported that most people would have screening for the bacterium if the opportunity existed.

In order to evaluate participants' awareness and acceptance of screening or treatment for *Helicobacter pylori* infection, two short questions were asked of our participants before they were prepared by the nurses for their gastroendoscopy. Almost half of the participants knew they have been tested and/or treated for *H. pylori* infection; the remainder did not or was unsure. In all cases the screening would have been done by their general practitioner before being referred for an upper gastroendoscopy procedure. Currently, screening for *H pylori* does not occur at a state or national level within Australia [417]. Based on the Maastricht III consensus, patients are usually not offered *H. pylori* screening unless they are present with active peptic ulcer disease; uninvestigated dyspepsia without bleeding, anaemia, unexplained weight loss, progressive dysphagia, early satiety, recurrent vomiting, odynophagia, family history of gastric cancer or a previous oesophagogastric malignancy [for patient age less than 45 years old]; low grade MALT lymphoma; after endoscopic resection of early gastric cancer or first degree relative with gastric cancer [418]. As *H. pylori* population screening would involve range diagnostic options (invasive methods or serologic tests), its cost, convenience and accuracy may vary.

H. pylori screening is more preferred in the older age groups although the benefits of eradicating the bacterium in an asymptomatic population have yet to be established [419]. *H. pylori* screening is not recommended for any populations considered to be at

low-risk of gastric cancer, such as Australians and New Zealanders [420]. Screening for *H. pylori*, which has been linked to the future development of gastric cancer, is now considered to be an important tool in cancer prevention [421]. It has the potential to be cost-effective in the prevention of gastric cancer, particularly in high-risk populations [422]. Therefore, screening and eradication of *H. pylori* in populations at high-risk of gastric cancer are warranted [423], and this may include the indigenous populations of Australia and New Zealand [424-425].

5.1.3 *Helicobacter pylori* infection status

5.1.3.1 Specimen collection and transport

H. pylori commonly reside in the stomach, particularly in the antral region [4]. At endoscopy, a sample from an area of a normal looking tissue should be taken from the antral region, approximately 2 cm away from the pylorus. Taking an area that has been affected by erosions or ulceration may limit the number of *H. pylori* present due to the damage to the epithelium or deprivation of the mucosa layer [426].

Upon collection, our antral biopsy specimens were inoculated in sterile broth consisting of brain heart infusion broth 2% (BHI) with 20% glycerol before being transferred to the laboratory for *H. pylori* culture. All of our biopsies were sent to the laboratory within three hours after the specimen collections for bacterial culture. All the specimens in BHI broth were kept on dry ice before been transported to the laboratory. As *H. pylori* is very sensitive to dry conditions, rapid specimen transportation is crucial in order to maximize the diagnostic yield [427]. Previous reports had suggested the use of various transport and storage conditions to improve *H. pylori* recovery from gastric biopsies including Stuart`s media and glycerol containing media [428-429]. It has been shown that the recovery rates of *H. pylori* from gastric biopsies can be correlated with the storage time prior to culture, regardless of whether the specimen is kept in BHI or not [430]. In general, transport at a low temperature is essential to get sufficient *H. pylori* recovery from gastric tissue specimens.

5.1.3.2 Bacterial culture

Transit time upon specimen arrival at the laboratory has been recognized as one of the major limitations for *H. pylori* recovery, therefore biopsies should be cultured within 4 to 24 hours [429]. Ideally, at least four biopsies should be cultured but it has been reported that about 95% of *H. pylori* can be yielded from just one antral biopsy [431]. *H. pylori* is a relatively slowly growing bacterium and could be overgrown by the other organisms found in the biopsy [432].

Overall, about 23% of samples we tested were positive for *H. pylori* infection based on positive cultures. A prolonged incubation period and correct atmosphere conditions for *H. pylori* also helped in yielding a satisfying outcome in our study. At first, the agar plates containing biopsies smears were left for about three days in a dedicated CO_2 incubator with daily inspection to see any growth. Some plates showed *H. pylori* growth after five days while others required longer incubation periods. According to Yin et al [433], prolonged incubation is necessary for certain strains of *H. pylori*, although a 10 days incubation was sufficient for *H. pylori* growth on a non-selective media and shorter times were required with selective media [434]. All the plates with positive growth were re-cultured for a second and third time in order to get a clean and pure *H. pylori* colony for subsequent identification analyses.

By using dry ice to immediately freeze samples upon endoscopy and keep them frozen until they went into the -80°C freezer, we were able to culture *H. pylori* from the biopsies (3 positives from 10 biopsies) even though the samples had been stored for more than three months. Evidence has shown that the bacteria recovery is better at a storage temperature of -70 °C than -20 °C [428]. It has been reported that freezing biopsy specimens might result in decreased *H. pylori* colony counts [435], however others have suggested that the loss is related to the initial number of organisms rather than the freezing duration [436]. Meanwhile, long term frozen specimens require a longer incubation period – around 7 to 11 days – due to temperature-related stress that

results in the *H. pylori* growing slowly [437]. However, in our study the use of a specialised laboratory that only focused on *H. pylori* work, dry ice to preserve the integrity of biopsies, shorter times between sample collection and analysis, combined with prolonged culture plate incubation periods contributed to good *H. pylori* recovery rates.

5.1.3.3 Polymerase chain reaction

One limitation in using a culture method to yield *H. pylori* is that the bacterium has a patchy distribution in the stomach [438]. Therefore, using bacterial culture alone may fail to detect the *H. pylori* especially if a small biopsy is used. *H. pylori* may also exist in coccoid forms and these forms may escape detection by culture [439]. Culture provides a good outcome in detecting *H. pylori* infection but does require a certain length of time for the bacteria isolation and subsequently delays the treatment implementation [440].

For those reasons, all our specimens were also tested for presence of *H. pylori* by polymerase chain reaction [PCR] detecting the *vacA* and *16S rRNA* genes. All of the *H. pylori* culture-positive specimens were also tested positive using PCR. This resulted in the number of samples confirmed as *H. pylori* positive increasing to 30.7%, demonstrating that the PCR assay was a more sensitive method compared to culture; which is in agreeance with the literature. In determining *H. pylori* infection status, PCR is regarded as having the highest diagnostic sensitivity, followed by histology, culture and the rapid urease test [440]. However, the use of PCR in detecting *H. pylori* infection from gastric biopsy specimen requires good laboratory expertise and technical handling [441].

A good technique for the DNA extraction from tiny biopsies is mandatory to ensure the collection of an adequate amount of DNA while making sure that the DNA is not damaged and remains uncontaminated to avoid any false-positives from the fragmented DNA. There are a number of methods of DNA extraction from biopsies including the boiling method [443] and phenol-chloroform-isoamyl-alcohol extraction [444]. The

boiling method yields much less DNA than phenol-chloroform extraction [445] but the latter is time consuming, toxic and may contaminate the resultant nucleic acid [446]. In this study, EDNA-Hispex^{TM} (Saturn Biotech, Western Australia) was used to extract the DNA from the biopsy samples and the absorbance method was used to determine bacterial DNA concentration and level of DNA purification. By using the EDNA-Hispex^{TM}, we were able to extract between 55 to 200 ng/µL of nucleic acid, more than enough for the PCR assay. Although the PCR assay used has the ability to detect copies of nucleic acid sequences at a very low level, a suitable PCR condition must be established to optimize the DNA fragment amplification [447]. For instance, the presence of *in vivo* Taq polymerase inhibitors may influence the assay result [448], hence the 'Hot Start' activation was applied in our PCR assay. The 'Hot Start' method avoids any non-specific DNA amplification by inactivating the Taq polymerase at lower temperature to improve the PCR performance [449-450]. Using a 35 cycles yielded a sufficient product in our PCR assay although it is recommended to use longer cycles - up to 45 cycles- to detect low copy number targets [450].

In view of greater accuracy of detecting the *H. pylori* by PCR is depending on the primers used [451], the suitable choice of primers must be planned carefully to avoid any false positive or false negative results. We used two different set of primers that gave two specific bands: – 109 bp (*16s rRNA*) and 700 bp (*vacA*) and a pure *H. pylori* DNA sample (*H. pylori* 26695, courtesy of Ondek Pty Ltd, Australia) was employed as the internal control. We observed that all our PCR positive samples showed positive bands for both *16S rRNA* and *vacA*. The *16s rRNA* was chosen to differentiate *H. pylori* from other *Campylobacter* strains [452] while *vacA* gene is present in all *H. pylori* strains [453]. Both *vacA* and *16S rRNA* have been reported to have equal sensitivities, 99% and 98% respectively, in detecting *H. pylori* from biopsies [380]. Additionally, the *16S rRNA* gene PCR assay has been shown to have a very high sensitivity, in that it is able to detect as little as 0.01 pg of *H. pylori* DNA or approximately five organisms [454]. A satisfactory PCR assay is accepted when the internal control and/or the *H. pylori* band are visible on the agarose gel [455]. False negative and false positive samples may be wrongly interpreted if no internal control is present.

In our study, the PCR assays were performed using 0.1 µg of purified *H. pylori* DNA with the optimal $MgCl_2$ concentration of 2.5mM. The PCR assay was repeated twice to avoid any false positive or false negative results. We also observed a stronger specific signal for DNA extracted from a pure *H. pylori* culture compared that from *H. pylori*-positive specimens obtained from biopsy. Unlike Chisholm et al [380], we did not observe the presence of non-specific bands that are believed to originate from the pool of DNA from human biopsies. This may be due to the high purification level of DNA in our study. It is important as any presence of non-specific bands may interfere with the assay sensitivity due to competition between co-amplification of the targeted and non-targeted DNA fragments.

5.1.3.4 Histology and rapid urease test

Histology was not a routinely used to investigate *H. pylori* status in our study. Out of the 10 samples from our study cohort that were sent to the hospital pathology laboratory, only one was confirmed positive by histology. The histology was requested by the gastroenterologist upon the endoscopy for various reasons. The biopsies for histology examination were embedded in fixed paraffin and transported in phosphate buffered formalin to the hospital pathology laboratory.

In practice, paraffin-embedded specimens are usually stained with haematoxylin–eosin for morphologic examination and detection of *Helicobacter*-like organisms [456]. The histological identification of *H. pylori* infection includes modified Giemsa, Warthin-Starry, Gimenez, Genta and immunohistochemically staining using polyclonal antibodies to *H. pylori*. The latter is regarded as the gold standard for histology due to its high specificity for *H. pylori* identification [457]. Histology also has been regarded as the least accurate method in detecting *H. pylori* as it is depended on the examiner`s expertise [458] and sampling variation [459]. There were discrepancies in whether histology is reliable in detecting *H. pylori* in view of its sensitivity and specificity as sampling of single biopsy may result in diminished sensitivities [460-462]. However, it

has been reported that sensitivity and specificity of histology may reach up to 95% especially when two or more biopsies are used [463]. Although the cost of conducting histology and PCR are similar, the former method requires considerable time to provide a result as opposed to short turn-around time using PCR [464].

Nearly 47% of our participants who underwent rapid urease test (CLOtest) were confirmed positive for *H. pylori*. The rapid urease test is conveniently conducted in the endoscopy suite and is recommended as the first choice as the result is obtained within hours [465]. The test is based on the principle of urease that has been released by *H. pylori* catalyzing the hydrolysis of urea into ammonia and subsequently increasing the local pH [466]. It has been reported that the rapid urease test has a high specificity but low sensitivity [467]. However, a comparison study showed that CLO test kit (Delta West Pty Australia) could detect the presence of *H. pylori* from the biopsies that tested negative by culture [468]. This discrepancy may be due to the fact that the rapid urease test sensitivity is dependent on the bacteria load in the biopsies as well as the total level of urease production and number of biopsies used [469].

5.1.4 P-glycoprotein expression and *Helicobacter pylori*

5.1.4.1 Overview

It has been thought that gender-differences in P-glycoprotein expression contributed to the disparity in level of P-glycoprotein expression [470]. In this study, we demonstrated that gender had no influence to the human gastrointestinal P-glycoprotein expression which is in agreement with other researchers [471, 472]. Maclean and colleagues [473] also reported no gender specific differences in P-glycoprotein expression observed along rats' intestines. Unlike intestinal P-glycoprotein, hepatic P-glycoprotein expression has been shown to be two-fold higher in males than females [474]. For instance, the elimination of verapamil, a P-glycoprotein substrate, is faster in women than men [475]. The difference in verapamil pharmacokinetics may be due to P-glycoprotein expression in hepatocytes of women, which in turn increases the drug`s exposure to metabolism by

CYP3A4 [476]. Hence, gender may contribute part of variable drug responses and drug toxicities of P-glycoprotein substrates but the exact mechanism is still controversial.

Given the potentially important effects that age may have on P-glycoprotein expression in intestine which may subsequently influence the pharmacokinetics of certain drugs, we also studied the difference of P-glycoprotein expression across age groups. Our study did not demonstrate any significant changes in P-glycoprotein expression levels across the various age groups based on analysis of antral and duodenal P-glycoprotein samples. There is a report suggesting that P-glycoprotein expression levels in the upper part of the intestine increase with age in type 2 diabetes obese mice associated with monosodium glutamate treatment [477]. However, we could not find any similar literature pertaining to the association between age and human intestinal P-glycoprotein expression.

Unlike the gastrointestinal tract, altered P-glycoprotein expression and function associated with ageing has been demonstrated in blood brain barrier and peripheral blood lymphocytes [478]. Pilarski et al [479] demonstrated that P-glycoprotein on T and B lymphocytes to be lowest during childhood then increased towards adulthood before decreasing again after age 60. Another study in healthy subjects aged 0 to 86 years showed that P-glycoprotein expression in peripheral blood lymphocytes was highest in cord blood and declined progressively with age [480]. Decreases in P-glycorprotein expression at the blood brain barrier have also been associated with ageing [481]. For example, increases in the volume of distribution of verapamil in the range of 18% to 38% were observed with normal ageing within several brain regions, such as frontal, temporal, medial temporal and anterior and posterior cingulate regions. The increased volume distribution was suggested to be caused by the combination of higher permeability and decreased P-glycoprotein function associated with ageing [482-483].

In this study, we observed that P-glycoprotein expression was significantly higher in the duodenum than the antrum. This finding is in agreement with the others who have investigated P-glycoprotein activity along the gastrointestinal tract [484-485]. It is essential to understand the pattern of P-glycoprotein expression along gastrointestinal

tissue with respect to the orally administered drugs, particularly for those given in controlled compound release preparations [486]. The small intestine represents the principal site of absorption for most ingested compounds including therapeutic and toxic substances [487]. The expression of P-glycoprotein has been observed to increase progressively by up to 5-fold from the proximal to the distal region of the small intestine and its corresponding *mdr1 mRNA* has also been detected [473,488]. The expression of P-glycoprotein has also been found to increase from the duodenum to colon. Like P-glycoprotein, other transporters such as *BCRP\ABCG2* have also been found throughout the small intestine and colon. Unlike P-glycoprotein, *MRP2* expression has been reported to be highest in the duodenum and subsequently to decrease to become undetectable towards the terminal ileum and colon [489].

Originally it was thought that it may be useful to use blood samples to predict the P-glycoprotein expression in liver or intestine in our study; however results from the literature are inconclusive regarding the value of using P-glycoprotein expression in peripheral blood mononuclear cells [490]. For example, Albermann and colleagues [491] reported that there was no correlation between hepatic and intestinal P-glycoprotein expression with peripheral blood mononuclear cells expression. For that reason, peripheral blood mononuclear cells were not used as an indicator for the transporter level in the intestine in our study. Instead, P-glycoprotein levels were directly determined from biopsy specimens.

In our study, the levels of P-glycoprotein expression in the antrum and duodenum were not found to be influenced by proton pump inhibitor use prior to endoscopy. In this clinical setting, proton pump inhibitors such as omeprazole, lansoprazole and pantoprazole are commonly used to treat patients with acid related diseases including peptic ulcer disease and GORD [492]. The proton pump inhibitors are characterized as substrates and/or inhibitors for P-glycoprotein [493]. Therefore, proton pump inhibitors may influence both intestinal first-pass metabolism and hepatic clearance [494]. As most of our participants have been treated with proton pump inhibitors prior to their

endoscopy, it was important to determine whether this exposure had had any influence on P-glycoprotein expression.

5.1.4.2 P-glycoprotein expression in presence of *Helicobacter pylori*

Our study showed that P-glycoprotein expression increased proportionally along the gastrointestinal tract. P-glycoprotein along with many other proteins such as tight junction and trefoil peptides that are expressed by epithelial cells are directly or indirectly involved in maintaining the gut's protective barrier [495]. Disturbances of the mucosa epithelial cells may lead to the development of mucosa inflammation, which triggers a variety of cellular responses in intestinal cell layers including P-glycoprotein expression which is amongst the first to defend the mucosa layers.

In this study, we found that P-glycoprotein expression in the antrum was increased significantly in the presence of *H. pylori*. *H. pylori* mostly resides within the gastric mucous layer and the bacteria is exclusively detected in the surface mucous layer, which becomes thinner in the infected stomach [496]. *H. pylori* is considered to be an extracellular pathogen and its attachment to gastric epithelial cells resembles that of the enteropathogenic *Escherichia coli* [497]. The bacterial attachment to human antral primary epithelial cells has been described by Heczko et al [498]. They reported that *H. pylori* was observed to display various morphological changes (between spiral, U-shaped, donut and coccoid forms) as well as bacterial membrane enlargement during attachment giving rise to the unique features of *H. pylori* infection in the gastric mucosa. *H. pylori* might also induce cell cycle arrest by *p53* expression interference and subsequently reduce the ability of damaged cells *in vivo* to repair significant cell injury [499].

In this present study, we also demonstrated that the subjects who were *H. pylori*-positive and have received multiple courses of eradication therapy had higher P-glycoprotein expression (antrum over duodenum) compared to the control group (i.e. *H. pylori* negative). *H. pylori* induces P-glycoprotein expression in the antrum. As P-glycoprotein

is a transporter that facilitates excretion of substrates from the systemic circulation into the gut lumen [500], the high levels of expression seen in the study subjects could be explained as an attempt by the gut mucosa to protect itself against infection. It could also be a result of a stress-related induction of P-glycoprotein as has been suggested to occur in other tissues in the body such as in blood brain barrier [501]. The high levels of expression of P-glycoprotein in gut mucosa may also limit the absorption of drugs that are known to be P-glycoprotein substrates following oral administration, whilst at the same time protecting the host against any orally ingested toxins [502].

In keeping with our data, there have been other reports related to P-glycoprotein regulation in the presence of endotoxin-induced inflammation in human and animal intestinal cells. For example, P-glycoprotein is believed to prevent the accumulation of inflammation-inducing bacteria such as *Helicobacter spp*. as demonstrated in knock-out mice with colitis [503]. Further, human *MDR1 mRNA* expression has been reported to increase in Caco-2 cells treated with proinflammatory cytokines and macrophages, yet the *mRNA* expression of CYP3A4 was found to be decrease [504]. In contrast, the activity of intestinal P-glycoprotein in rats was found to be reduced during acute inflammation [505]. Similarly, interleukin-10 deficient enterocolitis mice also had reduced P-glycoprotein expression along the intestine [506]. As inflammation usually follows enterotoxin bacterial invasion, these last two reports suggest the act of inflammation may counter any direct increase of P-glycoprotein expression. Nevertheless, with *H. pylori* infection, we found that the efflux protein was found expressed more in the antrum which may be a mechanism to protect the membrane mucosa.

5.1.4.3 Exploitation of P-glycoprotein expression in *Helicobacter pylori* eradication therapy

Knowing that P-glycoprotein expression is increased in the presence of *H. pylori* raises the question is higher P-glycoprotein expression responsible for the *H. pylori* eradication failure or resistance? Babic et al [305] reported that therapeutic success could be

influenced by P-glycoprotein activity as their subjects with higher basal P-glycoprotein tended to fail the eradication therapy. In a later study, Babic et al [507] reported *H. pylori* infection was associated with higher levels of P-glycoprotein expression, and those patients who experienced treatment failures had significantly higher levels compared to those who were treated successfully. They suggested that P-glycoprotein could be one of the causes treatment failure in patients with *H. pylori* and that is important to assess the P-glycoprotein expression before the starting the eradication therapy.

Although increased P-glycoprotein levels may lessen the oral bioavailability of antibiotics which are substrates for P-glycoprotein (**Figure 5-1)**, this action may potentially be beneficial in the case *H. pylori* eradication. More antibiotic will be available in the extracellular gastric mucus where *H. pylori* normally resides thus enhancing their local bacteriostatic or bacteriocidal effects. For example, macrolides produce bacteriostatic effect on *H. pylori* by inhibiting the ribosomal subunit in the bacteria cell to block the protein synthesis which later stops the bacteria from reproducing [508]. Macrolides at high concentrations may also demonstrate bactericidal activity [509]. As more P-glycoprotein gets expressed on the lumen cell mucosa it is possible that there are higher macrolide concentrations extracellularly leading to a bactericidal effect on the *H. pylori.*

In our study, more than half of our subjects who had failed eradication therapy showed cross-resistance between metronidazole and clarithromycin. The dual resistance between macrolides and metronidazole is quite common and frequently reported [510-513]. Clarithromycin resistance is the main risk factor for the failure of first line therapy to eradicate *H. pylori* [514]. The efficacy of metronidazole-based triple therapies was reduced by 18-38% with metronidazole resistance [515]. About 90% of our resistance subjects were cured after been treated with second-line regimen consisting of rifabutin, amoxicillin and rabeprazole. Recently, Tay and colleagues [516] had demonstrated that *H. pylori* was successfully eradicated in 95.2% of their subjects whom received novel quadruple

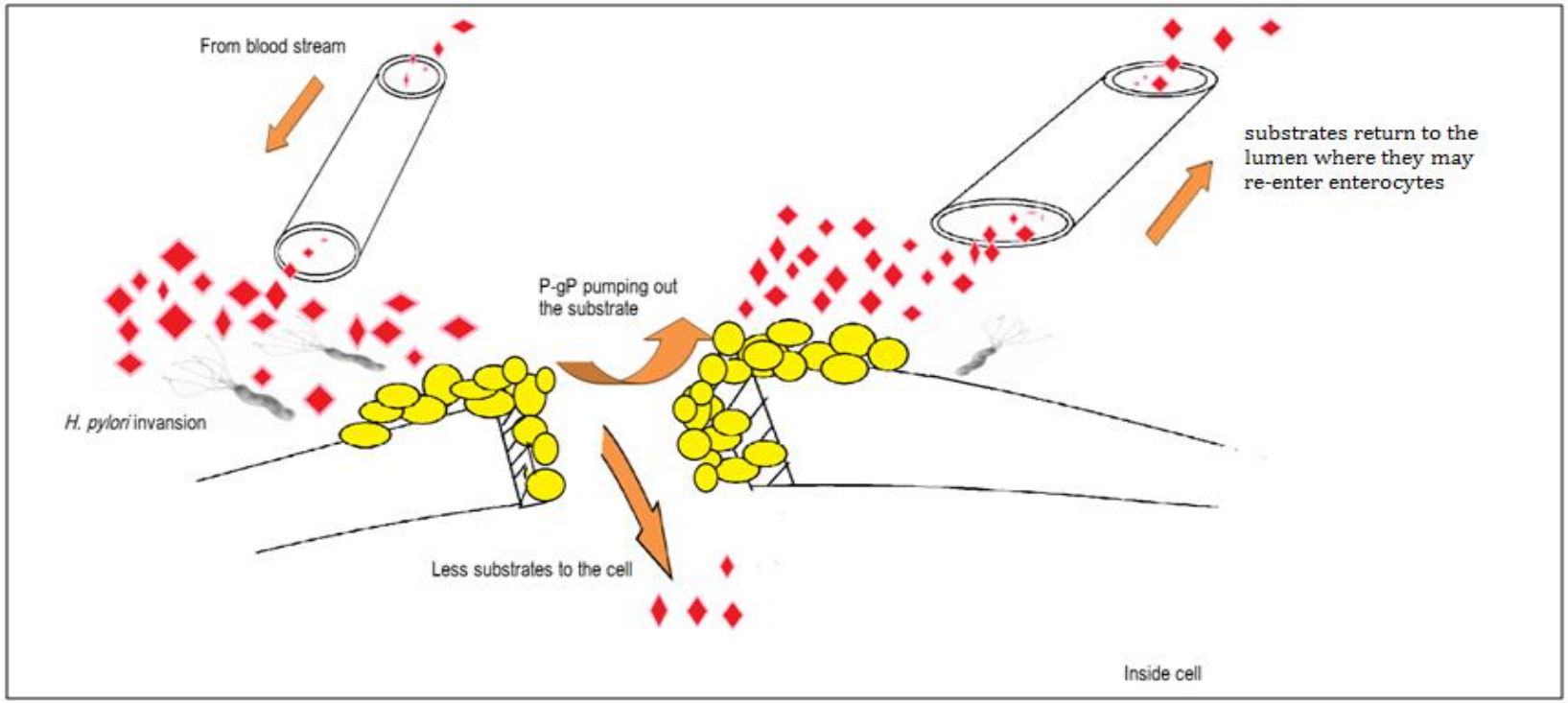

Figure 5-1: Illustration of the possible implications of higher P-glycoprotein expression in the presence of *Helicobacter pylori* (original by M. Omar)

quadruple therapy combinations of proton pump inhibitor, amoxicillin, rifabutin and ciprofloxacin. The eradication rate went down slightly to 94.2% by using the alternative quadruple therapy of proton pump inhibitor, bismuth subcitrate, rifabutin and ciprofloxacin. Rifabutin is a derivative of rifamycin *S* [517] and is suggested to be an inducer for both P-glycoprotein and *CYP3A4* as it is structurally related to rifampin [518]. Interestingly, rifabutin can inhibit *H. pylori* at very low concentrations [519] and its effects are not influenced by gastric pH [520]. With the current finding that *H. pylori* induces P-glycoprotein, high level of gut P-glycoprotein expression may then assist antibiotic eradication therapy for *H. pylori* as a protective mechanism which may be exploited through the use of P-glycoprotein inducers, such as rifabutin, in eradication regimens.

Whilst use of rabeprazole in *H. pylori* eradication was found to achieve similar eradication rates as omeprazole or lansoprazole when given with amoxicillin or clarithromycin [521], rabeprazole gives more consistent and better symptom relief to the patients [522]. Amoxicillin has low *H. pylori* resistance rates [523]. It gives rapid accumulation on the antral mucosa [524] and the minimum inhibitory concentration reduces when the gastric pH increases [525]. Amoxicillin may destroy the bacterial membrane, causing transmembrane efflux system impairment, trapping the macrolides inside the cell [526]. High levels of intracellular macrolides would then prevent any final attempt by the bacteria to prevent damage to its ribosome.

Although the resistance towards the *H. pylori* eradication may not occur as a result of P-glycoprotein alone, the association of over-expression of these drug efflux transport protein and resistant subjects cannot be dismissed. Our study contributes to the findings that subjects with *H. pylori* infection do have elevated P-glycoprotein levels and therefore may be have an increase of risk of drug interactions that involve P-glycoprotein substrates.

5.1.5 *MDR1 C3435T* polymorphisms

5.1.5.1 Overview

MDR1 C3435T polymorphism has been associated with numerous diseases including gastric cancer [11] and breast cancer [527]. In this present study sought to investigate the association between this particular P-glycoprotein gene polymorphism and *H. pylori* infection, and its potential impact on eradication therapy.

For the *MDR1 C3435T* polymorphisms, the distribution of *CC, CT* and *TT* alleles in both *H. pylori*-positive and *H. pylori*-negative groups were similar to that predicted from the literature [528-529] in that about half of the study population carry *3435CT* allele and the other half carry either *3435CC* or *3435TT* allele. To the best of our knowledge, there is no other study available describing the *MDR1 C3435T* genotype distribution among Australians. **Table 5.1** outlines the comparison between Australians recruited from our study and other ethnic groups regarding the allele frequencies for the *MDR1 C3435T* polymorphisms.

Interestingly, a similar *MDR1 C3435T* distribution has also been observed in New Zealander and Asian (Malay and Chinese) populations. Close geography between Australia and New Zealand with other parts of South East Asia may explain this pattern of genotype distribution possibly due to high migration between these countries. The Singaporean population also showed the same pattern of *MDR1 C3435T* genotype distribution, where half of the studied population carries either heterozygous or homozygous allele, however the *3435CC* frequency was lower than *3435TT* [529]. However, Li et al [529] study did not represent all ethnic groups from Singapore rather only those of Chinese origin were recruited; these Chinese are predominantly descendents of migrants from south China. Nevertheless, the described distribution of a functional single-nucleotide polymorphism in the human *MDR1C3435T* gene within the different populations may be helpful for individualizing pharmacotherapy.

Table 5-1: Comparison between Australians and other ethnic groups regarding the allele frequencies for the *MDR1 C3435T* polymorphisms

Population		Sample size	Genotype frequency (%)			Reference
			3435CC	*3435TT*	*3435CT*	
Australians		91	22%	31%	47%	Current study
Asians	Malay	99	25%	28%	46%	[528]
	Chinese	98	25%	32%	44%	
	Indians	93	18%	43%	39%	
Chinese		265	32%	20%	48%	[529]
Iranians		126	53%	61%	42%	[530]
Indians		147	12%	52%	36%	[531]
Ecuadorians		317	25%	22%	53%	[532]
Romanians		102	25%	22%	54%	[533]
Singaporean		224	28%	22%	50%	[534]
New Zealander		160	21%	27%	52%	[535]
Japanese		114	35%	12%	53%	[536]
Polish		122	42%	17%	41%	[537]
French		81	35.8%	42%	22.2%	[538]
Spanish		408	26%	22%	52%	[539]

5.1.5.2 *MDR1 C3435T* polymorphisms and *Helicobacter pylori* infection

In our study, P-glycoprotein expression in the antrum in *H. pylori*-positive and *H. pylori*-negative groups were found to be lowest in the *3435TT* genotype, followed by *3435CT* and *3435CC* genotypes, respectively. Overall, all the *MDR1 C3435T* genotypes showed an increased level of P-glycoprotein expression in the presence of *H. pylori* infection. Interestingly, the difference was most marked in *3435TT* genotypes sub-group. This observation may explain the findings of Tahara et al [11] who reported that *3435TT* genotypes have lower risk of gastric cancer. In their study, subjects with *3435TT* genotype were found to be less likely to be infected with *H. pylori* or develop gastric cancer. Reduced prevalence of gastric cancer in the *3435TT* genotype sub-group may then be a reflection of their ability to hyper-express P-glycoprotein in the presence of *H. pylori* infection thus enhancing its protective effect.

All the *MDR1 C3435T* genotypes in our resistant subjects also showed increases in P-glycoprotein expression in the presence of *H. pylori*, with the *3435TT* carriers showing the greatest increases ($p = 0.056$). Unfortunately, our inability to recruit larger numbers of patients diminished the power of the study and our ability to demonstrate statistically significance differences. Nevertheless, this finding supports our earlier theory that *3435TT* carriers are capable of inducing more P-glycoprotein compared to other *MDR1* genotypes in the presence of *H. pylori* infection. Earlier, Tahara et al [11] reported that *3435TT* carriage was associated with a higher degree of atrophy and intestinal metaplasia. These authors agreed that *MDR1 C3435T* polymorphism influences *H. pylori*-related inflammation in the stomach, especially in older subjects. Meanwhile, *3435CC* carriage has been seen at higher frequencies amongst patients with advanced gastric cancer compared with healthy volunteers [540]. Interestingly, Gawrońska-Szklarz et al [541] demonstrated that the *3435TT* genotype was associated with successful eradication after the first cycle of triple therapy. Therefore, the ability to induce more P-glycoprotein among *3435TT* carrier may be beneficial in reducing

inflammatory conditions in the gut mucosa that are caused by the *H. pylori* and achieving better therapy outcomes.

Our proposition requires further investigation as Sugimoto et al [12] showed that patients with *3435CT* genotype made up nearly 50% of those patients with gastric cancer and no significant difference was observed in gastric cancer risk across the three genotypes. Similarly, Sabahi and colleagues [542] also demonstrated that no significant association between the gastric cancer and *MDR1* genotypes in an Iranian population. Additionally, Salagacka et al [308] demonstrated that no significant difference was found in the genotype distribution between subjects with peptic ulcers and the healthy population, although the authors did observe a positive trend towards higher incidence of this disease among *3435TT* carriers.

As our study focused neither on peptic ulcer disease nor gastric cancer but rather on the presence of *H. pylori* and its influence on P-glycoprotein expression in the antrum and duodenum, this could explain the conflicting results between our study and other studies. Regardless, the increased capacity to induce P-glycoprotein in the presence of *H. pylori* among *3435TT* carriers seen in our study has not been previously reported and this information could be important in understanding of why patients response differently towards their infections and eradication therapy. The use of *MDR1 C3435T* genotyping has yet to be fully explored, although it has been suggested earlier that the different distribution of the functional alleles of this gene within the different populations or diseases may be helpful in individualizing pharmacotherapy. However, these particular genotypes may be influenced by the ethnicity [543] and pervasive environmental factors such as alcohol consumption [544] adding to the difficulties in achieving a conclusive result on this matter.

The risk of diseases such as renal and breast cancer have been demonstrated to be influenced by *MDR1 C3435T*, but little is known about *H. pylori*-related diseases [545]. As opposed to gastric cancer risk, a strong association between *MDR1 C3435T* and ulcerative colitis has been observed in a German population [546], similar to another

study in Iranian patients with ulcerative colitis [547]. Ulcerative colitis is an inflammatory bowel disease of unknown etiology and studies has been done to investigate whether *Helicobacter* strains may be a cause for this disease [548]. *H. pylori* has been isolated from the ulcerative colitis patients, however its presence was not found to be associated with the disease. This might be due to the fact that *Helicobacter* strains rarely colonize the lower part of human gastrointestinal tract, although several studies have demonstrated that some *Helicobacter* strains present in the gastrointestinal tract of mice can induce inflammation of the colon [549-550]. Using a more sensitive PCR assay, another study found that *H. pylori* strains were more frequently isolated in the intestinal mucosa of subjects with ulcerative colitis-like Crohn's disease than in intestinal mucosa of the control group [551]. Though no other *Helicobacter* species were isolated from the intestinal mucosa, these authors agreed that this observation could be secondary to the genesis behind the Chron`s disease rather than present of *H. pylori* alone.

It was reported earlier that *3435TT* allele was associated with higher plasma levels of *MDR1* substrates [189]; this may be beneficial in achieving better treatment outcome for some drugs whilst for others may increase the risk of toxicity. In a Japanese population, lansoprazole plasma levels were found to be influenced by the *MDR1 C3435T* polymorphisms [552]. In this study the *3435TT* carriers showed the highest lansoprazole plasma level among the three *MDR1* genotype groups during 15 days of drug administration, however interestingly no difference was seen in intragastric pH. It was concluded that *MDR1 C3435T* polymorphisms only influenced the drug pharmacokinetics rather than pharmacodynamics of lansoprazole.

Gawronska-Szklarze et al [541] have reported the eradication rate of *H. pylori* was also found higher in Caucasians with *3435TT* genotype than that in *3435CC* subjects. However, the influence of ethnicity in *MDR1 C3435T* polymorphism was once again highlighted when a lower eradication rate was reported among *3435TT* carriers when an *H. pylori* eradication regimen consisting of lansoprazole, clarithromycin and amoxicillin were administered in a group of Japanese subjects [314]. These findings were supported

by Oh et al [351] who reported the lowest cure rates for *H. pylori* were achieved among *3435TT* carriers in Korea. These variations in the observations from studies from around the world suggest that *MDR1 C3435T* may not be the only predictor of the success of *H. pylori* eradication therapy. Hence, larger epidemiology studies are warrant to investigate the relationship between P-glycoprotein polymorphism and *H. pylori* eradication rates.

5.1.6 CYP2C19 polymorphisms

5.1.6.1 Overview

Most proton pump inhibitors will undergo hepatic biotransformation via the hepatic cytochrome P450 2C19 isoenzyme [553]. The impact of the CYP2C19 pathway on the metabolism of proton pump inhibitors is most pronounce for omeprazole and esomeprazole followed by pantoprazole, lansoprazole and rabeprazole respectively. The differences for omeprazole and esomeprazole compared with other proton pump inhibitors are the result of their significant inhibition of CYP2C19, which is mainly responsible for their metabolic clearance [554].

5.1.6.2 CYP2C19 genotype among resistance subjects

In our study, only the pattern of distribution according to five different allelic patterns of CYP2C19 genotypes among resistance subjects was examined. Of these 38.1% of subjects were categorized as extensive metabolizers while another 33.3% were the passive metabolizers. In passive metabolizers, a splice defect in exon 5 (*CYP2C19*2*; single base change G>A), and a premature stop codon at position 636 of exon 4 (*CYP2C19*3*; single base change G>A) are commonly observed where it will subsequently result in a truncated protein and an inactive enzyme. Therefore, in these subjects the area under the plasma concentration-time curve of omeprazole and esomeprazole should be markedly increased and the clinical effect of these proton pump inhibitors may be greater [555].

There was no conclusive evidence from our study as to whether the different CYP2C19 genotypes contributed to the success of *H. pylori* eradication therapy as it was limited by small number of subjects in each subgroup. The CYP2C19 genotyping was investigated based on the fact we were not able to demonstrate that *MDR1 C3435T* was the only factor that was influencing the outcome of *H. pylori* eradication. It would have been better if we were able to recruit a larger number of resistant subjects; however, this was not possible given the limited operating days of the Helicobacter clinic and small number of referrals for *H. pylori* treatment resistance.

Nevertheless, based on the available literature, the efficacy of proton pump inhibitors in the treatment of *H. pylori* infections could be related to the CYP2C19 genotypes [556]. Furuta et al [555] suggested that CYP2C19 polymorphism affecting proton pomp inhibitor metabolism could impact on the chance of a cure in *H. pylori*-positive patients who were treated with a two week dual therapy regimen with omeprazole and amoxicillin. The cure rates for *H. pylori* infection in their study were only 28.6% in the extensive metabolizers as compared to 100% in the passive metabolizers. The influence of the CYP2C19 genotype on *H. pylori* eradication regimens containing omeprazole has also been demonstrated by report by Tanigawara and colleagues [557]. In a group of 108 subjects who were treated with either quadruple treatment without a proton pump inhibitor, dual therapy with omeprazole and amoxicillin or triple treatment with omeprazole, amoxicillin and clarithromycin, they found that the *H. pylori* eradication rates for the extensive metabolizers were only 50% and 86% for the dual and triple treatments, respectively as opposed to 100 % in the passive metabolizers treated with omeprazole and antibiotics.

In our study, rabeprazole was used to replace omeprazole or esomeprazole to eradicate *H. pylori* among resistance subjects. Rabeprazole is a newer proton pump inhibitor and although triple therapy with this proton pump inhibitor combined with amoxicillin and clarithromycin is effective, the eradication rate is not dependent on CYP2C19 genetic polymorphisms [558-560]. Previously, Horai et al [561] reported that CYP2C19 polymorphisms influenced the pharmacokinetic and pharmacodynamic of rabeprazole.

However, a meta-analysis by Padol et al [562] stated that the impact of CYP2C19 polymorphism on *H. pylori* eradication rates was only clinically important when subjects were prescribed with omeprazole as one of the components in their dual or triple therapy but not in the case of lansoprazole or rabeprazole. Such contradicted reports raise the question as why no impact on *H. pylori* eradication therapy have been demonstrated.

Additionally, the *CYP2C19*2* polymorphism occurs almost exclusively in Caucasians while the *CYP2C19*3* occurs primarily in Asians [563]. In Caucasians and African-Americans, about 2 to 3% of individuals can be characterized as passive metabolizers. In Asian populations, the percentage of passive metabolizer is about 15 to 20% [555]. Therefore, the large interindividual and interethnic variability in the pharmacokinetics of proton pump inhibitors may also be explained *CYP2C19* genetic polymorphism.

5.2 Phase 2: *Helicobacter pylori* attachment to gastrointestinal cell lines

5.2.1 Bacterial attachment study with Caco-2 cell lines

The ability of bacteria to adhere and colonize the gastrointestinal cells is important in their pathogenesis [564]. The bacteria also need to produce certain extracellular substances to help them invade cells to overcome any host defence mechanisms. Several agents including cyclosporine and its analogue valspodar (PSC-833) are capable of modulating and decreasing P-glycoprotein expression *in vitro* [565]. The approach of this current study was to abruptly block the P-glycoprotein expression in the Caco-2 cells with a use of P-glycoprotein inhibitor with the purpose of understanding the bacterial attachment to the human gastrointestinal cells as has been done previously in the laboratory [389]. The extent of *H. pylori* attachment to Caco-2 cell lines were represented by the intensity of bacterial fluorescence that corresponded to the amount of bacteria that was unwashed from the human cell layers coating the wells.

In this study, a constant increase of *H. pylori G27* and *H. pylori J99* attachment to the control Caco-2 cells were observed over the 4 hour of incubation period. When PSC-833 was introduced to the cell lines, further bacterial attachment was measured for both *H. pylori* strains. PSC-833 is a potent inhibitor of P-glycoprotein and displays a competitive manner by blocking the ATPase pump to inhibit P-glycoprotein function [567-568]. Defective efflux transport of P-glycoprotein subsequently allows more bacteria to attach to the cell lines. Further, it is known that the first step in bacterial infection is adherence and growth on the enteric epithelium surface with or without subsequent invasion of the cell [566]. Upon colonization, there is a transient period between 1 to 20 days of post-infection prior to the establishment of *H. pylori* persistence [569]. Among the strategies utilized by *H. pylori* to survive and persist in the gastric lumen are the inhibition of the gastric epithelial cell apoptosis and compensatory gastric epithelial hyperproliferative responses [570]. An intracellular location of *H. pylori* in human gastric epithelial cells is probably uncommon in *H. pylori* colonization [571], however *H. pylori* also have been observed inside the infected epithelial cell lines [572]. Chu et al [573] found that *H. pylori* invaded the epithelial cell lines and multiplied either on the plasma membrane or in double-layer vesicles. For these reasons, P-glycoprotein is important as defence mechanism in the earlier stage during the bacterial attachment in which this efflux transporter is exquisitely positioned to resist further bacterial invasion or production of substances that are allegedly harmful. Our earlier results support the hypothesis that normal response to *H. pylori* infection is the increased expression of P-glycoprotein presumably to provide a protective barrier.

The Caco-2 cells are known to express high levels of P-glycoprotein mRNA and protein [574]. Rahimifard et al [575] demonstrated that a number of *H. pylori* strains were able to attach to various cell lines including HepII, HeLa, SW742, AGS, HT29/219, HT29 and Caco-2 cells. Interestingly, in that study, negligible *H. pylori* attachment was seen with Caco-2 cells. Taking into consideration that Caco-2 cells express high levels of P-glycoprotein in comparison to other cell lines, the minimal *H. pylori* attachment to Caco-2 cells may explain the early response to the bacterial colonization by P-glycoprotein in gastric epithelium lining. As demonstrated in our study, both strains of *H. pylori* showed

an increasing bacterial attachment to the control Caco-2 cells over the 4 hour incubation period. When PSC-833 was used to block P-glycoprotein function in Caco-2 cells, the bacterial attachment was considerably increased, in comparison with the corresponding control cell lines supporting that P-glycoprotein expression by the gut is important in maintaining homeostasis in the gastrointestinal tract through modulation of intestinal responses to *H. pylori* infection.

Similar trends in bacterial attachment were observed when *E. coli* W and *S. aureus* were used in our study. Both bacteria were selected as positive controls as their attachment to Caco-2 cells were previously demonstrated to be significantly increased (up to 3 fold) when PSC-833 was introduced [389]. As *E.coli* W and *S. aureus* are commonly present in the gastrointestinal tract, it was concluded that P-glycoprotein expression may prevent the bacterial attachment to the human gastrointestinal tract, avert the bacterial toxicity and subsequently reduce the risk of gastrointestinal diseases. In another report, Caco-2 cell lines were infected with enterohaemorrhagic *E. coli* O157 to determine whether the bacteria could penetrate the cells monolayers and translocate its verotoxin from the intestinal tract lumen to the underlying tissue to cause the haemolytic uraemic syndrome [576]. The strain of *E. coli* tested in that study was found not to penetrate the Caco-2 cell monolayer system to cause the infection. This finding can be explained by those of our study and Crowe [389] in which increased P-glycoprotein expressions was shown to restrict bacterial attachment, and hence reduce the potential for invasion. These findings emphasize the important role of P-glycoprotein as a protection barrier in the gut.

S. aureus has been reported to adhere to the human colonic mucosa and its treatment could be complicated with emergence of antibiotic resistance among some strains [577]. Increased intestinal colonization is one of the causes for *S. aureus* treatment resistance [578]. As the first line of defence, intestinal epithelial cells make up a selectively permeable epithelial barrier function, preventing the uncontrolled passage of antigens and potentially harmful microorganisms from the intestinal lumen into the blood and internal tissues [579]. Nevertheless, on the basis of the findings in our study, high levels

of P-glycoprotein expression on the gastrointestinal cells are critical to function as barrier for initial bacterial attachment.

5.2.2 Bacterial attachment study with LS174T cell lines

The LS174T cell lines were established in 1974 from a moderately well differentiated primary colon adenocarcinoma by trypsinization of the parent cell line, LS180 [580-581]. Certain drugs such as amprenavir, nelfinavir and ritonavir have been shown to strongly induce the P-glycoprotein level in LS174T cell lines [582]. The expression of P-glycoprotein is mediated by human pregnane X receptor (PXR), a member of the nuclear receptors superfamily encoded by NR112 [583]. In normal tissues, PXR is highly expressed in the liver and intestine to regulate *CYP3A4* and *MDR1* genes [574]. It has been reported that PXR is endogenously expressed in LS174T cells and rifampicin, a human PXR agonist induces *MDR1* expression [575-576]. Similar to other studies [389,587], we were able to increase the level of P-glycoprotein expression in LS174T cells. However, both the control LS174T cells and rifampicin induced LS174T cells exhibited P-glycoprotein levels below that of the Caco-2 cells.

As positive controls, both *E. coli W* and *S. aureus* demonstrated a significantly reduced attachment to LS174T cells when rifampicin was used to induce the P-glycoprotein expression. Our data matched that from study by Crowe [389] which demonstrated a decrease in bacterial attachment to the cell lines with rifampicin enhanced P-glycoprotein expression. The decrease in bacterial attachment was completely reversed by the use of PSC-833, a P-glycoprotein inhibitor, leading to an increase in bacteria binding to the LS174T cell lines. Further, the greater influence on *S. aureus* attachment with LS174T could be due to effects on both bacterial adherence and invasiveness. *S. aureus* demonstrated a higher fluorescence level of bacterial attachment to the LS174T cells compared to *E. coli* due to it be an invasive pathogen compared to non-invasive *E. coli* which remains on the outer surface of the cells [375].

Unlike *E. coli W* and *S. aureus*, the attachment of *H. pylori* G27 and *H. pylori* J99 in our present study seemed to be unaffected by the level of P-glycoprotein expression in LS174T cell lines. The differences in *H. pylori* attachment to the LS174T cell lines with that in the Caco-2 study may be explained by the amount of P-glycoprotein expression present in the cell lines themselves. As shown in our Western Blot Analysis, LS174T cells had less P-glycoprotein than Caco-2 cells even though rifampicin was used to induce the *MDR1* expression. *H. pylori* might be able to escape the barrier function formed by the P-glycoprotein that has been shown to limit the bacterial attachment, in the absence of high P-glycoprotein expression. It is assumed that *H. pylori* would begin to attach to gastric epithelium lining once the P-glycoprotein expression falls below a certain threshold. Moreover, *H. pylori* is known to have the ability to undermine the innate and adaptive immune systems of the host by assuming different shapes during the initial colonization process [588]. *H. pylori* also has the ability to produce urease to elevate the pH of its environment. In that way, *H. pylori* may entirely escape the host defence mechanism. Without high P-glycoprotein expression available to prevent the bacteria from attaching to the gastrointestinal cells, a cascade of *H. pylori* proliferation and toxin secretion will facilitate disease development [589].

The differences in *H. pylori* attachment to the LS174T cells and Caco-2 cells may also be explained by the different levels of mucin mRNA expression in these cell lines. Only part of the colonizing *H. pylori* attach directly to the epithelial cells as most of them stay in the mucus layer of the superficial gastric mucosa and bind to the highly glycosylated mucins [590]. The aggregation of *H. pylori* around mucins will protect the bacteria from the surrounding environment and immune factors in the gastric mucosa layer [591]. The gel-forming mucin, MUC2 is expressed in goblet cells of native intestinal epithelium and intestinal metastasis of gastric mucosa but not in the normal gastric epithelium [592]. In contrast, the MUC5AC gene is mainly expressed in the gastric mucosa. The LS174T cells expressed a relatively high MUC2 but low MUC5AC mRNA level. Meanwhile, the MUC5AC mRNA was highly expressed in the Caco-2 cells and its MUC2 mRNA level was too low. Altered expression of MUC2 was reported to be related to the development of colorectal cancer where MUC2 suppression was found to induce the expression of molecules associated with LS174T cell invasion [591].

The exact underlying mechanism behind how P-glycoprotein prevents bacterial attachment remains unknown. P-glycoprotein may act as a conduit for cytoplasmic agent such as endogenous antimicrobial peptides to appear on the plasma membrane of cells [375]. If it is true, the action may be cell line specific, in that bacterial attachment would be inhibited depending on type of factors mediated by P-glycoprotein in those cells. It is possible that LS174T and Caco-2 cells have different expression of these yet to be determined factors. In general, *H. pylori* has been established as the major causative agent of human gastritis and is an essential factor in peptic ulcer disease and gastric cancer. Our study has demonstrated that the attachment of *H. pylori* to gastrointestinal cell lines is influenced by level of P-glycoprotein expression, which along with other factors such as the production of adherence factors and the ability to change shape during stress which may modulate the bacteria`s survival in the gastrointestinal tracts.

CHAPTER 6

SUMMARY OF FINDINGS, CONCLUSIONS, RESEARCH LIMITATIONS AND FUTURE RESEARCH RECOMMENDATIONS

CHAPTER 6 SUMMARY OF FINDINGS, CONCLUSIONS, RESEARCH LIMITATIONS AND FUTURE RESEARCH RECOMMENDATIONS

6.1 Summary of findings

The primary aim of the study was to determine whether P-glycoprotein expression in the human gastrointestinal tract influences *H. pylori* eradication therapy. Specifically, the study aimed to answer the following questions:

- Is there an association between P-glycoprotein expression and *H. pylori* infection?
- What is the relationship between P-glycoprotein expression and its genetic variant, particularly *MDR1 C3435T?*
- What is the influence of P-glycoprotein expression on *H. pylori* treatment resistance?
- Is the extent of *H. pylori* binding to gastrointestinal epithelial cell lines influenced by the presence of potent P-glycoprotein inducers and inhibitors?

This study was divided into two phases – Phase 1 (*Helicobacter pylori* and P-glycoprotein expression) and Phase 2 (*Helicobacter pylori* attachment to gastrointestinal cell lines). For Phase 1, a total of 91 participants from Sir Charles Gairdner Hospital, Nedlands, Western Australia were recruited from October 2010 to July 2011. For each patient, antral and duodenal biopsies along with venous blood samples were obtained for the laboratory analysis. For Phase 2, Caco-2 and LS174T cells were used to determine *H. pylori* attachment to the gastric epithelial cells within certain incubation periods in the present of the P-glycoprotein inhibitor (PSC-833) and the inducer (rifampicin).

It was found in Phase 1 that P-glycoprotein expression was induced by *H. pylori* in the upper gastrointestinal tract. Further, the *3435TT* carriers demonstrated a greater capacity to induce P-glycoprotein expressions in presence of *H. pylori* which may explain their

observed reduce risk of developing gastric cancer. The *H. pylori* treatment resistance subjects also demonstrated higher P-glycoprotein expression in the antrum. In the Phase 2 of the study, it was found that initial binding of *H. pylori* was reduced depending on level of P-glycoprotein expressed on the gastrointestinal cell lines.

6.2 Conclusions

P-glycoprotein expression in the upper gastrointestinal tract is induced by *H. pylori* and there is a positive association between P-glycoprotein expression and *MDR1 C3435T* polymorphisms. This is also true in *H. pylori*-treatment resistant subjects although no link between treatment resistance and P-glycoprotein expression was demonstrated. However, based on the fact that we were able to demonstrate that attachment of H. pylori to gastrointestinal lines is influenced by P-glycoprotein expression, we would suggest that the use of a P-glycoprotein inducer, such as rifabutin, may be beneficial in their eradication regimens.

6.3 Research limitations and future research recommendations

This study provided a new insight on the ability of *3435TT* carriers to induce P-glycoprotein expression in the presence of *H. pylori* to the greater extent than *3435CC* and *3435CT* carriers. This finding may provide a possible explanation for differences in observed complication rates, notably the development of gastric cancer, based on genotype. However, as genetic polymorphism varies inter-individually and inter-ethically, larger scale epidemiology studies need to be undertaken in order to determine the exact influence of *MDR1 C3435T* on *H. pylori* infection treatment outcomes and complications.

This study also postulated that the use of a P-glycoprotein inducer, such as rifabutin may be beneficial in the *H. pylori* eradication regimens among the resistance subjects. It is important to note that in this study, the baseline P-glycoprotein expression was not measured before the resistance subjects first commenced their *H. pylori* eradication

therapy. Before the recruitment, P-glycoprotein expressions among resistance subjects may have already been elevated by chronic *H. pylori* infection. Hence, to determine if in fact a relationship does exist between P-glycoprotein expression and *H. pylori* eradication regimen efficacy, a large study needs to be conducted in which P-glycoprotein expression is measured at baseline and at the time at which the patients is deemed to be resistant. Further, it is important that *CYP2C19* polymorphism is also evaluated in a larger population with treatment resistance, as this may influence proton pump inhibitor efficacy.

BCRP2 is expressed abundantly on the human liver, placenta and intestines, however to date there are no literature available on the influence of *H. pylori* to the BCRP2 expression level in the intestines. Determining whether *H. pylori* infection could up-regulated ABCG2 expression would be beneficial as this particular protein has broad and overlapping substrate specificities with P-glycoprotein which may also relevant in clinical drug resistance. Future studies should address the influence of *H. pylori* on BCRP2, along with other protein transporters in human intestines, as well as implication of polymorphism in the protein gene affecting the pharmacokinetics of substrates used in *H. pylori* eradication therapy.

In this study, it was found that resistant subjects showed higher relative antral P-glycoprotein expression compared to the *H. pylori*- negative group but they expressed similar protein level with *H. pylori*-positive-treatment naive subjects. Further, in the resistant group, all patients showed an increasing trend towards elevated P-glycoprotein levels in the presence of *H. pylori* irrespective of their C3435T polymorphism. It was difficult to conclude that P-glycoprotein alone causes treatment resistance, therefore studies on other ABC transporters that also present in the intestines should be undertaken in future.

P-glycoprotein (MDR1) is polymorphic and there is strong linkage between its SNPs such as C1236T, G2677A/T and C3435T. The influences of C1236T and G2677A/T polymorphisms on drug response and disease outcome have been reported especially in

organ transplant patients. As C1236T and G2677A/T are closely related to C3435T, it would be beneficial to perform studies on the effect of other SNPs to the P-glycoprotein expression as well as *H. pylori* eradication therapy in future.

One of the postulates in this study is that early attachment of *H. pylori* to the human gastrointestinal tract may be restricted by the level of P-glycoprotein expression as demonstrated with tissue culture. However, it is known that *H. pylori* infection usually a life-long and may asymptomatic, therefore it would be beneficial to use an animal model to determine the influence of P-glycoprotein expression on bacterial attachment over a long period. Importantly, if attachment varies dependent on environmental changes and/or virulence factors which may influence P-glycoprotein expression. It would be important to use multiple strains of *H. pylori* as it can be argued that different strains of *H. pylori* and the molecular basis of the pathogen virulence may influence P-glycoprotein expression.

APPENDICES

Appendices

Appendix I: List of P-glycoprotein substrates, inducers and inhibitors

Substrate	Inducer	Inhibitor
Actinomycin D	Alfentanil	Acetylsalicylic acid
Aldosterone	Amiloride	Amiodarone
Alpha-methyldigoxin	Astemizole	Atovarstatin
Amiloride	Atovaquone	Bromocriptine
Amitriptyline	Azelastine	Carvedilol
Amoxicillin	Azidopine	Ceftriaxone
Amprenavir	Azithromycin	Cefoperazone
Atorvastatin	B-carotene	Chlorambucil
Azelastine	Bepridil	Chloropromazine
Beta-acetyldigoxin	Biricodar	Cisplatin
Bisantrene	B-carotene	Clarithromycin
Bunitrolol	Carbamazepine	Cyproheptadine
Carbamazepine	Chloroquine	Colchicine
Carvedilol	Clotrimazole	Cyclosporine
Celiprolol	Cortisol	Daunorubicin
Cetrizine	Darunavir	Delavirdine
Chloropramazine	Desethylamiodarone	Dexamethasone
Chloroquine	Desipramine	Diltiazem
Cimetidine	Dexamethasone	Doxorubicin
Ciprofloxacin	Dipyidamole	Elacridar
Citalopram	Disulfiram	Emetine
Colchicine	Doxazosin	Erythromycin
Corticosteroids	Elacridar	Etoposide
Cortisol	Emetine	Fluorouracil
Cyclosporine	Erythromycin	Fluoxetine

Substrate	Inducer	Inhibitor
Daunorubicin	Felodipine	Flupenthixol
Debrisoquine	Fenofibrate	Hydrocortisone
Dexamethasone	Fentanyl	Hydroxyurea
Digitoxin	Flavonoids	Indinavir
Digoxin	Fluphenazine	Insulin
Diltiazem	Fucidin	Itraconazole
Dipyridamole	Glyburide	Ketoconazole
Docetaxel	Gramicidin D	Lansoprazole
Domperidone	Haloperidol	Mefloquine
Doxepine	Hydrocortisone	Methadone
Doxorubicin	Kempferol	Metrotrexate
Doxycycline	Levothyroxin	Miconazole
Emetine	Lidocaine	Midazolam
Enoxacin	Loperamide	Mitoxantrone
Epirubicin	Loratadine	Morphine
Erythromycin	Lovastatin	Nelfinavir
Estradiol	Maprotiline	Nevirapine
Etoposide	Mefloquine	Nicardipine
Fexofenadine	Methadone	Nifedipine
Glyburide	Mibefradil	Omeprazole
Gramicidin	Midazolam	Paclitaxel
Grepafloxacin	Mitomycin C	Pantoprazole
Hydrocortisone	Nelfinavir	Paroxetine
Imatinib	Nicardipine	Pentazocine
Indinavir	Nitrendipine	Phenobarbital
Irinotecan	Norverapamil	Phenothiazine
Itraconazole	Ofloxacin	Phenytoin
Ivermectin	Omeprazole	Prazosin
Ketoconazole	Paclitaxel	Probenecid
Lansoprazole	Pantoprazole	Progesterone

Substrate	Inducer	Inhibitor
Levodopa	Phenobarbital	Propafenone
Levofloxacin	Phenothiazines	Propanolol
Loperamide	Phenytoin	Quinidine
Losartan	Pimozide	Quinine
Lovastatin	Piperine	Rabeprazole
Mefloquine	Probenecid	Reserpine
Methylprednisoline	Progesterone	Retinoid acid
Mibefradil	Promethazine	Rifampicin
Mithramycin	Propafenone	Ritonavir
Mitomycin C	Propanolol	Saquinavir
Mitoxantrone	Quercetin	Sertaline
Morphine	Quinidine	Sirolimus
Nelfinavir	Quinine	Spironolactone
Nicardipine	Reserpine	Tacrolimus
Nifedipine	Retinoic acid	Tamoxifen
Nizatidine	Rifampicin	Trazodone
Nortriptyline	Saquinavir	Valsopodar
Olanzapine	Setraline	Verapamil
Ondansetron	Simvastatin	Vinblastine
Paclitaxel	Sirolimus	Vincristine
Paroxetine	Spironolactone	Yohimbine
Pentazocine	St John Wort`s	-
Phenobarbital	Tacrolimus	-
Phenothiazine	Tamoxifen	-
Phenytoin	Tariquidar	-
Prednisolone	Telithromycin	-
Progesterone	Terfenadine	-
Quetiapine	Testosterone	-
Quinidine	Tetrabenzine	-
Rantidine	Tetrafenadine	-

Substrate	Inducer	Inhibitor
Rapamycin	Trifluopromazine	-
Rifampin	Troglitazone	-
Risperidone	Valinomycin	-
Ritonavir	Valspodar	-
Rhodamine 123	Vinblastine	-
Saquinavir	-	-
Sertaline	-	-
Sirolimus	-	-
Sparfloxacin	-	-
Tacrolimus	-	-
Talinolol	-	-
Tamoxifen	-	-
Taxol	-	-
Teniposide	-	-
Terfenadine	-	-
Tetracycline	-	-
Topiramate	-	-
Topotecan	-	-
Triamcinolone	-	-
Trimipramine	-	-
Valinomycin	-	-
Valspodar	-	-
Vecuronium	-	-
Venflaxine	-	-
Vinblastine	-	-
Vincristine	-	-

[Reference: 112-116]

Appendix II: Ethics approval from Sir Charles Gairdner Hospital Research Ethics Committee

Government of Western Australia
Department of Health

Ethics Ref: 2010-082
Ext 2999

Sir Charles
Gairdner Hospital

2 September 2010

Dr Hooi Ee
Gastroenterology
7th Floor g Block
Sir Charles Gairdner Hospital
Hospital Ave
NEDLANDS WA 6009

Dear Dr Ee

APPLICATION TO CONDUCT HUMAN RESEARCH AT SCGH:
TRIAL No: 2010-082
TRIAL TITLE: P-glycoprotein, Helicobacter pylori and Eubacterium lentum: Is there an inter-relationship and does this contributes to the risk of digoxin-macrolide interactions (Phase 1)

On behalf of the Sir Charles Gairdner Group Executive I give approval to conduct your research project at Sir Charles Gairdner Hospital based on the favourable reviews provided to me by the Research Governance Unit and the Sir Charles Gairdner Group Human Research Ethics Committee. This approval is granted until 30 August 2012, and on the basis of compliance with all requirements laid out in your application and with the provision of reports as required by the RGU and approving HREC in giving their favourable opinion (attached).

The responsibility for the conduct of this study remains with you as the Principal Investigator. You must notify the Research Governance Unit of any relevant issues arising during the conduct of the study that may affect continued favourable opinions by the hospital or by an HREC.

Please quote Study number 2010-082 on all correspondence associated with this study.

Yours sincerely

SUE DAVIS
Acting Executive Director of Nursing Services
Sir Charles Gairdner Group
North Metropolitan Area Health Service

Appendix III: Reciprocal ethics approval from Curtin University Human Research Ethics Committee

Curtin University

Memorandum

To	Professor Jeffery Hughes, Pharmacy
From	A/Professor Stephan Millett, Chair, Human Research Ethics Committee
Subject	Protocol Approval **HR 117/2010**
Date	15 September 2010
Copy	Dr Andrew Crowe Marhanis Salihah Omar Dr Hooi Ee, Pharmacy Graduate Studies Officer, Faculty of Pharmacy

Office of Research and Development

Human Research Ethics Committee

TELEPHONE 9266 2784
FACSIMILE 9266 3793
EMAIL hrec@curtin.edu.au

Thank you for your application submitted to the Human Research Ethics Committee (HREC) for the project titled "*P-glycoprotein, Helicobacter pylori and Eubacterium lentum: Is there an inter-relationship and does this contribute to the risk of digoxin-macrolides interactions (Phase 1).*". The Committee notes the prior approval by Sir Charles Gairdner Group Human Research Ethics Committee (Study Number: 2010-082) and has reviewed your application consistent with Chapter 5.3 of the *National Statement on Ethical Conduct in Human Research*.

- You have ethics clearance to undertake the research as stated in your proposal.
- The approval number for your project is **HR 117/2010.** *Please quote this number in any future correspondence*.
- Approval of this project is for a period of twelve months **15-09-2010** to **15-09-2011**. To renew this approval a completed Form B (attached) must be submitted before the expiry date **15-09-2011**.
- If you are a Higher Degree by Research student, data collection must not begin before your Application for Candidacy is approved by your Faculty Graduate Studies Committee.
- The following standard statement **must be** included in the information sheet to participants:

  This study has been approved by the Curtin University Human Research Ethics Committee (Approval Number HR 117/2010). The Committee is comprised of members of the public, academics, lawyers, doctors and pastoral carers. Its main role is to protect participants. If needed, verification of approval can be obtained either by writing to the Curtin University Human Research Ethics Committee, c/- Office of Research and Development, Curtin University of Technology, GPO Box U1987, Perth, 6845 or by telephoning 9266 2784 or by emailing hrec@curtin.edu.au.

Applicants should note the following:

It is the policy of the HREC to conduct random audits on a percentage of approved projects. These audits may be conducted at any time after the project starts. In cases where the HREC considers that there may be a risk of adverse events, or where participants may be especially vulnerable, the HREC may request the chief investigator to provide an outcomes report, including information on follow-up of participants.

The attached **FORM B** should be completed and returned to the Secretary, HREC, C/- Office of Research & Development:
When the project has finished, or

- If at any time during the twelve months changes/amendments occur, or
- If a serious or unexpected adverse event occurs, or
- 14 days prior to the expiry date if renewal is required.
- An application for renewal may be made with a Form B three years running, after which a new application form (Form A), providing comprehensive details, must be submitted.

Regards,

A/Professor Stephan Millett
Chair Human Research Ethics Committee

Appendix IV: Participant information sheet and consent form

Sir Charles Gairdner Hospital

PARTICIPANT INFORMATION SHEET

Relationship between drug transport protein (p-glycoprotein) and ulcer causing bacteria *Helicobacter pylori*

P-glycoprotein, *Helicobacter pylori* and *Eubacterium lentum*: Is there an inter-relationship and does this contributes to the risk of digoxin- macrolide interactions (Phase 1)

Miss Marhanis Salihah Omar, Prof Jeff Hughes, Dr. Hooi Ee

Please take time to read the following information carefully and discuss it with your friends, family and general practitioner if you wish. Ask us any question if some part of the information is not clear to you or if you would like more information. Please do this before you sign this consent form.

Contact persons:
Should you have questions about the study you may contact:

Prof Jeff Hughes, School of Pharmacy, Curtin University	9266 7367
Miss Marhanis Salihah Omar, School of Pharmacy, Curtin University	9266 2208
Dr Hooi Ee, Department of Gastroenterology/Hepatology	9346 3677

As a study participant you will be provided with a copy of the Participant Information Sheet and Consent Form for your personal records. You may decide to be in the study or not take part at all. If you do decide to take part in this study, you may stop at any time. However, before you decide, it is important that you understand why this research is being done and what it will involve.

Whatever your decision, this will not lead to any penalty or affect your regular medical care or any benefit to which you are otherwise entitled. Details of our obligations to you may be found in the "West Australian Public Patients' Hospital Charter", copies of which we can provide to you.

What is the purpose of the study?
This study aims to determine whether the presence of the ulcer causing bacteria *Helicobacter pylori* in the gut influences an individual's expression of the drug transport protein known as P-glycoprotein. This protein is very important in a range of drug interactions which may occur with some of the antibiotics (macrolides such as clarithromycin) which are used to eradicate it.

Why is this study suitable to me?
You have been selected to participate in the study because you are undergoing an endoscopy to investigate possible peptic ulcer disease.

How long will I be in this study?
The study is to be conducted over a 12 month period; however your direct involvement will be limited to time required to undertake the endoscopy.

What will happen if I decide to be in this study?
You will be given a questionnaire by the doctor on the day of the endoscopy procedure. You need to answer four (4) short questions. Please read and answer all the questions appropriately.

Before the procedure, the doctor will ask about your medical and medication history. You will be required to have 10 mL (two teaspoons) of your blood taken before the endoscopy procedure. You will undergo the endoscopy procedure as per normal practice; however four additional biopsies (small samples of tissue from your gut) will be taken for the purpose of the study. Your medical record will be screened in order to get information about final diagnosis from the endoscopy.

Where will my tissue and blood samples be stored?
All the tissue and blood samples will be stored in School of Pharmacy, Curtin University of Technology, Western Australia.

Will my tissue and blood samples be used for other genetic study without my knowledge or consent?
No. Your tissue and blood samples only will be used for genetic analysis that is directly related to this study. Your genetic material and information will not be released for other uses without your prior consent.

How long will my tissue and blood samples be stored for?
All the tissue and blood samples will be stored up to 12 months and then will be disposed according to Curtin University Biological Safety Policy 2009.

Are there any reasons I should not be in this study?
If you have any form of bleeding disorder such as hemophilia or you are taking warfarin, you will be excluded from the study.

What are the possible risk and discomfort of taking part in this study?
The major risk of an endoscopy procedure is bleeding. However the chance for you to bleed from the procedure is only 1 in 50 000 per tissue sample taken.

When you provide your blood sample, there is a potential risk of excessive bleeding or local bruising. Once the blood has been collected, the puncture site is covered to stop any bleeding.

What are the possible benefits of taking part?
Your participation may assist in finding causes of therapy failure in patients with peptic ulcers who are treated for *Helicobacter pylori* infection.

Will my taking part in this study be kept confidential?
All data collected will be treated confidentially. All data will be grouped and no reference will be made to individual participant. Once data collection is completed, your name will be removed from the questionnaire and data collection forms. A new label consists of a unique number will be used to replace your details for blood and tissue samples

collected before being transferred to Curtin University for the laboratory analysis. Similarly, the questionnaire will be labelled to match your blood and tissues samples.

At the completion of the study the de-identified records will be held in the School of Pharmacy; Curtin University of Technology a locked archive for a period of 5 years, after which time they will be destroyed. Only the investigators and the Human Research Ethics Committee will be able to see study records.

Your intervention obtained through this study will remain strictly confidential at all times. Personal data, which may be sensitive, (e.g. date of birth, medical and medication history) will be collected and processed but only for research purposes in connection with this study. The study data will be analyzed here and at other sites, but will not leave this site in a form that could allow you to be identified. The result of the research will be made available to other health professionals through health care journals or meetings, but you will not be identifiable in these communications. By taking part in this study you agree not to restrict the use of any data even if you withdraw. Your rights under any applicable data protection laws are not affected.

Will I find out the results of the study?
You may contact the researchers if you wish to know the result of the study. The results of the study are intended for publication and will be reported as a partial fulfillment of the requirements of a Doctor of Philosophy degree through Curtin University of Technology, Bentley, Western Australia.

Who has reviewed the study?
The Sir Charles Gairdner Group Hospital Human Research Ethics Committee has reviewed this study and has given its approval for the conduct of this research trial. In doing so this study conforms to the principles set out by the National Statement on Ethical Conduct in Research and according to the Good Clinical Practice Guidelines.

Sir Charles Gairdner Hospital

CONSENT FORM

Relationship between drug transport protein (p-glycoprotein) and ulcer causing bacteria *Helicobacter pylori*

P-glycoprotein, *Helicobacter pylori* and *Eubacterium lentum*: Is there an inter-relationship and does this contributes to the risk of digoxin- macrolide interactions (Phase 1)

Investigators: Miss Marhanis Salihah, Prof Jeff Hughes,Dr. Hooi Ee

Name:_____

Date of Birth: _____

1. I have been given clear information (verbal and written) about this study and have been given time to consider whether I want to take part.
2. I have been told about the possible advantages and risks of taking part in the study and I understand what I am being asked to do.
3. I have been able to have a member of my family or a friend with me while I was told about the study. I have been able to ask questions and all questions have been answered satisfactorily.
4. I know that I do not have to take part in the study and that I can withdraw at any time during the study without affecting my future medical care. My participation in the study does not affect any right to compensation, which I may have under statute or common law.
5. I agree to take part in this research study and for the data obtained to be published provided my name or other identifying information is not used.

If you are unclear about anything you have read in the Participant Information Sheet or this Consent Form, please speak to your doctor before signing this Consent Form.

Name of Participant	Signature of Participant	Date
Name of Researcher	Signature of Researcher	Date

The Sir Charles Gairdner Group Human Research Ethics Committee has granted approval for the conduct of this study. If you have any concerns about the ethics or code of practice of the study, please contact the Executive Officer of the Sir Charles Gairdner Group Human Research Ethics Committee on (08) 9346 2999.

This study has been approved by the Curtin University Human Research Ethics Committee (Approval Number HR 117/2010). The Committee is comprised of members of the public, academics, lawyers, doctors and pastoral carers. Its main role is to protect participants. If needed, verification of approval can be obtained either by writing to the Curtin University Human Research Ethics Committee, c/- Office of Research and Development, Curtin University of Technology, GPO Box U1987, Perth, 6845 or by telephoning 9266 2784 or by emailing hrec@curtin.edu.au.

Study participants are to receive a copy of the Participant Information Sheet and Consent Form for their personal record.

Journal of Digestive Diseases

Journal of Digestive Diseases 2012; 13; 414–420 doi: 10.1111/j.1751-2980.2012.00606.x

Original article

P-glycoprotein expression in *Helicobacter pylori*-positive patients: The influence of *MDR1* C3435T polymorphism

Marhanis OMAR,* Andrew CROWE,* Richard PARSONS,* Hooi EE,† Chin Yen TAY‡ & Jeffery HUGHES*

*School of Pharmacy, Curtin University, †Department of Gastroenterology & Hepatology, Sir Charles Gairdner Hospital, and ‡School of Pathology and Laboratory Medicine, University of Western Australia, Perth, Western Australia, Australia

OBJECTIVE: The aim of this study was to determine whether the presence of *Helicobacter pylori* (*H. pylori*) infection and multidrug resistance protein 1 (*MDR1*) C3435T polymorphism had an influence on P-glycoprotein (P-gp) expression in the upper gastrointestinal tract.

METHODS: A total of 76 patients who underwent upper gastroendoscopy at Sir Charles Gairdner Hospital in Western Australia from October 2010 to July 2011 were enrolled in the study. Antral and duodenal biopsies were collected for P-gp examination. Blood samples were taken and analyzed for *MDR1* C3435T polymorphism. *H. pylori* infection status was confirmed by culture and polymerase chain reaction.

RESULTS: A significant difference was found in P-gp expression between *H. pylori*-positive and *H. pylori*-negative patients ($P = 0.028$). For the *MDR1* C3435T polymorphism, the TT genotype had a significantly lower P-gp expression compared with the CC genotype in antral specimens ($P = 0.041$). The homozygous TT genotype with *H. pylori* infection was also significantly different in P-gp expression compared with *H. pylori*-negative patients ($P = 0.029$).

CONCLUSIONS: P-gp expression in the upper gastrointestinal tract is associated with *H. pylori* infection, and the TT genotype appeared to be associated with lower P-gp expression than the CC genotype in the stomach.

KEY WORDS: *Helicobacter pylori*, multidrug resistance, P-glycoprotein, peptic ulcer disease, single base polymorphism.

INTRODUCTION

The adenosine triphosphate (ATP)-binding cassette (ABC) transporter superfamily is one of the largest protein families and is essential for transporting specific substances including amino acids, sugars, inorganic ions and complex polysaccharides across the extracellular and intracellular lipid membranes.[1] ABC transporters employ the energy released from ATP hydrolysis to pump the substrate across the membrane against concentration gradients.[2] In humans there are 48 members of the ABC superfamily.[3] The ABC transporters may also be found in bacteria, yeast, protozoa, insects and animals.[4]

P-glycoprotein (P-gp), also known as multidrug resistance protein 1 (MDR1) or ABC subfamily B member 1

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Conflict of interest: none.

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(ABCB1), is a large transmembrane glycoprotein of approximately 170 kDa which is encoded by the *ABCB1* gene and belongs to a large superfamily of highly conserved ABC transporters.[5] In humans, P-gp is expressed on the luminal surface of the intestinal epithelium, renal proximal tubule, bile canalicular membrane of hepatocytes, placenta and blood–brain barrier, which acts as a defense mechanism against harmful xenobiotics.[6] A high concentration of P-gp is found on the gastrointestinal (GI) tract epithelium which actively pumps out potentially cytotoxic substances. P-gp may prolong GI absorption time due to the repeated cycles of absorption and efflux along the intestinal tract, hence it increases drug exposure to cytochrome P450 3A4 (CYP3A4) in the systemic circulation as well as removing metabolites from the body more quickly, and the metabolites generated by CYP3A4 have high affinity for P-gp.[7,8]

The role of P-gp in the GI tract as part of the xenobiotic defense could be demonstrated by *Helicobacter spp.*, as these microflora have been used to explore the pathogenenesis of bacterial-induced intestinal disorders.[9,10] Multidrug resistance gene deficient (*mdr1a*-/-) mice lacking P-gp are more susceptible to developing inflammatory bowel disease (IBD) as well as severe diarrhea and weight loss when infected with rodent helicobacter such as *Helicobacter bilis* and *Helicobacter hepaticus*. Increased inflammation will further elevate oxidative damage, leading to the changes in cell survival. The high incidence of colitis in these gene knockout mice could be prevented by antibiotic treatment; thus it has been suggested that P-gp prevents the accumulation of inflammation-inducing bacteria and bacterial products. Interestingly, a relatively high level of P-gp expression has been detected in more than half of the gastric cancer cases caused by *Helicobacter pylori* (*H. pylori*).[11]

The influence of a single base polymorphism in exon 26 (C3435T) was first described by Hoffmeyer *et al.*,[12] who found that homozygous TT genotypes were associated with lower P-gp expression in the duodenum than homozygous CC genotypes. In Japanese patients TT genotypes have been linked with a reduced risk of gastric cancer with *H. pylori* infection.[13] However, Sugimoto *et al.*[14] reported that there was no relationship between the *MDR1* C3435T polymorphism and the risk of developing *H. pylori*-related gastric cancer or peptic ulcer, and another report[15] suggested that genotypes of CT and TT increased the prevalence of *H. pylori* infection compared with the CC genotype.

The study was aimed to determine whether *H. pylori* infection had an influence on P-gp expression in the upper GI tract and to investigate the relationship between *MDR1* C3435T polymorphism and P-gp expression.

PATIENTS AND METHODS

Participants

This study was approved by the Ethics Committees of both Sir Charles Gairdner Hospital and Curtin University, Western Australia, and conformed to the provisions of the World Medical Association's Declaration of Helsinki in 1995 (as revised in Tokyo 2004). All participants who attended the Endoscopy Unit, Sir Charles Gairdner Hospital, Perth (Western Australia, Australia) for an upper gastroendoscopy from October 2010 to July 2011 were invited to participate in the study. Patients who were deemed to be in poor condition upon endoscopy by their doctors were considered ineligible to participate. All participants were enrolled after giving their informed written consents. Antral and duodenal biopsies were collected during the endoscopies and used for the determination of *H. pylori* infection and P-gp expression.

Status of *H. pylori* infection

H. pylori infection was determined by culture or polymerase chain reaction (PCR). In the upper gastroendoscopy procedure, two specimens each from the duodenum and antrum were collected by the endoscopists and immediately placed in a 2% brain-heart infusion broth with 20% glycerol and kept at −80°C. All samples were cultured for *H. pylori* and incubated at 37°C under microaerobic conditions (90% CO_2, 4% nitrous oxide, 2% hydrogen) for 5–7 days on a Columbia Blood Agar Base (Oxoid, Adelaide, South Australia, Australia) with or without *H. pylori* selective supplement (*H. pylori*-selective supplement by Oxoid Australia Code: SR0147, Adelaide, South Australia, Australia). The isolates were identified based on colony morphology, gram stain, oxidase, catalase and rapid urease test. Samples were considered to be *H. pylori* positive if the result of either the PCR or the culture was positive.

PCR analysis was carried out in a 25-μL reaction volume consisted of 1 μL of genomic DNA and a 24-μL reaction mixture containing 10× PCR buffer, 25 mmol/L $MgCl_2$, 5 mmol/L deoxyribonucleotide triphosphates (dNTP), 5 mmol/L of each

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Table 1. Conditions for two different polymerase chain reaction (PCR)

Target genes	Primer	PCR conditions
16S rRNA	F: 5′-CTGGAGAGACTAAGCCCTCC-3′ R: 5′-ATTACTGACGCTGATTGTGC-3′	95°C for 3 min, 35 cycles at 95°C for 20 s, 60°C for 20 s and 72°C for 1 min, and 72°C for 5 min
*Vac*A	F: 5′-ACAACCGTGATCATTCCAGC-3′ R: 5′-ATACGCTCCCACGTATTGC-3′	95°C for 30 s, 95°C for 18 s, 55°C for 30 s for 30 cycles, 68°C for 90 s, 68°C for 5 min

F, Forward; R, Reverse.

oligonucleotide primers and thermostat *Taq*DNA polymerase in PTC-100 Programmable Thermal Controller (MJ Research, Waltham, MA, USA). Two different sets of primers were used for PCR (Table 1). About 10 μL of amplified samples were electrophoresed on 2% agarose gel in a Tris, acetic acid and ethylenediaminetetraacetic acid (TAE buffer). The gel was stained with 30 μL GelRed Nucleic Acid Gel Stain (Biotium, Hayward, CA, USA) in 100 μL TAE buffer, and the amplified bands were visualized under ultraviolet light.

Protein analysis

Antral and duodenal biopsies prepared for protein analysis were homogenized in 300 μL of ice-cold tissue lysis buffer (0.05 mol/L Tris hydrochloride, 20% glycerol, 2 mmol/L ethylenediaminetetraacetic acid [EDTA], 0.2 mmol/L phenylmethylsulfonyl fluoride and general protease inhibitors). The concentration of protein was measured by the Lowry method. A Western blot kit (Western Breeze Chemiluminescent Kit–Anti-Mouse; Invitrogen, Carlsband, CA, USA) was used to determine the expression of P-gp with β-actin as a loading control. Caco-2 cell homogenate from our own lab was used as positive control for the detection of P-gp. The immunoblots were normalized to β-actin expression, a constitutive enterocyte-specific protein whose level of expression appears to be relatively constant across individuals. ChemiImager 4400 (Alpha Innotech [now known as ProteinSimple] San Leandro, CA, USA) was used to capture chemiluminescent signals, which were typically between 10 and 15 min exposures to detect an immunoreactive band of 170 kDa. The pixel intensity value designated as integrated density value (IDV) in spot density tools from AlphaImager 4400 can represent P-gp expression level.

MDR1 C3435T polymorphism genotype

Genomic DNA was extracted from venous blood using red cell lysis buffer (Tris 10 mmol/L, potassium chloride 10 mmol/L, EDTA 2 mmol/L, $MgCl_2$ 4 mmol/L and 2.5% Triton X-100), 1 mL of white cell lysis buffer (Tris 10 mmol/L, potassium chloride 10 mmol/L, EDTA 2 mmol/L, $MgCl_2$ 4 mmol/L and 0.75% sodium dodecyl sulfate), proteinase K 10 mg/mL and 10 mol/L ammonium acetate solution. The supernatant was decanted into 4 mL of absolute ethanol into 15 mL of absolute ethanol in a new conical tube mixed by gentle inversion until the DNA became visible as white strands or clumps. The supernatant was discarded, the pellets were washed with 1 mL of 70% ethanol and each sample was rehydrated with 100 μL Tris EDTA (TE) buffer. The DNA quantification was measured by BioSpec-Nano UV-VIS spectrophotometers (Shimadzu Corporation, Kyoto, Japan) with TE buffer (made in our own lab; Tris 0.1 mol, EDTA 1 mmol/L, pH 7.4) as a blank solution.

PCR for the *MDR1* C3435T polymorphism was carried out in 25-μL reaction volume of 1 μL of genomic DNA and 24 μL of reaction mixture containing 10× PCR buffer, 25 mmol/L $MgCl_2$, 5 mmol/L dNTP and 5 mmol/L of each oligonucleotide primer (forward: 5′-TCTTTTCAGCTGCTTGATGG-3′ and reverse: 5′-AA GGCATGTATGTTGGCCTC-3′). *Taq*-Ti Heat-Activated DNA polymerase (1000 units; Fisher Biotec, Perth, WA, Australia) was added to each reaction volume and Caco-2 cell homogenate was used as a positive control. The amplification cycles consisted of 95°C for 3 min, 35 cycles at 95°C for 20 s, 60°C for 20 s and 72°C for 1 min with a final extension at 72°C for 5 min. Enzymatic digestion with 2 U of *Mbo* I (New England BioLabs, Ipswich, MA, USA) was performed to analyze C3435T polymorphism, yielding products of the homozygous TT genotype (197 bp), the homozygous CC genotype (158 bp) and two bands for the heterozygous CT genotype (197 bp and 158 bp, respectively). After incubation overnight at 37°C, the amplification of DNA was analyzed by agarose gel electrophoresis using standard procedures in which 10 μL of amplified samples was electrophoresed on 2% agarose gel in a TAE buffer. The gel was stained with GelRed Nucleic Acid Gel Stain (30 μL in 100 μL TAE buffer; Biotium, Hayward, CA, USA) and the

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Table 2. Characteristics of the participants

	Helicobacter pylori status			*MDR1* genotypes			
	Negative ($N = 54$)	Positive ($N = 22$)	*P* value	CC ($N = 15$)	CT ($N = 38$)	TT ($N = 23$)	*P* value
Gender, *n* (%)			0.77				0.50
Male	25 (46.0)	11 (50.0)		9 (60.0)	16 (42.1)	11 (47.8)	
Female	29 (54.0)	11 (50.0)		6 (40.0)	22 (57.9)	12 (52.2)	
Use of PPI, *n* (%)			0.38†				0.88†
None	27 (50.0)	14 (63.7)		7 (46.6)	21 (55.3)	13 (56.5)	
Esomeprazole	6 (11.1)	1 (4.5)		1 (6.7)	4 (10.5)	2 (8.7)	
Lansoprazole	1 (1.9)	0		0	1 (2.6)	0	
Omeprazole	0	1 (4.5)		1 (6.7)	0	0	
Pantoprazole	20 (37.0)	6 (27.3)		6 (40.0)	12 (31.6)	8 (34.8)	

P values are calculated by χ^2 test unless otherwise specified. †Fisher's exact test. MDR, multidrug resistance; PPI, proton pump inhibitor.

amplified bands were visualized under ultraviolet light.

Statistical analysis

SAS 9.2 (SAS Institute, Cary, NC, USA) was used for all the analyses. Standard descriptive statistics (frequency and percentages for categorical variables, medians, means, standard deviation and range for variables measured on a continuous scale) were used to summarize the data of the participants. A *P* value less than 0.05 was considered to be statistically significant. Differences in these variables between *H. pylori*-positive and *H. pylori*-negative participants were examined. Differences in the distribution of the participants' variables across *MDR1* C3435T were compared using a χ^2 test. P-gp expressions were compared between specimens from the antrum and duodenum using a paired *t*-test. A regression model was used to identify the association of P-gp levels in specimens of the antrum and duodenum with *H. pylori* infection status and the *MDR1* genotype. The tables quote raw medians of the P-gp expression but *P* values, including those from the paired *t*-test, were based on the log-transformed data.

RESULTS

P-gp expression and C3435T polymorphism screening were determined in all 76 participants. Their characteristics are shown in Table 2. Of these participants, 22 (28.9%) were confirmed to be *H. pylori*-positive. A significant difference between the P-gp expressions of the antral and duodenal specimens was observed (218 *vs* 797, $P < 0.0001$; Fig. 1).

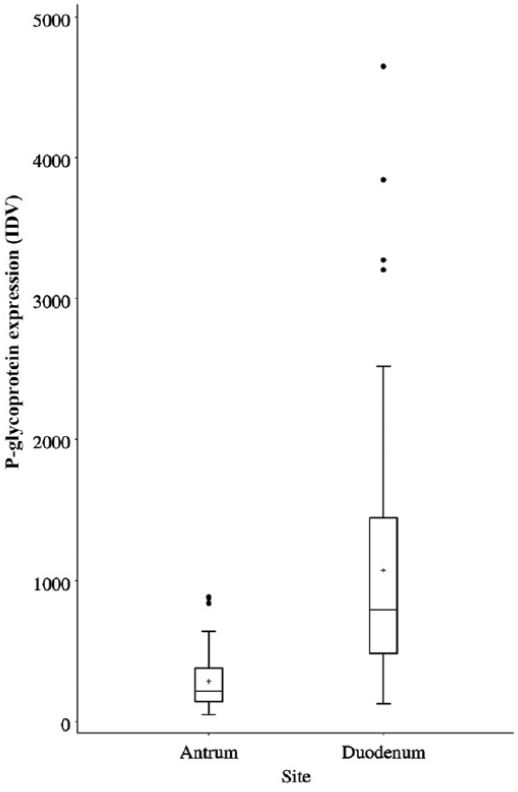

Figure 1. Comparison of P-glycoprotein expressions in antral and duodenal specimens. IDV, integrated density value.

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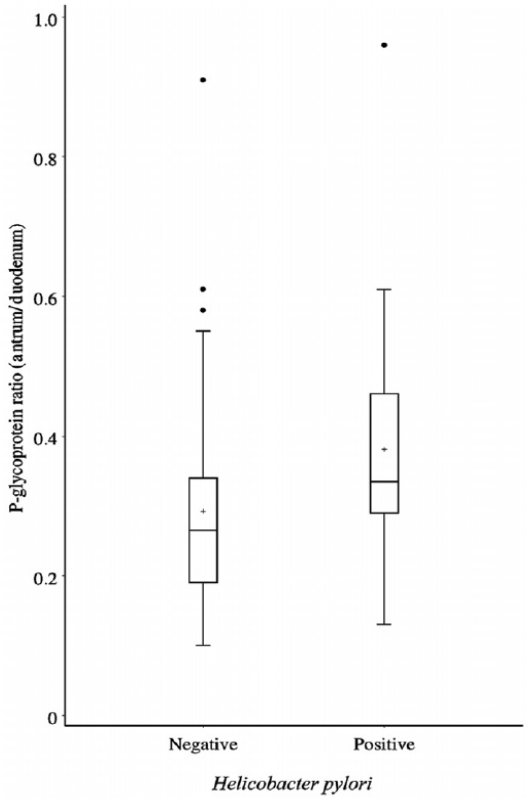

Figure 2. Comparison of P-glycoprotein expressions (antrum to duodenum ratio) in *Helicobacter pylori*-positive and *Helicobacter pylori*-negative participants.

A significant difference in P-gp expression between *H. pylori*-positive and *H. pylori*-negative participants was detected (mean ratio of antrum to duodenum 0.38 *vs* 0.29, $P = 0.028$; Fig. 2), suggesting that the expression level of antral P-gp was proportionally greater in the presence of *H. pylori*. The ratio of P-gp expression was not influenced by age, gender or proton pump inhibitor (PPI) use prior to the endoscopy ($P < 0.05$).

A regression model was used to identify the difference between *MDR1* genotype and P-gp levels of the antrum (Table 3). Heterozygous carriers were demonstrated to have an intermediate P-gp level compared with the homozygous genotypes (CC and TT), although the difference was not statistically significant ($P = 0.1049$). However, based on the paired *t*-test, the TT genotype appeared to have a significantly lower P-gp expression compared with the CC genotype ($P = 0.041$).

Figure 3 illustrates the distribution of homozygous (CC and TT) and heterozygous (CT) genotypes with *H. pylori*-positive and *H. pylori*-negative in relation to P-gp expression (antrum to duodenum ratio). There was an increasing trend in P-gp expression linked to the presence of *H. pylori* infection for all the genotypes; however, only the homozygous TT genotypes demonstrated a statistically significant difference in the P-gp expression between *H. pylori*-positive and *H. pylori*-negative groups (0.41 *vs* 0.24, $P = 0.029$).

DISCUSSION

About 50% of the general population are infected by *H. pylori*,[16] most of whom are asymptomatic. Less than 10% of infected individuals developed peptic ulcer disease, gastric cancer or mucosa-associated lymphoid tissue lymphoma. We found that P-gp expression was higher in the duodenum than the antrum ($P < 0.0001$) and this profile was in good agreement with the activity of P-gp along the intestinal tract described by other authors[17,18]. However, with the presence of *H. pylori* infection in the antral specimens, the ratio of antral to duodenal P-gp expression increased, suggesting that P-gp expression in antrum was induced by *H. pylori* infection. The increase of P-gp would suggest that cytotoxic substances released by *H. pylori* may efflux from the gastric cell mucosa, contributing to the protective role of P-gp. In addition, we also found that age or gender had no influence on P-gp level, in agreement to the previous reports,[19,20] although changes of P-gp expression in the aging population have been reported by Pilarski *et al.*[21] and Machado *et al.*[22]

P-gp plays a major role as a drug efflux transporter and influences the regulation of drug absorption, distribution and elimination.[23] PPIs such as omeprazole, lansoprazole and pantoprazole are characterized as substrates as well as inhibitors for P-gp[24] and are commonly used in the treatment of acid-related diseases, including peptic ulcer disease and gastroesophageal reflux disease. It has been reported[25] previously that patients who had high basal P-gp levels tend to fail *H. pylori* eradication. With PPIs as P-gp substrates, patients with a high P-gp expression may fail the therapy due to less drug entering the epithelial cells. In addition, participants with a chronic increase in P-gp due to the presence of *H. pylori* infection would also be more likely to exhibit the pharmacokinetic effects

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Table 3. P-glycoprotein (P-gp) expression in antrum and *MDR1* (C3435T) genotype

Site	*MDR1*	*n*	P-gp mean (SE)	Overall *P* value	Pairwise *P* value	
					CT	TT
Antrum	CC	15	383.4 (61.4)	0.1049	0.075	0.041
	CT	38	274.3 (31.6)			0.600
	TT	23	246.7 (36.7)			

SE, standard error.

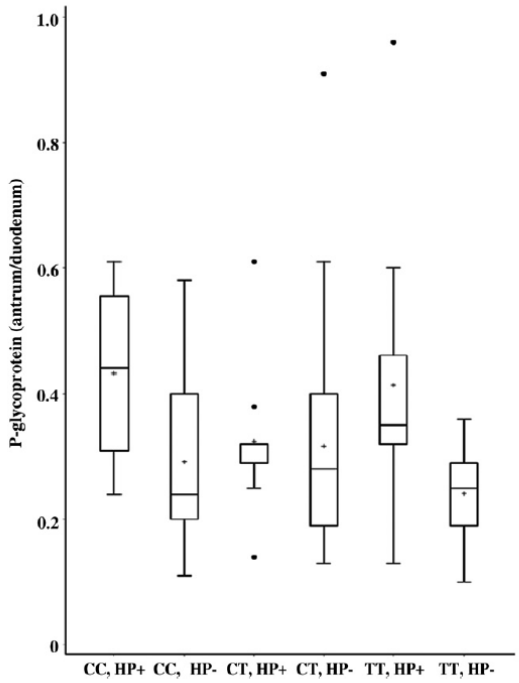

Figure 3. P-glycoprotein expression (ratio of antrum to duodenum) in *Helicobacter pylori*-positive (HP+) and *Helicobacter pylori*-negative (HP-) participants according to the genotypes (CC, TT and CT).

of other medications they may take if macrolides antibiotics are incorporated in *H. pylori* eradication, as these drugs are P-gp inhibitors.[26]

In this study, P-gp expression in the antrum was found to be the lowest in the TT genotype, followed by the CT and CC genotypes, respectively. All the genotypes showed an increased P-gp expression in the presence of *H. pylori* infection; interestingly, the difference was most marked in the TT genotype. P-gp expression was increased significantly in TT genotype only with *H. pylori* infection, and this result might explain the findings of Tahara *et al.*[13] who reported that TT genotype has lower risk of gastric cancer. Assuming that P-gp is protective against gastric cancer with the presence of *H. pylori*, this might explain the reduced prevalence of gastric cancer in the TT genotype subgroup. This proposition requires further investigation as Sugimoto *et al.*[14] showed that patients with CT genotype made up nearly 50% of the patients with gastric cancer, no significant difference was observed in gastric cancer risk across the three genotypes.

The variation of P-gp expression demonstrated in this study has a significant impact on the therapeutic efficacy of many drugs, especially those with P-gp substrates. Therefore, it would be beneficial to initiate a risk assessment study in the future to examine any association between those who are taking multiple drugs which are P-gp substrates, inhibitors or inducers and relate it to the patients' genotypes and *H. pylori* infection status.

CONCLUSION

P-gp expression is induced by *H. pylori* infection in the upper GI tract and the TT genotype appeared to have a significantly lower P-gp expression compared with the CC genotype in the stomach. TT genotype may have a great capacity to induce P-gp expression in the presence of *H. pylori*, which may explain the reduced risk in such individuals of developing gastric cancer.

ACKNOWLEDGMENT

We would like to thank Professor Barry MARSHALL from the University of Western Australia, Dr Simon FOX and Erin BOLITHO from the School of Pharmacy, Curtin University, Western Australia, Jim BLANCHARD and all staff from the Gastroenterology Unit, Sir Charles Gairdner Hospital, Perth, for their contribution to this study.

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Appendix VI: Abstracts for conferences attended

Abstract 1

Oral presentation at 7th conference on New Frontiers in Microbiology and Infection - *Helicobacter pylori* - from basic science to clinical issues. Villars sur Ollon, Switzerland [2- 6 October 2011]

Expression of P-glycoprotein in *Helicobacter pylori* positive patients

Marhanis Omar, Andrew Crowe, Hooi Ee[1], Jeff Hughes
School of Pharmacy, Curtin University, Western Australia.

Background: Proton pump inhibitor and antibiotics had successfully treats up to 90% of *Helicobacter pylori*-positive cases. However, there are therapy resistant cases reported. Since P-glycoprotein, a drug efflux transporter protein has defensive roles in drug distribution and mechanism, it was of interest to examine its possible role that contributed to successful of *H. pylori* eradication therapy. Aim: To determine whether *H. pylori* and *MDR1 C3435T* polymorphism influence P-glycoprotein expression upper gastrointestinal tract Method: 76 subjects were recruited from October 2010 to July 2011 upon their informed consent. *H. pylori* status was confirmed from culture and polymerase chain reaction. Subjects then were divided into *H.pylori*-positive and –negative groups. Their antral and duodenal biopsies were taken for Western Blot Analysis. RLFP-PCR was used to determined *MDR1 C3435T* polymorphism from blood DNA. Results: There is a significant difference between P-glycoprotein in antrum and duodenum [$p < 0.0001$] of all participants. Positive correlation was detected between P-glycoprotein expression and *H. pylori* status [$r^2 = 8.7\%$, $p = 0.0185$]. P-glycoprotein expression was found higher in subjects with *H. pylori*-positive. For *MDR1 [C3435T]* polymorphism, subjects with TT genotype had a significantly lower P-glycoprotein expression than CC genotype in their antral biopsies. Conclusion: P-glycoprotein expression is associated with the presence of *H. pylori* in upper gastrointestinal tract. TT genotype appeared to be associated with lower P-glycoprotein expression than CC genotype in stomach.

Abstract 2

Poster presentation at Keystone Symposia on Proteomics, Interactomes, Stockholm, Sweeden [7-12 May 2012]

Do *Helicobacter pylori* and *MDR1 C3435T* polymorphism influence P-glycoprotein expression in human ?

Marhanis Omar, Andrew Crowe, Jeffery Hughes
School of Pharmacy, Curtin University, Western Australia 6102 Australia

P-glycoprotein is a large transmembrane glycoprotein of approximately 170kDa that belongs to a large superfamily of highly conserved ATP-binding cassette [ABC] transporters. Various in-vivo and in-vitro studies have shown that certain gastrointestinal bacteria or drugs would inhibit or induce P-glycoprotein expression. The aim of this study was to determine if presence of *Helicobacter pylori* and multidrug resistance 1 protein *[MDR1] C3435T* polymorphism influence P-glycoprotein expression in the upper gastrointestinal tract. A total of 76 subjects who underwent an upper gastroendoscopy procedure were recruited. Antral and duodenal biopsies were collected for P-glycoprotein analysis. Blood samples were analyzed for *MDR1 [C3435T]* polymorphism. In this study, P-glycoprotein expression was found higher in subjects with *H. pylori*-positive. For *MDR1 [C3435T]* polymorphism, subjects with TT genotype had a significantly lower P-glycoprotein expression than CC genotype in their antral biopsies. The homozygous TT subjects with *H. pylori* also demonstrated a significant difference in their P-glycoprotein expression compared to *H pylori*–negative subjects. As for conclusion, the level of P-glycoprotein expression in the upper gastrointestinal tract is associated with *H. pylori* infection and TT genotype appeared to be associated with lower P-glycoprotein expression than CC genotype in stomach.

Abstract 3

Poster presentation at Joint ASCEPT-APSA 2012 Conference, Sydney Australia [2-5 December 2012].

P-glycoprotein expression level in treatment - resistant *Helicobacter pylori* patients

Marhanis S Omar, Andrew Crowe & Jeffery Hughes. School of Pharmacy, Curtin University

Introduction. There appears to be an increasing incidence of *Helicobacter pylori* becoming more resistant to antibiotic therapy, which is resulting in a reduction in complete *H. pylori* eradication in patients.Aims. We aimed to assess the P-glycoprotein expression levels among subjects who were *H. pylori*-positive and received multiple courses of eradication therapy [resistant group] to determine whether the presence of *H. pylori* increased the expression of this efflux protein. The profile of the *MDR1 C3435T* polymorphism also been investigated. Methods. Eleven subjects were recruited for this study during their hospital visit for upper gastrointestinal examinations. *H. pylori* infection status was confirmed by rapid urease test and bacterial culture. Antibiotic sensitivity testing was performed by E-test. P-glycoprotein expressions from the antral and duodenal biopsies were measured by Western Blot. Genotyping for *MDR1 C3435T* of each resistant subject was performed using polymerase chain reaction and restriction fragment length polymorphism analysis. The data was compared with two other groups, recruited from our previous study, namely *H. pylori*-negative [n = 54] and *H. pylori*-positive but treatment naive [n = 22]. Results. The resistant group did show higher P-glycoprotein expression levels [antrum over duodenum ratio] compared to the *H. pylori*- negative group [p = 0.0361]. The levels of P-glycorprotein expression in the resistant group was observed to be similar to *H. pylori*-positive but treatment naive group [p=0.319]. In the resistant group, all three *MDR1 C3435T* genotypes showed an increasing trend of P-glycoprotein expression with the presence of *H.pylori*. Most subjects demonstrated resistance to clarithromycin [72%], metronidazole [63.6%] or both [54.5%]. Discussion. *H. pylori* infection induces the expression of P-glycoprotein in antrum. Increasing P-glycoprotein at the gut level may assist antibiotic therapy for *H. pylori* if the drug regime chosen consisted of P-glycoprotein substrate due to the increased duration and drug levels outside the cells where the bacteria resides.

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