Study Title: Surveillance of antibiotic resistance, treatment effectiveness, treatment side effects, and bacterial genotypic/phenotypic analyses on clinical *Helicobacter pylori* isolates

STUDY PROTOCOL

Trial No.: 2013-007

Principle investigator: Dr Barry J Marshall
G75 Gastroenterology and Hepatology Department
G Block
Sir Charles Gairdner Hospital
Hospital Avenue
Nedlands 6009
Western Australia

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PROTOCOL SYNOPSIS

Title:	Surveillance of antibiotic resistance, treatment effectiveness, treatment side effects, and bacterial genotypic/phenotypic analyses on clinical *Helicobacter pylori* isolates
Trial Number:	2013-007
Number of Patients:	400 consecutive non-randomised patients and 1051 participants from previous study (Trial no. 99-026)
Population:	Male or female, ages 18-75 years, who have generally failed multiple *H. pylori* treatments prior to presenting at our clinic.
Objectives:	1. Continue monitoring the antibiotic resistance profile of *H. pylori* isolated from each patient. 2. Continue monitoring the treatment side-effects and effectiveness. 3. To fully characterise, both genotypically and phenotypically, isolates taken from patients who have failed multiple triple therapy treatments. • identify mutations contributing to antibiotic resistance. • identify novel genomic characteristics harboured by Australian Clinical *H. pylori* isolates. • identify ubiquitous genomic information across all sequenced isolates. 4. Detect presence of other *Helicobacter* species, eg *H. suis, H. heilmannii,* and *H. felis, etc*, isolated from patients using genome sequencing technology.
Biological Samples:	Three small stomach biopsies will be requested from the gastroenterologist during the endoscopy examination. In rare cases, patients may be asked to provide a 10 ml aliquot of blood, 10ml of gastric juice, 2ml of saliva, 10ml of urine and a stool specimen.
Study Centers:	Sir Charles Gairdner Hospital, Perth

Background

Helicobacter pylori has infected half the world's population and its infection is associated with several gastric diseases, including non-cardia gastric adenocarcinoma, gastric lymphoma, and peptic ulceration. Although the incidence of gastric cancer has declined in developed countries, it remains one of the most common types of cancer worldwide [1-3]. The prevalence of *H. pylori* infection varies significantly between developed (10-30%) and developing countries (70-90%) [4]. In general, *H. pylori* prevalence is higher in lower socio-economic groups, institutionalised individuals and those who have migrated from the developing world [5,6]. *H. pylori* is a fast mutating organism and according to Multi-locus sequence typing (MLST) database, almost every *H. pylori* strain, even among the same family, is unique. Such high allelic diversity is probably due to the high rate of intraspecies genetic recombination, and a long evolutionary history of the species [7]. In addition, the natural competence property of *H. pylori*, that allow the organism to uptake similar DNA from the environment, contributed to its diversity and potentially aid its resistance to antibiotics.

Antibiotic treatment and eradication of *H. pylori*, is complex, usually involving multiple antibiotics and a proton pump inhibitor (PPI). Treatment failure is generally attributed to lack of compliance with the drug regimen or infection by antibiotic resistant strains [8]. In Australia the prevalence of *H. pylori* was reported to be between 25-35% [9,10] and the rate of failure to eradicate *H. pylori* has recently gradually increased [11-13]. In addition, there has been a significant increase in the number of migrants coming to Australia from the neighbouring developing countries [14]. It is therefore reasonable to expect an increase in the number of *H. pylori* cases in Australia.

The current first line triple therapy, recommended by the first Maastricht consensus report [15], consists of a PPI, amoxicillin (AMX) and clarithromycin (CLR) or metronidazole (MTZ) and has generally been adopted worldwide. The current efficacy of this treatment is almost 80%, however due to the increase in prevalence of CLR resistance, the failure rate of this treatment can be as high as 30% [16]. Prevalence of bacterial resistance varies in different geographical areas, and it has been shown to correlate with the consumption of antibiotics in the general population [12,17]. For example, careful use of macrolide antibiotics in Northern European countries resulted in a reduced rate of *H. pylori* CLR resistance as compared to Central and Southern European countries, where CLR is widely prescribed [11,13,18,19]. During the last two decades, widespread use of antibiotics, such as CLR for respiratory infections, MTZ for anaerobic bacterial infections and levofloxacin for urinary tract infections, has increased the occurrence of primary *H. pylori* resistance [11,12,17].

The current gold standard for the detection of *H. pylori* is direct detection in histological sections of the gastric biopsy and successful culture. However, even in excellent laboratories approximately 10% of the infections are not detected due to difficulties in culturing and processing histological sections of the gastric biopsy. To date, no single test is ideal in the identification of *H. pylori* because of the length of time required to perform the test, lack of sensitivity, and/or reproducibility.[1] In addition, despite of the high cure rate in personalised treatment regimes reported in our recent publication (Tay *et al*, 2012), a handful of patients remain uncured. It is not known whether patients are re-infected with the same strain that has acquired antibiotic resistance or whether, post-treatment, the patient has acquired the strain from the environment or family. Therefore, there is a need to continue monitoring the resistance pattern in *H. pylori*, using genomic

tools to understand the genotype of these non-culturable and multi-drug resistant *H. pylori*, and develop other, non-endoscopic, tests to overcome the problem of the invasive nature of the collection of gastric biopsy specimens. Using the blood, saliva, urine or stool specimens of the patient to detect *H. pylori*. Ongoing developments in Next Generation Sequencing (NGS) technologies are likely to become the diagnosis and monitoring of all pathogens, including viruses, bacteria, fungi and parasites. The genome sequence of an isolate, theoretically, contains all, or nearly all, of the information required to direct treatment and to inform public health measures. With the dramatic reduced cost in genome sequencing, it is becoming possible to sequence every *H. pylori* isolated from patients.

STUDY OBJECTIVE:

1. To determine the antibiotic resistance profile of *H. pylori* isolated from each patient:

   Failure of *H. pylori* treatment is mainly due to antibiotic resistance. It is therefore important to continually monitor the resistance profile in Western Australia. The bacterial culture will be isolated from the biopsies provided by the gastroenterologist from the hospital. Antibiotic sensitivity testing will be performed using e-Test (Biodisk) for Amoxicillin (AMX), Clarithromycin (CLR), Metronidazole (MTZ), Tetracycline (TET), Rifampicin (RIF), and Ciprofloxacin (CIP). Resistance will be defined according to National Committee for Clinical Laboratory Standards (NCCLS) 20: AMX, minimum inhibition concentration (MIC) $\geq$ 2 μg/ml; CLR, MIC $\geq$ 1 μg/ml; MTZ, MIC $\geq$ 8 μg/ml; TET, MIC $\geq$ 1 μg/ml; RIF, MIC $\geq$ 4 μg/ml; and CIP, MIC $\geq$ 1 μg/ml.

2. To fully characterise, both genotypically and phenotypically, isolates taken from patients who have failed multiple triple therapy treatments:

   This aspect of the study intends to sequence and analyse as many *H. pylori* genomes as possible. The analysis of *H. pylori* genomes will aid the understanding of the pathogenicity of the organism, such as the association of the genotype with different disease outcomes. Identify novel genes or mutations responsible for antibiotic resistance. It is possible to use the genomic information to identify the key mutations that directly contribute to the antibiotic resistance. Strain-to-strain relationships can also be inferred. These data will be married with phenotyping information performed on each isolate. In particular comparative differences of genes that encode outer membrane proteins and endotoxin biosynthesis proteins will be analysed.

3. To identify the presence of other *Helicobacter* species, eg. *H. suis, H. heilmannii,* and *H. felis, etc* isolated from patients with positive diagnosis but negative *H. pylori* bacterial culture:

   According to our culturing experience, there are about 1-5% of biopsies containing non-culturable *H. pylori*. *H. pylori* is the most common pathogen in the human stomach. However, other *Helicobacter* subspecies such as *H. suis* (pig), *H. heilmannii* (cat) and *H. felis* (cat) have been reported to infect humans. Therefore, this study intends to utilise the whole genome sequencing technology to aid the identification of such infection.

4. To identify novel genomic characteristics harboured by Australian Clinical *H. pylori* isolates.

Our recent data suggest that there are a limited number of shared genes between all *H. pylori* isolates, however, the *pan*-genome is unlimited. By whole genome sequencing, we aim to further characterise the *pan*-genome of *H. pylori.* To achieve this, we will need access to bacterial isolates collected from previous study (Trial no. 99-026)

5. Identify ubiquitous genomic information across all sequenced isolates.
   As mentioned, *H. pylori* is a diverse organism that has a very small and limited core genome. These genes will be essential for its survival and potentially able to be used as a new drug target or development of novel diagnostic method. In addition, since majority of our participants will be carrying multi-antibiotic resistant *H. pylori*, this study will reveal common "hot spot" mutations that contribute to their resistance. To achieve this, we will need access to participants' medical records from previous study (Trial no. 99-026).

HYPOTHESES

1. Clarithromycin and Metronidazole resistance among *H. pylori* is the main reason for the failure of standard triple therapy.
   Clarithromycin and Metronidazole resistance among *H. pylori* is increasing and they are no longer recommended in Europe as the first line treatment for *H. pylori* infection. Therefore, one of the aim is to continue to monitor the antibiotic resistant rate in Western Australia by screening patients participating in our study.

2. Non-compliance is a reason for patients to fail standard triple therapy.
   H. pylori treatment usually involves multiple antibiotics and adverse side effects. Hence, it is common for patients to become non-compliant with the treatment regime which results in failing the standard triple therapy. This study will continue to survey the patient's side effect and examine its contribution to treatment failure.

3. Other closely related *Helicobacter* species adapted the human stomach.
   Based on previous study (Trial 99-026), about 1-5% of our patients' *H. pylori* infection were non-culturable. They were determined as *H. pylori* infection by positive blood and breath test. However, other *Helicobacter* spp. can also yield positive blood and breath test result. In addition, *H. heilmanii* (original host: dog/cat) infection has been frequently reported from time to time by pathologist. These other non-human *Helicobacter* spp. have shown to be difficult to culture. Thus, this study will utilize the NGS technology to aid the speciation of these non-culturable *Helicobacter* spp.

4. Genomic analysis will identify genotypic drivers of antibiotic resistance
   Gene mutation is known to be responsible to antibiotic resistance. However, not all the mutations have been reported. This study is aimed to utilise the NGS technology to expand the knowledge in antibiotic resistance by identifying all the mutations involved in antibiotic resistances.

5. Transmission of *H. pylori* in Western Australia occurs largely between families, not within families as expected.

   It has been hypothesised that *H. pylori* infection commonly occurred during early childhood and transmitted by mother. However, we have some bacterial genomic evidence that suggest that the spread of *H. pylori* may occur more frequently among a community than within a family. This hypothesis shall be tested by wider sample collection.

6. Ubiquitous pathways exist and are expressed in *H. pylori* during infection that can be utilised to develop a robust diagnostic method.

   The genomic data has allowed us to study all the genes of *H. pylori*. We expect to identify unique biochemical pathways existing in *H. pylori* and use it for the development of a more robust diagnostic method.

SIGNIFICANCE OF PROJECT

1. Complete eradication of *H. pylori* in patients at SCGH will reduce the financial burden and reduce the risk of increased rates of antibiotic resistance in *H. pylori* and other colonising bacteria:

   H. pylori treatment is complex and it can take months to eradicate. Due to antibiotic resistance and unconfirmed eradication, "reinfection" is commonly reported. This study aims to improve the treatment efficiency, compliance, and ultimately reduce the financial burden of both patients and the hospital.

2. Isolating and culturing other *Helicobacter* species from the human stomach could provide important information in understanding how *Helicobacter* evolve and cause infection:

   It is estimated that half of the worlds population is infected by *H. pylori*. The infection is associated with a wide spectrum of gastric diseases ranging from peptic ulcer to gastric carcinoma. *H. pylori* is classified as the class II pathogen and Class I carcinogen. However, the molecular mechanisms of how *H. pylori* infection leads to different disease outcomes is not yet fully understood. By sequencing the genome of clinical *H. pylori* isolates, this study will provide important information on the genetic elements required to cause *Helicobacter* induced pathogenicity.

3. Understanding specific mutations that contribute to antibiotic resistance will help reduce the diagnostic time frames:

   Since culturing *H. pylori* and applying antibiotic sensitivity testing is the only method that can accurately determine the antibiotic resistance, the process can take up to 4 weeks. Such diagnostic time can be greatly reduced by simple DNA testing. However, the targeted gene or mutation need to be known prior DNA testing. Hence, the genome sequencing data generated by this study has the potential to contribute to such development.

4. Genomic analysis of *H. pylori* may provide information on different disease causing isolates, potentially identifying more severe strains:

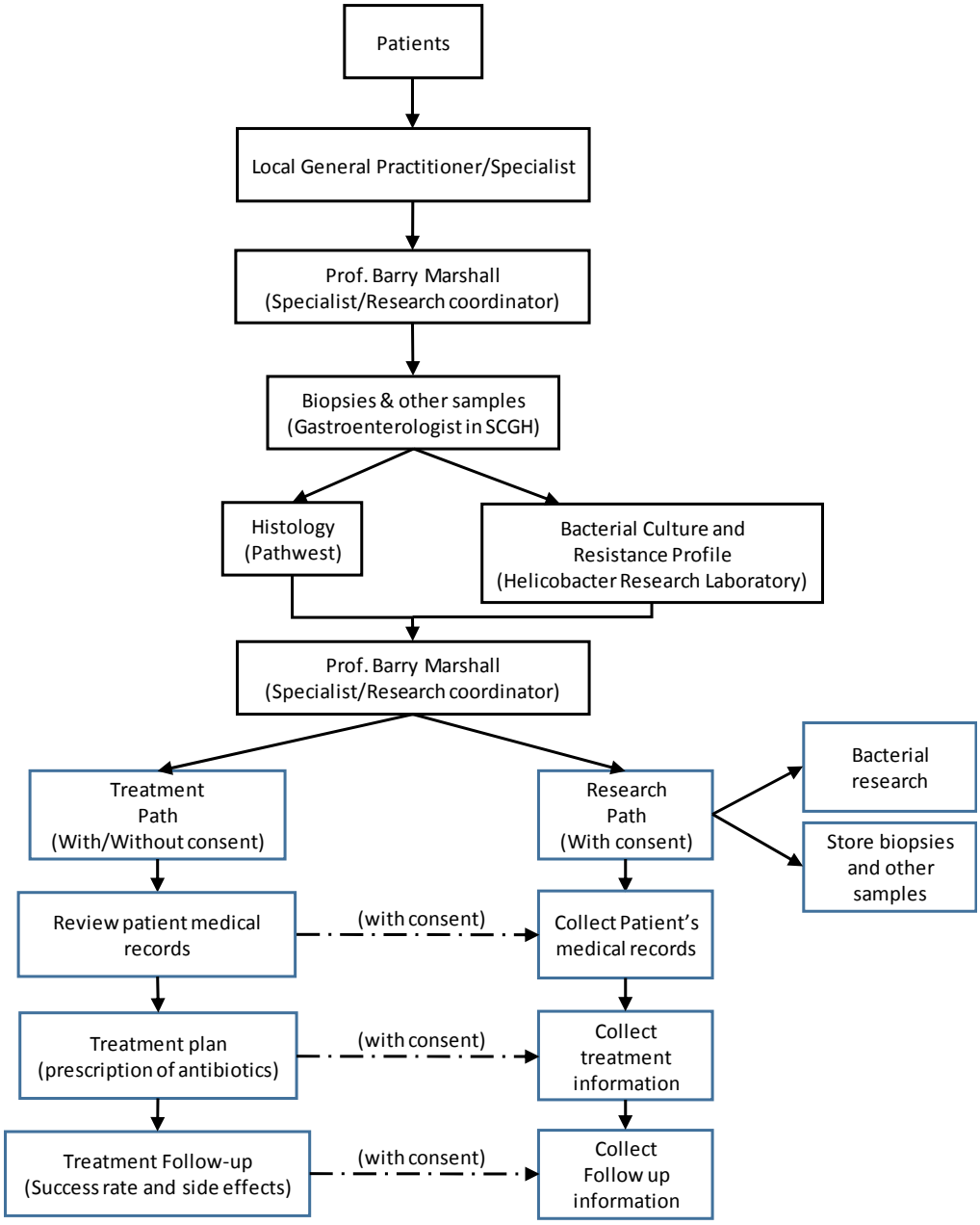
   As mentioned, not all *H. pylori* infection gives the same disease outcome. The mechanism is still not fully understood. Only a handful of

virulent genes were discussed to date. Hence, the ability to study full genome of all the clinical isolates will provide more information in determining the more severe *H. pylori* isolates.

5. Identification of environmental transmission of *H. pylori* will change the perceived wisdom on its transmission:

   According to some of our bacterial genomic studies, there is evidence that suggest the spread of *H. pylori* may have occurred more frequently among a community than within a family. This hypothesis shall be tested by collecting and analysing more samples.

PARTICIPANT ENROLLMENT FLOWCHART



STUDY PROTOCOL

Patient enrolment

Patients will be considered for this study if:

- they have failed at least one standard triple therapy regime
- are Urea Breath Test positive
- are referred from their General Practitioner

Day 1-20 -Gastro-endoscopy and culture of *H. pylori*

Patients will be required to fast for 12 hours prior to the appointment, having not taken antacid, H2 antagonists or Proton Pump Inhibitors in the preceding 24 hours (5-7 dyas). The gastro-endoscopy procedure will be performed in Sir Charles Gairdner Hospital according to the hospital standard routine procedure.
The study information and consent form will be provided and explained to the patients prior to the gastro-endoscopy procedure.
The procedure will be performed by the gastroenterologist on duty and Dr Barry Marshall.

During the gastro-endoscopy, the following tests will be performed.
6 biopsies will be collected from the patient

- 1 antrum biopsy will be used to perform the CLOtest
- 1 antrum biopsy will be taken for Pathwest to perform bacterial culture and an antibiotic sensitivity test.
- 1 antrum and 1 corpus biopsy will be taken for Pathwest to perform a histology test.
- **2 antrum and 1 corpus biopsy will be taken for the Helicobacter Research Laboratory and be used in this study.**

Helicobacter Research Laboratory will culture *H. pylori* strains from days 0-14. Further purification will be performed to remove any contaminants from days 7-21. The antibiotic sensitivities will be determined after purification from days 14-28.

Patient data collected in this study include:

- Gender
- Year of birth
- Place of birth
- street name and post code
- Ethnicity
- Disease status related to the digestive system

Day 21-59 - Reviewing of the patient's record

Based on the combined results provided by the Helicobacter Research Laboratory and Pathwest, Dr Barry Marshall will prescribe the requisite antibiotics. Patient will complete treatment. The antibiotic combinations were published in 2012 in Alimentary and pharmacology therapeutics [20].

Day 60 - Treatment follow up

After one month of antibiotic treatment, patients will required to have a urea breath test. If *H. pylori* eradication is successful, no further action will be recommended. If *H. pylori* eradication is not successful, the patient record will be reviewed again and a second line antibiotic combination will be prescribed [20]. A second endoscopy may be required depending upon the patient's symptoms. Patients will be followed up until the *H. pylori* infection is successfully eradicated.

PATIENT RECRUITMENT AND SPECIMEN COLLECTION

All patients attending Dr Barry Marshall gastrointestinal clinic for endoscopy, with permission, will be asked to be participants. Patients will normally be referred to Dr Barry Marshall gastrointestinal clinic after failing at least one (usually twice) antibiotic treatment. Prior attending the clinic, patients will have tested positive in urea breath test to show *H. pylori* infection. Patients will be consecutive attendants at the clinic and will vary in age, sex and ethnicity. Since this study is not requesting an extra endoscopy session or extra biopsies, our experience is that nearly all patients are very keen to participate. This is an intention to treat study and patients who will be followed up until proven successful eradication of *H. pylori.*

Pregnant women, children (less than 18 years old), and mentally disabled patients will not be recruited in this study. These patients will likely to be filtered out by their family doctors before referring to Dr Barry Marshall's clinic. According to the medicine handbook, some of the antibiotics used in *H. pylori* treatment may have adverse effect for children's development and other drug-drug interaction. Therefore, it will be unlikely for these patients to be recruited in this study.

Biopsies (6 Gastric Biopsies - 3 antrum and 3 corpus) will be routinely collected from the patients. Part of these biopsies will be requested from the gastroenterologist who perform the endoscopy prior sending to Pathwest for histology and bacterial culture examination. The requested biopsies will be used for bacterial culture. These samples will primarily be used for the clinical management of the patient. If any material is left, it will be stored at -80°C indefinitely in a secured freezer located in UWA, PC2 Laboratory, Helicobacter Research Laboratory. Patients may be requested to also donate 10 ml aliquot of blood, 10ml gastric juice, 2ml saliva, 10ml urine and a stool specimen. All donated biopsies, gastric juice, saliva, urine and stool samples shall belong to the research community.

RETENTION OF RECORDS

The Investigator shall maintain all the records until the patients decided to withdraw from the study.

A listing of all documents to be retained by the Investigator is found below:

- Single protocol and all dated amendments
- Documented informed consent for each patient
- Detailed medical histories for each patient
- Copies of test and examination results
- Copies of special reports on any serious adverse effect, death, or life-threatening problems

INFORMED CONSENT

Participants who are asked to participate in clinical research are entitled to choose whether or not to take part. Their decision is voluntary and they should be competent to comprehend what is involved. Their decision not to participate in the study will not affect the treatment they will be receiving.

Participants will receive adequate verbal and written information. The verbal explanation to the patient will be performed by the principal investigator or a medically qualified deputy. For non-English speakers, a translator will be provided by the hospital. The oral explanation will cover all the elements specified in the written information. The Investigator will inform the participant of aims, methods, anticipated benefits and potential hazards of the study including any discomfort it may entail. The participant will be given opportunity to clarify any points he/she does not understand and if necessary, ask for more information. Participants and investigators will be required to sign and date the Informed Consent form. It should be emphasized that the participant is at liberty to withdraw their consent to participate at any time from the study, without penalty or loss of benefits to which the patient is otherwise entitled.

Patients who are not competent to give consent (eg. Mentally disabled, highly dependent on medical care, terminal care, emergency care, intensive care, and unconscious) will not be eligible to participate. Since this study is not likely to increased understanding about, or improvements in, the care of this population, these patients will not be requested to participate in this study.

In the situation when the PCIF is sent to patients prior to their attendance, a cover letter will be provided with the information sheet, encouraging patients to contact the clinic with any queries prior to attending their appointment.

DISCLOSURE OF DATA/PUBLICATION

A. Individual patient medical information obtained as a result of this study is considered confidential and disclosure to third parties other than those noted below is prohibited:
    1. Such medical information may be given to the patients personal physician or to other appropriate medical personnel responsible for the patient's welfare.
    2. Data generated as a result of this study are to be available for inspection on request by regulatory health authorities.

B. Research findings will not be disseminated until the findings have been tested through peer review. In general, participants will not be informed about the finding outcome unless it is requested or is directly impacted by the research.

ACCESSING INFORMATION FROM PREVIOUS PARTICIPANTS

This study, is an extension of a previous study (TRIAL NO: 99-026), is also a low risk study. As explained, this study will continue monitoring the resistant profile of *H. pylori* in Western Australia and in addition, focus more on the genomics of the clinical *H. pylori* strains. In order to relate the bacterial genotype and the participants' clinical presentation, this study would need to access the medical records of participants in TRIAL 99-026. However, due to the large quantity and accessibility of records, it is not practical to obtain a new consent from all previous participants. In addition, the participants consented previously were informed that their biopsies would be stored for future studies. Therefore, it is very likely that participants would have consented if they had been asked.

A waiver of consent from previous participants are requested through HREC. This study will comply with the guideline in National Statement, Section 2.3. Private information required in this study only include, age, gender, post code, country of birth and disease outcome. This research is unlikely to adversely affect the participants. The participants will not be identified through this study. Participants will be allowed to withdraw any data or tissue they provided.

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