Evaluation of diagnostic and treatment strategies for the management of *Helicobacter pylori* infection

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By

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Summary

Helicobacter pylori infection is responsible for chronic gastritis and the development of gastrointestinal disorders such as gastric or duodenal ulcers, gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma. Patients who are infected with *H. pylori* are either diagnosed by non-invasive tests, for example the urea breath test (UBT), or invasively by means of endoscopic tests, such as the rapid urease test (RUT). Clarithromycin-based triple therapy was one of the first treatments recommended for *H. pylori* infection. However, in recent years the success rate of this treatment has fallen below the recommended 80% intention-to-treat (ITT) rate, mainly due to the emergence of antibiotic resistant strains of *H. pylori.* As a result, European guidelines recommend local surveillance of *H. pylori* antibiotic resistance to guide clinicians in their choice of therapy.

The overall aim of this study was to evaluate strategies for the management of *H. pylori* infection. Firstly, to ensure the most sensitive and specific tests are used routinely for the diagnosis of *H. pylori* in Ireland, the accuracy of non-invasive and invasive tests was assessed. Additionally, culture-based and molecular antimicrobial susceptibility testing was performed to determine the prevalence of *H. pylori* antibiotic resistance. Finally, antimicrobial susceptibility-guided tailored therapy was investigated for first-line and subsequent treatment of *H. pylori* infection.

In terms of diagnosis, the Premier Platinum HpSA Plus stool antigen test was found to be less accurate than the UBT, both for initial diagnosis and post-treatment testing in our population. It has been suggested that the delta over baseline (DOB) value in the UBT is indicative of bacterial load in the stomach and could potentially predict treatment outcome; however in our cohort of patients, it did not. The use of combined antral and corpus biopsies is recommended for culture, as it significantly improved culture rates when compared to the use of a single biopsy. The use of Dent's transport media significantly reduced contamination and increased bacterial yield. These methods could potentially improve the detection of antibiotic resistance.

Antibiotic resistance surveillance revealed that there has been a sharp increase in *H. pylori* resistance to commonly prescribed antibiotics in our centre. Specifically, primary clarithromycin and metronidazole resistance rates have increased significantly since rates were last evaluated in 2007-2009 in Ireland (36.1% and 56.9% at present by culture-based methods, respectively). A primary levofloxacin resistance rate of 16.7% was observed. In addition, resistance to amoxicillin, tetracycline and rifabutin has emerged in our patient cohort. Secondary resistance to

clarithromycin was significantly higher than primary resistance, as well as the rate of dual resistance to clarithromycin and metronidazole. There is considerable variation in resistance rates across different regions. While the rates reported in this study are high, they are similar to some recent studies conducted in other countries.

The GenoType HelicoDR assay (a PCR-based molecular method) was effective in detecting *H. pylori* in biopsy samples. However the assay displayed suboptimal sensitivity and specificity in detecting levofloxacin and clarithromycin resistance respectively. Until a more suitable molecular test has been validated, culture-based phenotypic testing should remain the method of choice for resistance surveillance and tailoring therapy in our cohort of patients. The GenoType HelicoDR assay was inadequate in assessing antibiotic resistance in stool samples from patients infected with *H. pylori.* In terms of virulence of the bacterium, it was found that less virulent strains of *H. pylori* (*CagA*-negative and *vacA* S1/M2 genotypes) are now the most prevalent in *H. pylori* strains in Ireland. There was a relationship between the less virulent strains of *H. pylori* and primary clarithromycin resistance.

During this study, guidelines were released which recommended extending the duration of *H. pylori* treatment to 14 days. Although not significant at the numbers tested, treatment duration of 14 days improved the efficacy of all treatments. The efficacy of first-line 14 day tailored treatment compared to standard triple therapy was 85.7% vs 83.3% respectively (ITT), with no statistically significant difference in efficacy observed. However, unfortunately the study did not meet the required sample size. The efficacy of second-line 14 day tailored treatment was 66.6% (ITT) and for subsequent rescue treatment (>2 failed therapies), the efficacy was 85.7% (ITT).

Based on the findings presented herein, the UBT is recommended as the gold-standard non-invasive test for both *H. pylori* diagnosis and assessment of eradication in Ireland. To maximise success of bacterial culture, combined antral and corpus biopsies and a specialised transport media should be used. Going forward, it will be vital to continue monitoring antibiotic resistance rates and success rates of treatment regimens in larger patient cohorts. If high primary clarithromycin resistance rates prevail and eradication rates are not acceptable, empirical clarithromycin-based triple therapy should no longer be used and alternatives will need to be strongly considered. Tailored therapy should be reserved for those having failed 2 previous therapies. On-going antimicrobial susceptibility testing is essential to (i) monitor resistance rates in a population and (ii) for tailored rescue treatment of *H. pylori* infection.

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Chapter 1 Introduction

1.1 *Helicobacter pylori*

Helicobacter pylori (*H. pylori*) is a gram negative bacterium that specifically colonizes the epithelium of the human stomach, in particular the gastric antrum (Figure 1.1). The bacterium was discovered in 1983 by Barry Marshall and Robin Warren, who were later awarded the Nobel Prize in Physiology and Medicine in 2005 for their discovery (1-3), *H. pylori* infects approximately 44-50% of the world's population (4, 5) and has developed adaptations in order to survive in the unique ecological niche of the stomach. One such adaptation is the production of large amounts of the enzyme urease, which hydrolyses urea to ammonia and carbon dioxide. Ammonia then protects the organism from acid damage by gastric juice (6, 7). Its major mode of transmission is unknown but possible routes of infection include either oral-oral or faecal-oral (8). *H. pylori* infection is typically acquired in childhood, within the first 5 years of life (9, 10).

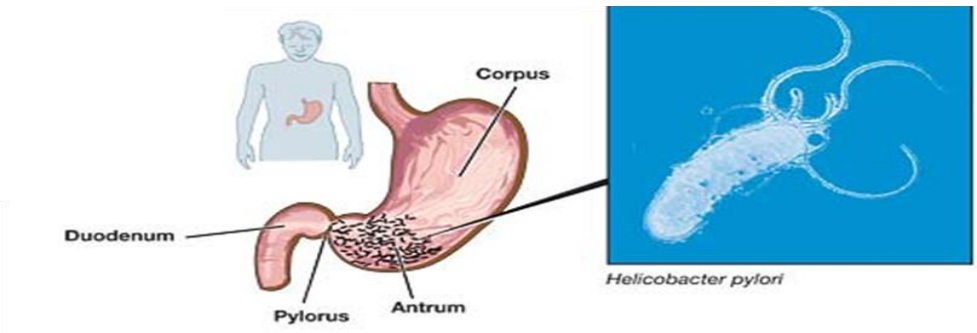


Figure 1.1: *H. pylori* is a spiral shaped flagellated bacterium (pictured right) which specifically colonizes the gastric epithelium of humans.

Most infected individuals will not develop any clinically significant complications; however *H. pylori* infection causes chronic active gastritis of varying severity in all infected subjects (11). *H. pylori* induced gastritis may cause dyspeptic symptoms in some patients (12, 13). Atrophic antral gastritis and intestinal metaplasia are also strongly associated with *H. pylori* infection (11, 14). Infection with *H. pylori* confers a 1-10% risk of developing peptic ulcer disease (PUD), which is the presence of gastric or duodenal ulcers (Figure 1.2) (4, 15). Duodenal ulcers are approximately 4 times more common than gastric ulcers (16). The estimates of incidence of gastric cancer and MALT lymphoma in *H. pylori*-infected individuals are 0.1- 3% and ≤0.01% respectively (17-19). In addition to gastrointestinal (GI) disorders, the infection is also associated with extra digestive diseases such as immune thrombocytopenic purpura (20), unexplained iron deficiency anaemia (21) and vitamin B12 deficiency (22). *H. pylori*-induced gastritis is the strongest single risk factor for cancers of the stomach (18), therefore the World Health

Organisation and the International Agency for Research on Cancer has designated *H. pylori* as a Class I carcinogen (23).

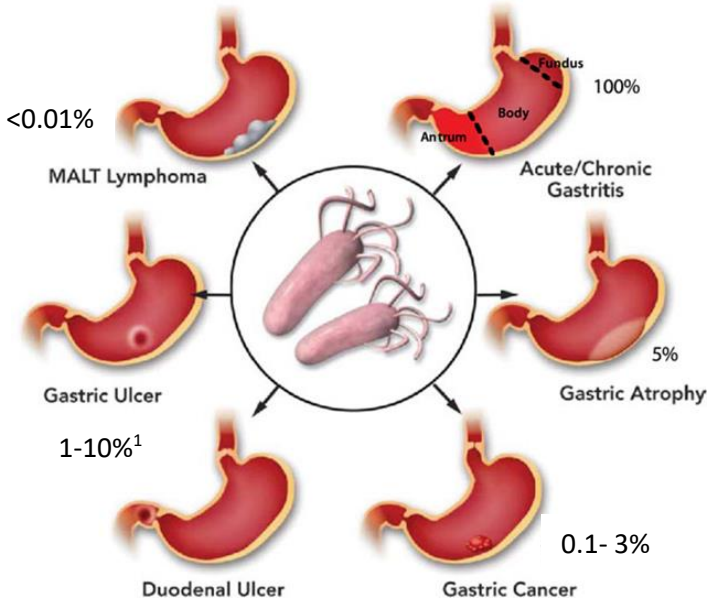


Figure 1.2: The GI disorders resulting from *H. pylori* infection. Gastritis is present in all *H. pylori* infected individuals. PUD (gastric or duodenal ulcer) is present in 1-10%, gastric cancer occurs in 0.1-3% and MALT lymphoma occurs in <0.01%. Figure adapted from Sachs et al. (24). [1]The figure of 1-10% represents incidence of both gastric and duodenal ulcer.

1.2 Prevalence of *H. pylori* infection

Zamani et al. conducted a systematic review and meta-analysis on population-based studies published from 2000-2017, to evaluate the prevalence of *H. pylori* globally (5). They reported an overall prevalence of 44.3% worldwide, ranging from 50.8% in developing countries to 34.7% in developed countries (5). They did not report on prevalence in Ireland specifically; however prevalence in the UK was 27%.

There are few published studies on the prevalence of *H. pylori* in adults in Ireland. In Northern Ireland in the 1980's, prevalence was 50.5% (25). In 1998, in the general population, Buckley et al. reported prevalence of 43% (26). A study conducted in Cork in 2006 reported a 47.1% detection rate in patients undergoing Urea breath test (UBT) (27). Finally, a study conducted in a Dublin-based Hospital in 2015 reported 22.7% prevalence in those referred for a UBT (28).

Since its discovery by Marshall and Warren in 1983 (2, 3), *H. pylori* prevalence has generally decreased in recent years in the developed world (29), however it is still high in indigenous populations and the developing world (30). Prevalence increases with older age and lower socio-economic status (4, 31), likely to be caused by poorer living conditions in previous decades (8). In a systematic review of global population-based studies, Hooi et al. reported the prevalence of *H. pylori* from 1970- 1999 and from 2000- 2016 to be 48.8% and 39.8% respectively (32). Specifically they reported prevalence of 34.2% in Western Europe in 2015 (32). Furthermore, systematic reviews reported a sharp reduction in the prevalence of *H. pylori* in European countries (33), with one study reporting that prevalence decreased by 19-28% throughout a period of 10-21 years (34). Prevalence in the Netherlands in 2013 was 48% in those born between 1935 and 1946, compared to just 16% in those born between 1977 and 1987 (35). A Polish study reported a prevalence of 35.8% in 2017, an almost two fold fall compared to previous studies (36). In Sweden, prevalence of *H. pylori* decreased from 37.9% in 1989 to 15.8% 2012 (37).

1.3 Virulence factors of *H. pylori*

High levels of mutations and recombination occur in the *H. pylori* genome and therefore the bacteria are highly heterogeneous and its virulence varies geographically. The most commonly studied virulence factors in *H. pylori* are encoded by the cytotoxin associated gene A (*cagA*) and the vacuolating associated gene A (*vacA*). They play important roles in gastric mucosal injury, such as gastric inflammation, peptic ulcer, atrophy, intestinal metaplasia, dysplasia, and malignancy (38-44). Virulence factors may play a role in determining treatment outcome (38, 45-49) and some studies have found an association with the presence of antimicrobial resistance (50).

1.3.1 CagA

CagA is a highly immunogenic protein encoded by the *cagA* gene, located at one end of the cag pathogenicity island (PAI) (51). CagA is injected into gastric epithelial cells through the type IV secretion system (T4SS) - an extracellular structure for transferring nucleic acids and proteins encoded by the cag PAI. Following injection, CagA undergoes tyrosine phosphorylation (52) and mimics a host cell protein by binding to and activating a number of inflammatory signalling pathways such as the NF-κB signalling pathway (51, 53). The presence of the *cagA* gene in *H. pylori* strains varies among different countries, and more than 90% of *H. pylori* strains are *cagA*-positive in East Asian countries (43). *CagA*-positive strains are associated with a greater inflammatory response and an increased risk of adverse clinical outcomes than in strains lacking

the *cagA* gene in Western countries (54-56). The distribution of virulence factor genotypes was investigated in Ireland in 2009 and the frequency of strains with a *cagA*-positive genotype was 68% (50). There is a well-known association between *cagA*-positive strains of *H. pylori* and PUD (38, 39).

1.3.2 VacA

The *vacA* gene is present in all *H. pylori* strains (57) and encodes the vacuolating cytotoxin, a potent toxin that is secreted by endocytosis into the extracellular space of the cells of the gastric epithelium. The toxin induces vacuolation in these cells, ultimately resulting in apoptosis (49). Specific allele variations of *vacA* exist and the distribution of these vary geographically (44). There are at least 4 variable regions in the *vacA* gene; in the signal (s) region, of which one of two alleles can be present: s1 or s2, and in the middle (m) region, of which one of two alleles can be present; m1 or m2. These cause differences in vacuolating activities among individual *H. pylori* strains (40). The *vacA* s1 and m1 genotypes produce a large amount of toxin and induce higher vacuolating activity in gastric epithelial cells, whereas s2 and m2 genotypes produce little or no toxin (40). *VacA* s2/m2 strains are reported to be rarely associated with the development of peptic ulcer and gastric cancer (58). The presence of *vacA* s1 and *vacA* m1 genotypes has been significantly associated with PUD and gastric cancer in a number of studies (40-42). In Ireland in 2009, the most dominant *vacA* type was s1/m2, followed by s1/m1 and s2/m2 (50).

1.4 Diagnosis of *H. pylori*

H. pylori infection can be detected by invasive and non-invasive means, using a variety of diagnostic tests. The appropriateness of a given test depends on patient factors such as age, symptoms and risk of gastric cancer and on population factors such as the prevalence of *H. pylori* infection.

1.4.1 Test and treat strategy

The test-and-treat strategy uses non-invasive tests to detect *H. pylori* in patients with dyspepsia and in cases where *H. pylori* is detected, appropriate treatment of the infection is carried out. A number of randomised controlled trials which compared the endoscope and treat strategy (discussed in Section 1.4.2) to the test and treat strategy found the test and treat strategy to be as effective (59-62). A meta-analysis conducted by Ford et al. found a small but significant benefit of the endoscope and treat strategy in relation to symptom improvement and patient satisfaction, however the test and treat strategy allowed a significant cost saving per patient compared to the latter (63). In light of these studies, current Irish (Irish *Helicobacter pylori*

Working Group Consensus) and European (Maastricht V/ Florence Consensus) guidelines recommend the 'Test and Treat' strategy in countries where the prevalence of *H. pylori* is at least 20% and when patients under 45 years present with uncomplicated dyspepsia with no alarm symptoms associated with an increased risk of gastric cancer (detailed in Section 1.4.2) (64, 65). As discussed, *H. pylori* prevalence rates have decreased in recent years in the developed world (5, 32, 34-37). The prevalence of an infection affects the predictive values of a given diagnostic test. As the prevalence of an infection increases, it is more likely that a patient who tests positive will truly have the infection than if the test is performed in a population with low prevalence (66, 67). For example, in a test with a positive predictive value of 60%, 60 out of every 100 positive test results will actually have disease, but 40 will not (these are false positive results). Therefore in a given population with a prevalence of disease of 25%, there are 150 false positive test results for every 1000 people tested (67). As the prevalence of *H. pylori* decreases, the 'Test and Treat' strategy must be used cautiously as the predictive values of the UBT will be affected and will become less accurate in this setting (68). The UBT, stool antigen test and serology are examples of non-invasive tests used worldwide in the diagnosis of *H. pylori* infection.

1.4.1.1 Urea breath test

The UBT is easy to perform and is used both to diagnose *H. pylori* infection and to assess eradication of *H. pylori*. To perform the test, the patient must be fasting for 12 hours. Firstly the patient ingests a test meal consisting of citric acid or fruit juice which delays gastric emptying (69). Then a solution containing ^{13}C-urea, which contains one carbon atom labelled with the non-radioactive isotope ^{13}C, is orally administered to a patient. In the presence of the *H. pylori*-produced enzyme urease, ^{13}C-urea will be hydrolysed into ammonia and $^{13}CO_2$ in the stomach. $^{13}CO_2$ is then absorbed into the circulation and exhaled by the lungs (Figure 1.3). If the patient is not infected, no labelled CO_2 is produced and most of the isotope is eliminated in the urine (69). Samples of exhaled breath are taken before administration of ^{13}C-urea and 30 minutes post-administration and are then analysed spectrophotometrically (70). A delta over baseline (DOB) value is obtained, which is the difference in the concentration of $^{13}CO_2$ in the baseline breath sample and the breath sample taken 30 minutes post ^{13}C-urea administration. The DOB value has been suggested to reflect the number of bacteria present in the stomach (71-78) and may potentially predict eradication therapy outcome (79-86), but evidence is controversial. The DOB value could potentially serve as a useful indicator to stratify patients with *H. pylori* infection (81); especially as histology and antimicrobial resistance information is unavailable in patients undergoing non-invasive testing for *H. pylori* infection. Guidelines currently recommend the use

of UBT in non-invasive diagnosis of *H. pylori* infection as well as to confirm eradication of the infection (64, 65, 87, 88). These recommendations are based on studies reporting sensitivities of 90-96% and specificities of 88-98% for the UBT (89-95). Studies have shown that the UBT has the highest accuracy compared to other non-invasive tests such as serology and stool antigen testing (91, 95). Vaira and Vakil reported that the UBT had higher overall accuracy (95% specificity and sensitivity) than serology tests (85% sensitivity and 79% specificity) and stool antigen testing (93.1% sensitivity and 92.8% specificity) (91). Additionally, the UBT is cost-effective and easy to perform (93, 95). A test-and treat strategy using the UBT is more cost-effective than a serology based strategy and empirical anti-secretory therapy (95).

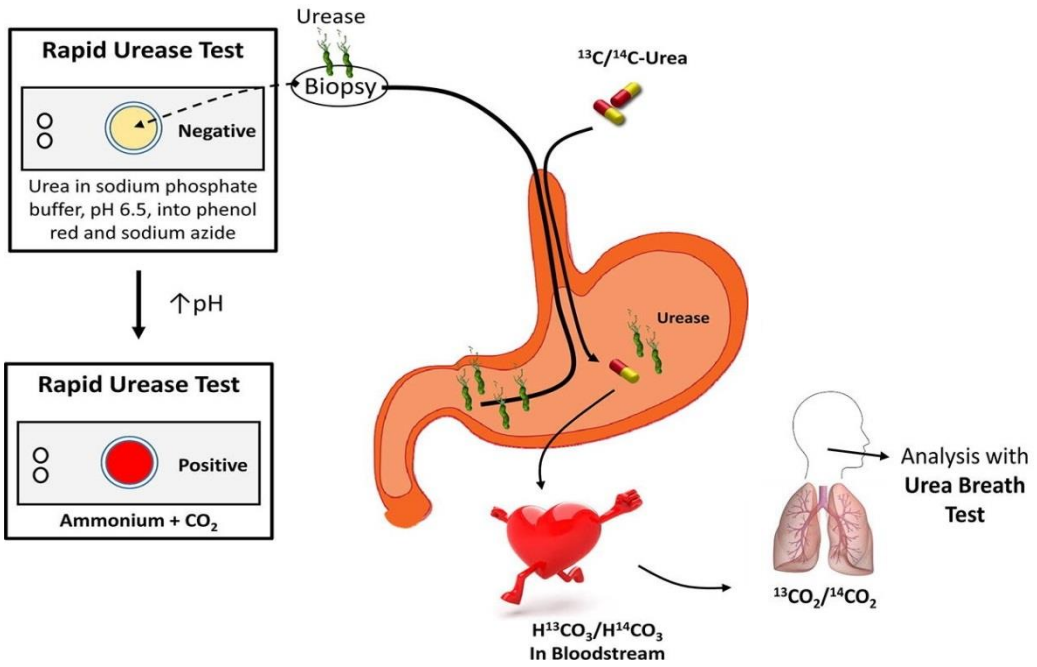


Figure 1.3: The urea breath test and rapid urease tests are based on the production of urease by *H. pylori*, which allows the organism to colonize the acidic environment of the stomach. Figure adapted from Graham and Miftahussururb (96).

1.4.1.2 Stool antigen test

Stool antigen testing involves the detection of *H. pylori* antigens in a stool sample from a patient. Stool antigen tests are typically monoclonal antibody-based enzyme immunoassays. In 2006, an Irish study compared the accuracy of three stool antigen tests- the Premier Platinum HpSA test, the IDEIA HpStAR, and the ImmunoCard STAT to the UBT for the primary diagnosis of *H. pylori* infection. They reported sensitivities ranging from 79-92% and specificities ranging from 92.5-100% (27). The Premier Platinum HpSA Plus test (Meridian Diagnostics) is the most commonly used stool antigen test. The performance of the Premier Platinum HpSA test was assessed in our

centre in 1999, with a sensitivity of 96% and specificity of 75% reported (97). Other studies have reported sensitivity and specificity values of 90-92.2% and 91-94.4%, respectively for the Premier Platinum HpSA Plus test (98, 99). The test has a simpler sampling method compared to UBT; only one stool specimen is required (instead of two breath samples) and it does not require expensive equipment. However stool antigen testing may be less acceptable in some societies (64). As such, patient compliance can be an issue and therefore the cost effectiveness of the test can be impacted (100). This assay can also be used to diagnose infection and to assess eradication following therapy. Recently, several new monoclonal rapid stool tests have appeared on the market, with reported sensitivities and specificities of 79-95% and 77.1-91.6% respectively. However none were found to be as accurate and reliable as UBT (101).

1.4.1.3 Serology

Serologic tests can also diagnose *H. pylori* infection non-invasively, by detecting IgG antibodies to *H. pylori* in the blood. As IgG antibodies persist in the blood for long periods of time, these tests are unreliable for assessing eradication of *H. pylori* (102). Irish and European guidelines recommend that only locally evaluated tests be used (64, 65), as the accuracy of the test varies according to antigenic composition of strains in different geographical areas (103). Serological testing can be useful for *H. pylori* diagnosis in patients with conditions such as GI bleeding, atrophic gastritis, MALT lymphoma and gastric cancer. The presence of these conditions can cause a low bacterial load in the stomach and as a result, with the exception of serology, the sensitivity of all other diagnostic methods is reduced (104-106). There is variability in reported sensitivity and specificity of serology (107-110). Feldman et al. compared the performance of 8 commercially available kits across 17 laboratories and reported high inter-laboratory variation and low diagnostic accuracy, with sensitivities ranging from 79.9%- 95.8% and specificities ranging from 86.5%- 98.6% (109). Serological testing for *H. pylori* infection is still widely used in the US (100).

1.4.2 Endoscope and treat strategy

The endoscope-and-treat strategy involves endoscopy, specifically an oesophago-gastro-duodenoscopy (OGD). Endoscopy allows early diagnosis of structural disease such as PUD and malignancies. Dyspepsia is a marker for the risk of these diseases (111). However it is not practical to perform endoscopy in all patients with dyspepsia. In an attempt to identify patients with dyspepsia who are at higher risk of harbouring these diseases, endoscopy is recommended in patients over 45 years who present with dyspepsia and alarm symptoms associated with an increased risk of gastric cancer (64, 65, 111, 112). Alarm symptoms include weight loss,

dysphagia, overt gastrointestinal bleeding, abdominal mass and iron-deficient anaemia (111). When an endoscopy is performed, *H. pylori* infection can be diagnosed invasively on a gastric biopsy. Commonly used tests to invasively diagnose *H. pylori* infection include histology, the rapid urease test (RUT) and culture.

1.4.2.1 Histology

Histological diagnosis of *H. pylori* is one of the most commonly used tests in countries where endoscopy is performed. Diagnosis of *H. pylori* by histology can reach a sensitivity and specificity of approximately 95% under optimal conditions (69). The histological characteristics of the gastric mucosa (inflammation, activity, atrophy, intestinal metaplasia) can also be reported. To generate reproducible and clinically useful diagnosis, the updated Sydney system of grading can be used for the classification of gastritis (113). According to this system, 2 biopsy specimens from the lesser and greater curvature of the antrum, 2 specimens from the corpus at the lesser and greater curvature and a specimen from the incisura should be obtained (113). The biopsies are formalin fixed immediately following collection and paraffin embedded upon arrival in the pathology laboratory (69). Several stains can then be used for histological detection of *H. pylori*; the Giemsa stain is most popular as it is simple to perform and results in good contrast which allows effective visualisation of the bacteria (114). Other stains include the Warthin Starry stain and immunohistochemistry (IHC). IHC is used in cases where no bacteria can be detected but chronic active gastritis is present, which can be caused by recent use of proton pump inhibitors (PPIs, see Section 1.4.4). Of all available stains used to detect *H. pylori*, IHC was found to have the highest sensitivity and specificity (115-117). However, IHC staining is more expensive than histochemical stains and centres may not have the resources to routinely perform IHC on every patient sample.

1.4.2.2 Rapid Urease Test (RUT)

The RUT has a sensitivity of 80-100% and a specificity of 97-99% (118-121); it is relatively cheap and quick to perform. A significant advantage of this test is that it provides a result before the patient is discharged from the day ward, allowing prompt treatment. The test involves placing a gastric biopsy specimen in a solution of urea and a pH indicator, e.g. phenol red. If *H. pylori* is present in the biopsy, its enzyme urease will convert the urea to ammonia. The ammonium ion increases pH, resulting in a colour change from yellow to red in this case, which indicates *H. pylori* infection (Figure 1.3). The majority of positive tests change colour after 20 minutes. After the 24 hours the test should be discarded as colour changes after this time are not reliable (96). A study found that the majority of endoscopy units read RUTs earlier than recommended, which

leads to a decrease in sensitivity (122). False-negative tests can also occur due to recent use of PPIs, antibiotics, or bismuth-containing compounds or with GI bleeding, atrophy or intestinal metaplasia (96, 104-106, 122). Therefore a negative RUT result should not be used to exclude *H. pylori* infection- a combination of histology and RUT for invasive diagnosis of *H. pylori* infection is recommended (64, 65, 87, 88). In the past, it was standard practice to use single antral biopsies in the RUT. However, it has been found that using combined antral and corpus biopsies in the RUT optimizes *H. pylori* detection and overcomes sampling error in a low prevalence population (106, 123-126).

1.4.2.3 Culture

Microbiological culture of the infecting *H. pylori* strain is also possible using a gastric biopsy. This method is particularly useful for antimicrobial susceptibility testing (Section 1.6). Culture is highly specific but sensitivity can vary, with reported positivity rates of between 55% and 93% (127, 128). Culture success depends on sampling site, transport media and growth conditions (69). This is due to the bacteria being fastidious in nature with very specific growth conditions. Bacterial culture is time-consuming and requires trained personnel. In order to maximise *H. pylori* yield, biopsy specimens should be transported in special transport media, to prevent contact with air and desiccation, and should be processed for culture as soon as possible following endoscopy, ideally within 6 hours (69). Transport media such as Dent's transport media or Portagerm pylori contain additional antibiotics to reduce contamination of oropharyngeal flora and to select for *H. pylori* growth (129-134). Biopsy specimens are streaked onto Columbia blood agar plates and incubated under microaerophilic conditions (86% N_2, 4% O_2, 5% CO_2 and 5% H_2) at 37°C for 7–10 days (69, 132, 135). Selective supplements may be added to the culture media to limit growth of contaminating flora (134). The Gram stain, a positive oxidase, urease and catalase test confirm the presence of *H. pylori* (136) as well as the growth of small, circular, smooth colonies. Once *H. pylori* reaches its growth plateau, it loses its spiral shape and becomes coccoidal and thus its viability is lost, due to lack of adequate nutrients (137). Therefore, it is recommended to subculture colonies as soon as they reach optimal size (69).

1.4.3 Molecular based methods

Molecular based methods (see also section 1.6.2) may be used to detect genes present in the *H. pylori* genome. These are of particular advantage as they also allow detection and quantification of genes associated with the pathogenesis of a particular strain of *H. pylori* (e.g. *cagA*) and

mutations which confer resistance to antibiotics (Section 1.5.1). Many studies have investigated the efficacy of these methods using biopsy specimens (138-144) and in recent years, stool specimens (145-150). Genes detected by PCR include those of the urease operon (*ureA* and *glmM*) (69) and 23S rRNA (138, 151, 152) which also allows detection of clarithromycin resistance. There are a number of commercially available assays which allow molecular detection of *H. pylori* as well as detection of mutations conferring resistance to certain antibiotics, such as the GenoType Helico DR assay (Hain Lifescience, Nehren, Germany), the ClariRes Realtime PCR assay (Ingentix, Vienna, Austria) and the RIDA®GENE *Helicobacter pylori* assay (R-Biopharm AG, Darmstadt, Germany). Molecular methods can also determine heritability and recrudescence of an infecting strain (100, 153).

1.4.4 Proton pump inhibitors and accurate diagnosis of *H. pylori*

The recent use of PPIs, antibiotics or bismuth containing compounds may jeopardize the accuracy of the UBT, stool antigen test and endoscopy-based methods. PPIs increase gastric pH which can then cause a decrease in the bacterial load of *H. pylori* and the bacteria may change from a typical morphology to a coccoidal form. This may also cause the bacteria to inhibit urease activity (154). These tests depend on the presence of a sufficient density of bacteria and false negative tests may occur whenever the bacterial load is reduced (155-158). It is recommended that PPIs should be stopped 14 days before a patient undergoes testing for *H. pylori* (64, 65). This may not be possible for many patients and 7-day withdrawal has been shown to be sufficient (159). To a lesser extent, this effect has also been observed with H2 antagonists (160). As mentioned, serological tests are the only diagnostic methods that are not affected by decreased bacterial load (158).

1.5 *H. pylori* resistance to commonly used antibiotics is increasing

A literature review conducted on studies published worldwide in 2016-2017 on antibiotic resistance noted a relentless rise in resistance, particularly to clarithromycin and levofloxacin (161). The presence of antibiotic resistance is important as it is one of the contributing factors which impact treatment outcome (162-166). A number of studies have shown that the presence of clarithromycin and metronidazole resistance can impact the efficacy of regimens that contain these antibiotics (164, 167-172). As such, local surveillance of existing and emerging antibiotic resistance is recommended and *H. pylori* therapy should be guided by local resistance patterns (64, 65, 88, 173, 174). Table 1.1 illustrates the emergence of *H. pylori* primary resistance in Europe. The most recent published study which assessed resistance rates locally in Ireland for the period 2007-2009 found a primary resistance rate to clarithromycin of 9.3%; a significant

increase on the 3.9% resistance rate found in a study done in 1997 (175). European- wide studies to assess antibacterial resistance rates of *H. pylori* were conducted in 1998 and 2008 (163, 176). In 1998, primary clarithromycin resistance was 9.9% (176), while in 2008 it was 17.5%, therefore the rate of clarithromycin resistance had almost doubled in that decade (163). In 2008, primary resistance to levofloxacin was 14.1% (163). As levofloxacin-based treatment was not used at the time, levofloxacin resistance was not investigated in 1998; however other studies have reported an emergence of levofloxacin resistance in other countries in recent years (163, 177).

Region	**Year**	**Resistance Rate (%)**			**Reference**
		CLA	**MET**	**LEV**	
Ireland	1997	3.4	35.6	ND	(178)
Ireland	2007-2009	9.3	29.1	11.7	(175, 179)
Europe	1998	9.9	33.1	ND	(176)
Europe	2008	17.5	34.9	14.1	(163)
Northern Europe	1998	4.2	33	ND	(176)
Northern Europe	2008	7.7	28.6	7.7	(163)
Southern Europe	1998	18.4	40.8	ND	(176)
Southern Europe	2008	21.5	29.7	13.1	(163)
England	2000	6.3	17.2	ND	(180)
England	2005	12.7	41.3	ND	
Wales	2000	5.6	13.8	ND	(180)
Wales	2005	7.5	26.3	ND	

Table 1.1: Phenotypic primary resistance rates reported in Europe ranging from 1997-2009. Northern Europe: Finland, Ireland, Lithuania, Norway, The Netherlands, UK; Austria, Belgium, France, Germany, Hungary, Poland; Southern Europe: Croatia, Greece, Italy, Portugal, Slovenia, Spain; [1] Primary resistance rates; CLA: clarithromycin; MTZ: metronidazole; LEV: levofloxacin; ND: no data presented in this study.

Another reason for the recommendation of antibiotic resistance surveillance is the variation of resistance rates observed between geographical regions. The findings of recent studies investigating *H. pylori* antibiotic resistance rates in a number of countries are illustrated in Table 1.2. Primary clarithromycin resistance ranges from 8.2% in Lithuania (181) to 22.4% in Spain (182) and 43.1% in Iran (183). Metronidazole resistance is high in most studies described and ranges from 23.2% in The Netherlands (184) to 79% in Poland (185). The impact of metronidazole resistance on success rates of *H. pylori* eradication is less substantial than clarithromycin resistance as it can be overcome by increasing the dose and duration of treatment (162, 186). Amoxicillin resistance is generally low but in The Netherlands, the Americas and the Eastern Mediterranean region, it ranges from 10-14% (169, 184). Levofloxacin resistance ranged from 13% in Austria (187) to 38.7% in Spain (182). Rifampicin resistance was 7.5% in Lithuania (181) and 44.2% in The Netherlands (184). Finally, tetracycline resistance is also generally low, with a rate of 2% in the Western Pacific and 10% in the Eastern Mediterranean regions (169).

Region	Year	AST method	Resistance Rate (%)					
			CLA	MET	AMO	LEV	RIF	TET
Americas (169)	2017	Both	10	23	10	15	ND	ND
Asia-Pacific (170)	2016	Both	17	44	3	18	ND	4
Austria (187)	2018	Genotypic	21	ND	ND	13	ND	ND
China (188)	2016	Both	28.9	63.8	3.1	28	ND	3.9
Eastern Med (169)	2017	Both	33	56	14	19	ND	10
France (189)	2018	Genotypic	36[1]	ND	ND	ND	ND	ND
Greece (190)	2018	Phenotypic	25.9	31.1	0	ND	ND	ND
Iran (183)	2016	Phenotypic	43.1[2]	73.8[2]	ND	ND	ND	ND
Italy (191)	2016	Phenotypic	35.9	40.2	ND	29.3	ND	ND
Lithuania (181)	2018	Phenotypic	8.2	32.8	0	ND	7.5	ND
Netherlands (184)	2015	Phenotypic	18.1	23.2	10.0[3]	13.0	44.2	2.3
Peru (192)	2017	Phenotypic	35.5	32.9	ND	ND	ND	ND
Poland (185)	2018	Phenotypic	32	79	ND	17	ND	ND
South Korea (193)	2018	Phenotypic	31.0	41.8	6.7	39.2[4]	ND	ND
South-east Asia (169)	2017	Both	10	51	2	30	ND	0
Spain (182)	2017	Phenotypic	22.4	27	0	38.7	33.3	0
USA (194)	2016	Genotypic	32.3[2]	ND	ND	ND	ND	ND
Western pacific (169)	2017	Both	34	47	1	22	ND	2

Table 1.2: Recent data on primary *H. pylori* antibiotic resistance rates worldwide. AST: antimicrobial susceptibility testing method; CLA: clarithromycin; MET: metronidazole; AMO: amoxicillin; LEV: levofloxacin; RIF: rifampicin/rifabutin; TET: tetracycline; Both: Resistance rates determined by both phenotypic and genotypic included; ND: No resistance data available for this

antibiotic; Eastern Med: Eastern Mediterranean; [1]Study included children; [2]Overall resistance [3]Resistance to ampicillin was investigated; [4]Resistanceto moxifloxacin was investigated.

1.5.1 Mechanisms of antimicrobial resistance

Antimicrobial resistance in *H. pylori* is mainly due to point mutations which occur within the genome (195). The mutations which confer resistance to commonly prescribed antibiotics such as clarithromycin, tetracycline, rifabutin and amoxicillin have been well characterised (196) and are outlined in Table 1.3. The upregulation of efflux pumps located in the cytoplasmic membrane of *H. pylori* is also a potential mechanism for antibiotic resistance (197).

1.5.1.1 Clarithromycin resistance

Clarithromycin is the most frequently used antibiotic to eradicate *H. pylori* infection as it is better absorbed in the gastric mucus layer and has unusual acid stability, making it more effective against *H. pylori* (198). Clarithromycin belongs to the macrolide family, binds to 23S rRNA and suppresses the activity of the bacterial ribosomal subunit, inhibiting protein synthesis (199). Single point mutations in the *H. pylori rrl* gene, which encodes the bacterial ribosomal subunit, confer resistance to clarithromycin (Figure 1.4) (200). The most common mutations are A2146C, A2146G and A2147G (Genbank Accession number NC_00915), which account for 90% of primary clarithromycin resistance in Western countries (201, 202). Other point mutations have been reported but appear to be rare and their clinical usefulness remains unclear (195). Resistance to clarithromycin could be due to previous consumption of clarithromycin for the treatment of other conditions such as respiratory tract infections (203). The molecular mechanism by which clarithromycin exerts its effects on *H. pylori* is outlined in Figure 1.4.

1.5.1.2 Metronidazole resistance

The mechanism of resistance to metronidazole is less clear, as many factors contribute to the development of metronidazole resistance (204, 205). Metronidazole must be activated before it can kill *H. pylori*. The enzyme flavodoxin is required to convert metronidazole into its active state in the bacterial cell. Metronidazole then exerts its action by generating free radicals which result in DNA damage (195). Mutations in the genes that encode the enzymes involved in this process can occur in metronidazole resistance. No point mutations have been clearly described but some have been reported. Specifically, the NADPH nitroreductase gene (*rdxA*) and/or the NADPH flavin oxoreductase gene (*frxA)* gene (206, 207), however *rdxA* mutations have also been shown to occur in susceptible strains of *H. pylori* (206).

1.5.1.3 Levofloxacin resistance

Levofloxacin belongs to the fluoroquinolone family of drugs. It exerts it's effects by inhibiting DNA gyrase, which is a topoisomerase, the enzymes which regulate unwinding of DNA (Figure 1.4) (208). The most frequent point mutations which confer quinolone resistance are at positions 87 and 91 of the *H. pylori gyrA* gene, which encodes DNA gyrase in *H. pylori* (209).

1.5.1.4 Amoxicillin resistance

Amoxicillin is a β-lactam that belongs to the penicillin family of antibiotics. It exerts its effects by binding to penicillin-binding proteins (PBPs) which inhibits bacterial wall synthesis. Resistance to amoxicillin occurs in *H. pylori* by mutations in PBP's, the most common being the *pbp-1A* gene (210). Other mutations have been described such as *pbp2, pbp3, hefC* and *hofH* (203).

1.5.1.5 Tetracycline resistance

Tetracycline exerts its effects by binding to the 30S subunit of the ribosome, resulting in impaired protein synthesis and bacterial growth (211). Resistance to tetracycline is most commonly characterised by a mutation which occurs on the 16S rRNA gene, the binding site for tetracycline (211)

1.5.1.6 Rifabutin resistance

Rifabutin binds to the β subunit of RNA polymerase, inhibiting both RNA and protein synthesis. Resistance occurs with a mutation in the *rpoB* gene which encodes RNA polymerase in *H. pylori* (212).

Antibiotic	**Mutation**	**Reference**
Clarithromycin	*rrl* gene	Hwang et al. (200)
Levofloxacin	*gyrA* gene	Moore et al. (209)
Tetracycline	16S rRNA	Glocker et al. (211)
Rifabutin	*rpoB* gene	Lawson et al. (212)
Amoxicillin	*pbp-1α* gene	Gerrits et al. (210)
Metronidazole	*rdxA* and *frxA* genes	Marais et al. (206)

Table 1.3: The most common reported mutations in the *H. pylori* genome which confer resistance to certain antibiotics.

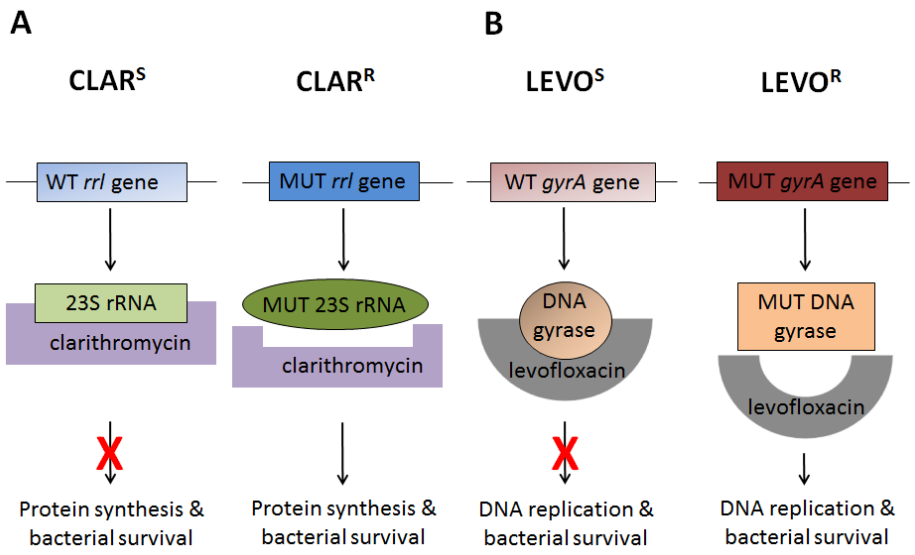


Figure 1.4: Molecular mechanism of clarithromycin and levofloxacin resistance. Point mutations in the *rrl* gene encoding a subunit of ribosomes and mutations in the *gyrA* gene encoding a subunit of the DNA gyrase enzyme confer resistance to clarithromycin and levofloxacin respectively. These mutations cause a conformational change in the proteins from which these genes are translated from and as a result the drugs cannot bind. CLAR^S: clarithromycin sensitive; CLAR^R: clarithromycin resistant; LEVO^S: Levofloxacin sensitive; LEVO^R: levofloxacin resistant; WT: wild type; MUT: mutant. Figure adapted from Smith et al., (136).

1.6 Antimicrobial susceptibility testing

H. pylori culture and susceptibility testing is useful because it allows assessment of prevalence of resistance in the population which allows clinicians to decide on a suitable empirical treatment for a given population. As mentioned, regular susceptibility testing is important as regional antibiotic resistance patterns and eradication rates should be considered when choosing first-line therapy. It also allows clinicians to tailor treatment for an individual patient, which is particularly important in refractory infection. If failure of first-line treatment of *H. pylori* occurs, there is a high likelihood that resistant *H. pylori* infection is present, in the range of 60–70% for clarithromycin (213). Susceptibility guided treatment is recommended in patients who have had more than 2 failed therapies (64, 65, 87, 88). Susceptibility testing can be carried out in two ways; via culture and phenotypic testing and molecular testing.

1.6.1 *H. pylori* culture and antimicrobial susceptibility testing

For *H. pylori* culture-based antimicrobial susceptibility testing, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (www.EUCAST.org) and the British Society for Antimicrobial Chemotherapy (http://bsac.org.uk) recommend the use of Etest strips (Biomeriux, Basingstoke, UK). These strips can quantitatively determine the minimum inhibitory concentration (MIC) of an antimicrobial agent required to inhibit growth of a bacteria. The strips have a concentration gradient of antibiotic on them and the MIC can be read directly from the strips. Results for this type of phenotypic testing can take 7-10 days to obtain and as mentioned in Section 1.4.2.3, because *H. pylori* is a fastidious bacterium, culture can be difficult. As culture rates as low as 55% have been reported (128), steps should be taken to maximise culture yield as described in Section 1.4.2.3.

1.6.2 Molecular testing

Molecular testing could be an attractive alternative to culture. A number of techniques have been described, such as PCR, restriction fragment length polymorphism (RFLP) fluorescent *in situ* hybridization (FISH) and most recently sequencing (195, 214-217). Molecular testing has been recommended to detect *H. pylori* and both clarithromycin and quinolone resistance when standard culture and sensitivity testing are not available (64, 65, 174). These techniques have the potential to detect minimal traces of *H. pylori* strains. Gastric biopsy samples can be rapidly analysed, with sensitivity results obtained 1-2 days following endoscopy. It is possible for these tools to detect hetero-resistance; the co-existence of *H. pylori* strains susceptible and resistant to the same antibiotic within the same patient (218).

Molecular based tests are available for the detection of resistance to clarithromycin and levofloxacin resistance (219). However commercial molecular-based tests to determine metronidazole and amoxicillin resistance are not yet available. Many factors contribute to the development of metronidazole resistance (204, 205). As mentioned in Section 1.5.1.2, studies have found that mutations in the *rdxA* and/or the *frxA* gene are the predominant resistance mechanisms (206, 207), however *rdxA* mutations can occur in susceptible strains of *H. pylori* (206). Additionally, development of a commercial test to detect metronidazole resistance is not considered necessary for developers of *in-vitro* diagnostic devices as metronidazole resistance can be overcome by increasing dose and duration of treatment (186, 220). Resistance to amoxicillin and rifabutin can be determined by sequencing (221).

The GenoType HelicoDR assay (Hain Lifescience, Nehren, Germany) is a multiplex PCR-based assay which enables the determination of resistance to clarithromycin and fluoroquinolones in DNA isolated from *H. pylori*. This DNA can be extracted either from bacterial cultures or biopsy material. Compared to the gold standard of culture and susceptibility testing, studies have reported sensitivity and specificity for detecting clarithromycin resistance in the range of 55-100% and 80-100% respectively; while for detecting quinolone resistance, 74-89% and 70-98.5% respectively (138, 140, 151, 222).

Currently, *H. pylori* antibiotic resistance surveillance is based primarily on patients undergoing invasive testing by means of endoscopy. However, most patients are diagnosed by non-invasive methods such as the UBT. The efficacies of molecular assays such as the *H. pylori* ClariRes Assay (Ingenetix) and The RIDA®GENE *Helicobacter pylori* assay (R-Biopharm AG, Darmstadt, Germany) for the molecular detection of clarithromycin resistance-mediating mutations in stool samples have shown promise (145, 215, 223-227). This approach is interesting as it would potentially allow resistance surveillance on those undergoing non-invasive testing for *H. pylori.*

These molecular tests are easily standardised and give reproducible results. As molecular techniques require only the gastric biopsy specimen rather than a bacterial culture, they also provide a prompt result. However, due to genetic variation, local prevalence of mutations needs to be considered, to effectively detect the majority of mutations in a particular population. Additionally, it is unclear whether mutations observed genotypically will be translated into a phenotypic change resulting in resistance in the bacteria (138); as such, molecular susceptibility testing needs to be validated by comparison with results of the gold-standard culture-based susceptibility testing. Additionally, the impact of the presence of resistance-mediating mutations on treatment outcome requires further study in different populations.

1.7 Treatment of *H. pylori* infection

Treatment for *H. pylori* infection is recommended in all symptomatic individuals. Eradication *of H. pylori* has been shown to provide a long-term cure for gastric and duodenal ulcers (228, 229), as well as being protective against progression of gastric cancer in patients with premalignant gastric lesions (230-236) and without premalignant lesions (237). Additionally, eradication of *H. pylori* was found to initiate regression of MALT lymphoma (238-241).

1.7.1 Clarithromycin-based triple therapy

Clarithromycin-based triple therapy was recommended at the first Maastricht Consensus on the management of *H. pylori* infection in 1997 (242) and has since been widely used throughout the

world (243, 244). As described in Table 1.4, clarithromycin-based triple therapy consists of a PPI and the antibiotics clarithromycin and amoxicillin (or in the case of penicillin resistance, metronidazole). However, the success rate of this treatment has fallen below the recommended 80% intention-to-treat rate in recent years (245, 246), with eradication rates for 7 day clarithromycin-based triple therapy reported as low as 55% reported in western Europe (186).

There are several factors that contribute to *H. pylori* treatment failure including low gastric pH, impaired mucosal immunity, dormant forms of *H. pylori*, and high bacterial load (162). The main factors are considered to be poor patient compliance (247-249), and the presence of antimicrobial resistant strains of *H. pylori* (162-166, 169, 171, 172). Resistance to clarithromycin can decrease the success rate of clarithromycin-based triple therapy by up to 70% (171).

No novel treatment has been developed as an alternative to triple therapy, but a number of alternative combinations of known antibiotics or extended treatment durations have been studied (161). However, the increasing complexity of these regimens may be associated with a reduction in compliance and increased risk of side effects (247).

The efficacy of clarithromycin-based triple therapy can be improved however, with a number of meta-analyses and observational studies reporting that increasing the duration to 14 days improves the efficacy of first-line triple therapy (250-254). PPIs- which inhibit acid secretion in the stomach- are used in conjunction with antibiotics in the treatment of *H. pylori* infection, as antibiotics are more stable in a less acidic environment. Studies have shown that using high-dose, newer generations PPIs such as 40 mg esomeprazole twice daily can also improve treatment efficacy (255-257).

Most guidelines recommend that regional antibiotic resistance patterns and eradication rates should be considered when choosing first-line therapy (64, 88, 173, 174). In areas of low clarithromycin resistance (<15%), 14 day clarithromycin-based triple therapy (with newer generation high dose PPIs) is still recommended by some consensus guidelines (64, 87, 174).

1.7.2 Alternatives to clarithromycin based triple therapy

1.7.2.1 Bismuth quadruple therapy

Bismuth quadruple therapy (BQT) consists of a new generation PPI, bismuth salt, tetracycline and metronidazole for a duration of 14 days (Table 1.4). In areas of high clarithromycin resistance, the choice of therapy should be based on both the frequency of metronidazole and of dual clarithromycin and metronidazole resistance (64). Dual resistance to clarithromycin and

metronidazole >15% will impact the efficacy of all non-bismuth therapies (258); therefore bismuth quadruple therapy is the recommended first-line treatment in this setting (64, 87, 173, 174). This is because the presence of antibiotic resistance has less impact on the efficacy of BQT than others. Luther et al. compared the efficacy of first-line BQT to clarithromycin-based triple therapy (245). They reported that the success of BQT was 89.4% and 80.6% in metronidazole-sensitive and resistant strains respectively, and success of triple therapy of 90.2% and 22.2% in clarithromycin-sensitive and resistant strains. Indeed BQT is unaffected by the presence of clarithromycin resistance (246, 259) and is only slightly affected by metronidazole resistance (260). Liang et al. assessed the efficacy of BQT in those who did not respond to previous treatment and reported greater than 90% eradication in patients resistant to metronidazole, fluoroquinolones, and clarithromycin (261)

As this regimen contains four agents to be taken multiple times per day, it may be difficult for patients to comply with. Thus a single capsule formulation named Pylera® which consists of 140mg bismuth subcitrate potassium, 125mg metronidazole and 125 mg tetracycline hydrochloride (Aptalis Pharma, Houdan, France Aptalis Pharma SAS) has been developed. Pylera® has shown promise as a first-line therapy in a number of studies, with reported eradication rates of up to 97% (186, 262, 263). A very recent meta-analysis examining the effectiveness of 10-day treatment with Pylera® and a PPI reported an eradication rate of approximately 90% both in first- and second-line therapy. This was regardless of the type and dose of the PPI, in patients with clarithromycin- or metronidazole-resistant strains, and in those previously treated with clarithromycin (264).

However, bismuth quadruple therapy is a not widely available in Ireland. De-Noltab (bismuth subcitrate potassium 120mg; Astellas Pharma, Leiden, The Netherlands), is not licensed or marketed in Ireland, and is considered an Exempt Medicinal Product. Pylera is licenced but not marketed in Ireland, making it currently very difficult for pharmacies to obtain (65).

1.7.2.2 Concomitant therapy

Concomitant therapy consists of a PPI and the antibiotics clarithromycin, amoxicillin and metronidazole. In contrast to sequential therapy (detailed in Section 1.7.2.7), as all three antibiotics are taken concomitantly, the complexity of the regimen is reduced (265). In regions with high clarithromycin resistance (15–40%) but low metronidazole resistance (<40%), non-bismuth therapy for a duration of 14 days should be used. Currently, concomitant therapy is recommended by some guidelines (64, 173, 174). A number of studies have shown this regimen to be the effective, with eradication rates of between 82-90% reported (265-270). Meta-analysis

studies have reported that the efficacy of concomitant therapy is duration dependent- with longer durations of 7-10 days showing better eradication (265, 271). Concomitant therapy has been shown to be the most effective non-bismuth therapy to overcome antibiotic resistance. It performs well in the presence of clarithromycin, metronidazole and dual resistance (272-274), as the other antibiotics in the regimen will cure the infection (275).

1.7.2.3 Dual therapy

High Dose Dual Therapy (HDDT) consists of higher dosages of amoxicillin (750 mg four times daily compared to standard 1g twice daily) and PPI (high dose, three times daily). Dual therapy has been recommended as an alternative treatment regimen in areas where there is high dual clarithromycin and metronidazole resistance (>15%) and when bismuth is not available (64). Studies in Asia have reported good eradication rates for HDDT, with Sugimoto et al. reporting 90% eradication in metronidazole resistant infection (276) and Yang et al. reporting 95% and 89% eradication in treatment naïve and previously treated patients respectively (277). A very recent study examining the efficacy of 14 day HDDT achieved an eradication rate of 91.7% and 95.7% by ITT and PP analysis respectively (278). Another very recent meta-analysis of randomised controlled trials comparing HDDT to BQT found that both therapies can achieve similar pooled per protocol eradication rates of 88.4% and 91.5% respectively. They also reported similar adherence to the regimens but side effects were more likely with BQT (279). On the other hand, some studies have shown that HDDT had no advantage over BQT (280) and that it does not perform well in areas with high clarithromycin resistance (281).

1.7.2.4 Sequential therapy

Sequential therapy consists of a PPI and amoxicillin taken for 5 days, followed by a PPI, clarithromycin and metronidazole taken for 5 days (Table 1.4). Although sequential therapy has been recommended in some countries (87, 174), it is not recommended as a first-line regimen in Ireland or Canada (65, 173). It is greatly affected by metronidazole resistance and dual clarithromycin-metronidazole resistance (88). A study conducted in this centre reported its eradication rate to be 69%, not significantly better than standard clarithromycin-based triple therapy (282). Additionally, a randomised control trial reported that although sequential therapy achieved a better eradication rate than 14-day triple therapy in those resistant to clarithromycin, overall eradication rate of sequential therapy was not superior to 14-day triple therapy (283). The complexity of sequential therapy detracts from its potential a first-line treatment option.

1.7.2.5 Hybrid therapy

Hybrid therapy combines the principles of sequential therapy and concomitant therapy, as it's similar to the first phase of sequential therapy and combines four antibiotics as in concomitant therapy. It consists of a PPI with amoxicillin for 14 days plus clarithromycin and metronidazole or equivalent for the final 7 days (284) (Table 1.4). Recent studies have been controversial in their findings on the efficacy of this treatment with one reporting a disappointing eradication rate of 77% (285) and a systematic review reporting eradication rates of up to 97% with excellent compliance (161, 286).

1.7.2.6 Levofloxacin and fluoroquinolone based therapy

With regard to second-line treatment, guidelines recommend that the treatment prescribed depends on what was given first-line and most recommend either 14 day BQT or levofloxacin based triple therapy (64, 87, 88, 173, 174). Levofloxacin-based triple therapy is recommended for second-line therapy, unless local levofloxacin resistance data indicate otherwise. A number of studies support the use of levofloxacin-based triple therapy following failure of standard clarithromycin-based triple therapy (287-289). One study reported a decline in eradication rate of levofloxacin-based triple therapy after 2012, likely due to rising rates of resistance (290). Other fluoroquinolone-based antibiotics such as moxifloxacin have been suggested as a more potent alternative to levofloxacin with the potential to overcome fluoroquinolone resistance (291-293).

1.7.2.7 Susceptibility-guided treatment

After failure of second-line treatment, treatment should be guided by susceptibility testing, either by culture or molecular methods (64, 87, 88). Tailoring an individual patient's treatment based on the antimicrobial susceptibility of their infecting *H. pylori* strain has been suggested as a way to limit development of secondary resistance. Several prospective, retrospective and meta-analysis studies have demonstrated that tailored treatment achieves a better eradication rate than empirically chosen treatments (294-303). Zhou et al. reported that in their population with high clarithromycin and metronidazole resistance (49% and 66% respectively), first-line tailored therapy achieved a significantly better eradication than triple therapy plus bismuth and concomitant therapy, with fewer side effects (297). The feasibility and cost-effectiveness of susceptibility-guided treatment is unclear however as most centres do not have the resources to carry out culture-based susceptibility testing (296, 304-306) and as discussed in Section 1.4.2.3, culture of the bacteria can be difficult. However, the use of genotypic susceptibility guided

treatment has shown promise, as results can be obtained within 24 hours and the tests can be relatively inexpensive. Papastergiou et al. investigated the efficacy of 7-day, genotypic susceptibility- guided treatment in a high-resistance setting and reported it can be highly effective (307)

1.7.2.8 Rifabutin based therapy

Rifabutin- based triple therapy consists of rifabutin, amoxicillin and a PPI. Guidelines recommend that rifabutin-based therapy should be reserved for third or subsequent treatments (65, 173, 174). Studies have demonstrated its effectiveness as a rescue strategy but its use is limited as it has potential adverse effects and is also used to treat tuberculosis (161).

Treatment	Description
Standard triple therapy	PPI, clarithromycin & amoxicillin (14 days)
Bismuth quadruple therapy	PPI, bismuth salt, metronidazole & tetracycline (14 days)
Sequential therapy	PPI & amoxicillin (5-7 days) followed by PPI, clarithromycin & metronidazole (5-7 days)
Concomitant therapy	PPI, amoxicillin, clarithromycin & metronidazole/tinidazole (3-14 days)
Hybrid therapy	PPI, amoxicillin (14 days) with clarithromycin and tinidazole (for the final 7 days)
Dual therapy	PPI and amoxicillin (7-14 days)
Levofloxacin-based triple therapy	PPI, levofloxacin, amoxicillin (14 days)
Rifabutin-based triple therapy	PPI, amoxicillin & rifabutin (7-14 days)

Table 1.4: *H. pylori* treatment regimens. Table adapted from Smith et al. (284).

1.7.2.9 The Irish *Helicobacter pylori* Working Group consensus for the diagnosis and treatment of *H. pylori* infection

The Irish *Helicobacter pylori* Working Group (IHPWG) consensus was established in 2016 with the aim to provide updated recommendations most appropriate for the treatment of *H. pylori* in adult patients in Ireland (65). The treatment decision schematic for first and second-line

treatment of *H. pylori* infection is detailed in Figure 1.5. Following completion of treatment, eradication of *H. pylori* infection should be confirmed by the UBT, stool antigen test or endoscopy.

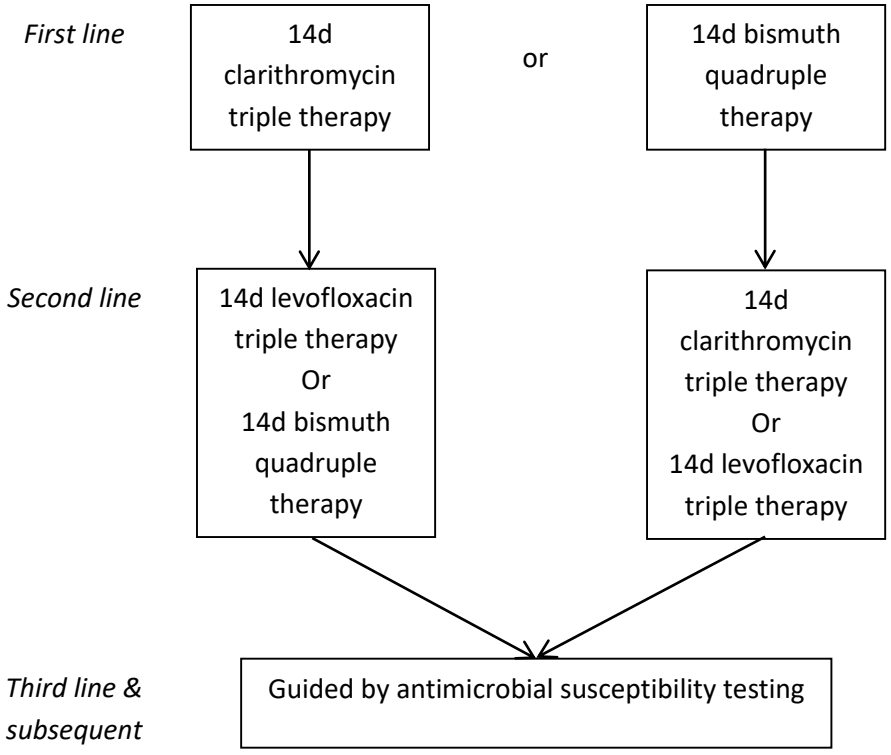


Figure 1.5: Treatment decision schematic as recommend by The Irish *Helicobacter pylori* Working Group. Figure adapted from Smith *et al.* (65)

1.8 Project aims

The overall objective of this study was to evaluate strategies for the diagnosis and treatment of *H. pylori*, with the long term goal of improving *H. pylori* eradication rates and reducing the burden of *H. pylori*-related disease. Specifically, we sought to;

1. Evaluate non-invasive and invasive tests for *H. pylori* and optimize their performance and diagnostic yield.
2. Assess primary and secondary resistance of *H. pylori* to commonly prescribed antibiotics in Ireland, by culture-based phenotypic susceptibility testing.
3. Assess the performance of the GenoType HelicoDR assay in the detection of *H. pylori* antibiotic resistance.
4. Evaluate the potential use of the GenoType HelicoDR assay in the non-invasive detection of *H. pylori* antibiotic resistance.
5. Provide an update on the prevalence of virulence factor genotypes in Irish *H. pylori* strains.
6. Assess the impact of virulence factor genotype on antibiotic resistance and treatment outcome.
7. Determine the efficacy of tailored treatment for both first-line and subsequent eradication of *H. pylori*.
8. Assess the impact of clarithromycin resistance on treatment outcome.

Chapter 2 Optimal Diagnosis of *Helicobacter pylori* Infection in Ireland

2.1 Introduction

H. pylori infection can be detected by invasive and non-invasive means, using a variety of diagnostic tests (described in detail in Section 1.4). Factors such as prevalence of infection, age and the presence of GI disease can affect the accuracy of a diagnostic test for *H. pylori* (68, 104, 106, 158, 308). Positive and negative predictive values which describe the accuracy of a diagnostic test depend on the prevalence of disease and may vary among populations (67). For example, as the prevalence of an infection increases, it is more likely that a patient who tests positive will truly have the infection than if the test is performed in a population with low prevalence (66, 67). This means that as the prevalence of *H. pylori* increases, the positive predictive value of a diagnostic test increases while the negative predictive value decreases. Indeed it has been reported that the 'Test and Treat' strategy must be used cautiously in populations with a low *H. pylori* prevalence as it becomes less accurate in this setting (68). Age has been shown to affect the accuracy of stool antigen testing (308) and the RUT, with one study reporting the sensitivity of the RUT in patients under 60 years as 75%, compared to 57% in those older than 60 years (104). The presence of certain GI conditions such as atrophy and intestinal metaplasia (104, 106) as well as the use of PPIs can reduce the sensitivity of invasive and non-invasive diagnostic tests (69, 155-158). With this in mind, it is important that local evaluation of diagnostic tests is performed regularly to ensure that the most sensitive and specific tests are used in clinical practice. In this chapter, the performance and diagnostic yield of commonly used tests for *H. pylori* infection in Ireland was evaluated.

The UBT is the current gold standard non-invasive test to diagnose infection and to assess eradication (64, 65), with high sensitivities of 90-96% and specificities of 88-98% reported (89-95). Stool antigen testing is a non-invasive test which involves the detection of *H. pylori* antigens in a stool sample from a patient. This test is an attractive alternative to UBT as it doesn't require expensive equipment and may be more cost effective (91). Studies have investigated the accuracy of stool antigen tests in Ireland (27, 97), however the most recent was published approximately 12 years ago (27). Therefore the diagnostic accuracy of the Premier Platinum HpSA Plus test compared to the UBT was evaluated in our patient cohort.

The delta over baseline (DOB) value is used to determine the outcome of the UBT. It has been suggested by a number of studies that the UBT DOB value is indicative of bacterial load in the stomach (71-78). Indeed, Perri et al. compared DOB results with histological and endoscopic findings and found that the DOB value significantly correlated with both intra-gastric bacterial

load and the severity of gastritis (74). On the other hand, some studies found no association between DOB value and bacterial density (quantified by histology and bacterial counts via culture) (309, 310). It has also been speculated that higher bacterial loads of *H. pylori* confer larger amounts of *H. pylori* antigens in the stool (311). Chang et al. found that both the UBT DOB value and HpSA test value correlated with the density of *H. pylori* and inflammatory activity (77). Based on the majority of studies reporting an association between DOB value and bacterial load (32-39), this theory that higher bacterial loads of *H. pylori* confer larger amounts of *H. pylori* antigens in the stool was investigated further in our patient cohort, by determining whether the UBT DOB value (as a possible surrogate marker for bacterial load) affects the accuracy of the Premier Platinum HpSA Plus test.

Additionally, it has been suggested that the UBT DOB value is associated with treatment outcome but the published literature on this is also controversial, with several studies finding that the DOB value can predict treatment outcome (79-84), and others disagreeing (85, 86). Eradication of *H. pylori* remains a challenge, and there are continual efforts to identify factors which predict treatment success. Therefore the association between UBT DOB values and HpSA test values and response to first line eradication of *H. pylori* infection in our patient population was investigated.

Commonly used invasive tests in Ireland include the RUT, histology and in centres with the necessary expertise, culture and molecular methods (detailed in Section 1.4.2). *H. pylori* culture and antimicrobial susceptibility testing is useful because it allows assessment of resistance to antibiotics which will allow optimum treatment of the infection. When resistance is assessed within a population, it also allows clinicians to decide on a suitable empirical treatment for that population. Indeed most guidelines recommend that regular susceptibility testing is carried out for this purpose (64, 65, 88, 173, 174). *H. pylori* is a fastidious bacterium and as a result, it can be difficult to perform bacterial culture, even by experienced personnel. While culture is highly specific, sensitivity can vary, with positivity rates of between 55% and 93% reported previously (127, 128). Culture success depends on the biopsy sampling site, transport media and growth conditions. Studies have shown that using combined antral and corpus biopsy samples improves culture success (69) and the accuracy of the RUT (106, 123, 125, 126). To optimise culture methods and improve the detection of *H. pylori* in our centre, the use of combined antral and corpus biopsies compared to single antral biopsy samples was evaluated for use in bacterial culture.

Another strategy is to use specialised transport media to store biopsy samples until processed for culture. Their purpose is to prevent contact with air and desiccation and some contain nutrients and antibiotics to reduce contamination and select for *H. pylori* growth (69, 312), for example Dent's transport media or Portagerm pylori (129-134). To optimise culture methods in our centre, the efficacies of 3 types of bacterial transport media were assessed; nutrient broth (a standard transport medium which is a commercially available liquid media used routinely in Irish Hospitals); Dent's transport medium (components detailed in Appendix 2), which is a liquid media made in-house and Portagerm pylori (Biomerieux, France), which is a commercially available semi-solid agar.

2.1.1 Aims of the study

1. To locally evaluate **non-invasive** tests and optimise their performance and diagnostic yield
    a. To prospectively evaluate the diagnostic accuracy of the Premier Platinum HpSA Plus test versus UBT for:
        i. the initial diagnosis of *H. pylori* infection and
        ii. assessing eradication of *H. pylori* infection
    b. To determine whether the DOB value of the UBT (a possible surrogate for bacterial load) impacts the accuracy of Premier Platinum HpSA Plus test
    c. To investigate whether UBT or Premier Platinum HpSA Plus test results can predict response to first line eradication of *H. pylori* infection.

2. To locally evaluate **invasive** tests and optimise their performance and diagnostic yield
    a. The use of combined biopsies for detection of *H. pylori* by culture and RUT was investigated
    b. Standard culture transport medium, Dent's Transport Medium and Portagerm pylori were assessed according to their ability to promote successful *H. pylori* culture.

2.2 Methods

2.2.1 Patient recruitment and ethics

All work related to this study was carried out in a tertiary referral teaching hospital (Tallaght University Hospital, formerly the Adelaide and Meath Hospital, incorporating the National Children's Hospital, Dublin) affiliated with Trinity College Dublin. Patients recruited to the studies outlined in this thesis attended Tallaght University Hospital (TUH) or Charlemont Medical Clinic, Dublin 2, Ireland (a private medical clinic). The study received ethical approval from the Joint Research Ethics Committee of the Adelaide and Meath Hospital, incorporating the National Children's Hospital and St James's Hospital (Ethics Committee Study Reference Number: 2013/23/04). This project received funding from the Health Research Board Ireland (HRB-HRA-2014-526). Patients over the age of 18 years who were either referred to the UBT clinic or scheduled to undergo endoscopy as a routine part of their care were invited to participate in the study. In patients referred to the UBT clinic, informed consent was obtained by the PhD candidate and in those undergoing gastroscopy, informed consent was obtained by either the PhD candidate or members of the clinical team. It was explained that the study was for research purposes and potential participants received a Patient Information Sheet and consultation prior to committing to the study. Participants were informed of their right to refuse to participate and of their right to withdraw from the study at any point. This was stated in the Patient Information Sheet and explained by the staff member seeking consent. Patients willing to participate in the study signed a consent form. Participants were given a copy of the Patient Information Sheet and signed Consent Form to take with them and an additional copy was placed in their hospital file. Patient information and consent sheets for each group of patients are contained in Appendix 1. A contact person and phone number was provided in the Patient Information Sheet, should the participant have any queries at a later stage. If a participant withdrew from the study, any identifiable information was destroyed and biological samples were disposed of appropriately.

2.2.2 Study population

Inclusion criteria for the group of patients referred for UBT and those undergoing endoscopy were (i) ability and willingness to participate in the study and to provide informed consent.
Exclusion criteria for both groups were (i) age less than 18 years, (ii) pregnancy or lactation, (iii) severe inter-current illness, (iv) recent antibiotic use (within 4 weeks) and (v) bleeding problems or use of blood thinning drugs (for endoscopy patients).

In addition to exclusion criteria listed above, for the group of patients referred for UBT, those who had been previously treated for *H. pylori* infection were excluded.

2.2.3 Non-invasive detection of *H. pylori*

2.2.3.1 ^{13}C UBT

Prior to the UBT, patients were advised to fast for at least 6 hours before the test. At time zero, the patient was given a test meal which consisted of 250 ml orange juice. To obtain two baseline breath samples, the patient was asked to exhale through a straw into two 12 ml gas collection tubes (Labco Limited, Lampeter, UK). 5 min after this the patient was administered a solution containing 75.0 mg ^{13}C urea (Pylobactell, Cambridge Healthcare Supplies Ltd.) dissolved in 30 ml water. 30 min post-dose, the patient was asked again to exhale through a straw into two 12 ml gas collection tubes. A blank sample was prepared using a breath sample from a person who had not received a dose of ^{13}C urea. All breath samples and the blank sample were analysed using a HeliFan Plus- ^{13}C Breath Test System (Fischer ANalysen Instrumente GmbH, Germany)**.** This protocol was carried out in accordance with manufacturer's guidelines. The interpretation of results is outlined in Table 2.1. Based on manufacturers suggestions of the UBT system used in this study (HeliFan Plus- ^{13}C Breath Test System, Fischer ANalysen Instrumente GmbH, Germany) (313) and validation tests performed at regular intervals by the physiologist based in the GI laboratory, a UBT DOB value of >4.0 ‰ was used as the cut-off to determine *H. pylori* positivity.

Evaluation Criteria: Delta Over Baseline (DOB)	
> 4.0 ‰	*H. pylori* positive
< 4.0 ‰	Normal

Table 2.1: Interpretation of UBT results.

2.2.3.2 Stool antigen test

Patients referred for a UBT were asked to provide a stool sample when attending their scheduled UBT. The sample was taken on the morning of or the day before the scheduled UBT and storage at 4 °C was recommended until transportation to the laboratory. The Premier Platinum HpSA Plus test (Meridian Bioscience Inc., OH, USA) was used to confirm *H. pylori* infection non-invasively in patient's stool samples. This protocol was carried out in accordance with manufacturer's guidelines (314). Firstly, 500 µl of Sample Diluent was added to a clean test tube. A small portion (6 mm diameter) of stool was transferred into the tube. The remaining

stool specimen was stored at -20 °C until required for DNA extraction. The tube was vortexed for 15 seconds. The sample was then centrifuged at 2750 x g (6000 rpm) for 5 min. The supernatant was recovered and the pellet disposed of. 100 µl of supernatant was transferred to the appropriate well in the assay plate. 100 µl of Positive Control (provided with the kit) and 100 µl Sample Diluent (negative control) was added to the appropriate wells. 50 µl of enzyme conjugate was added to each well. The plate was firmly shaken for 30 sec. The plate was sealed and incubated for 1 hour at RT. The plate sealer was removed and the wells washed 5 times using 1 X Wash Buffer. 100 µl of Premier Substrate Solution I was added to each well. The plate was firmly shaken for 30 sec and incubated for 10 min at 19-27 °C. 100 µl of Premier Stop Solution I was added to each well. The plate was shaken for 30 sec. Within 15 min, the results were then spectrophotometrically determined at 450 nm using an ELx800 Universal Microplate Reader (Bio-Tek Instruments Inc, VT, USA) The interpretation of results is outlined in Table 2.2. Patients were deemed to be infected with *H. pylori* if the absorbance value (at 450 nm) was ≥0.140.

Evaluation criteria: Absorbance value (@450 nm)	
≥0.140	*H. pylori* positive
≤0.140	Normal

Table 2.2: Interpretation of HpSA PLUS results

2.2.3.3 UBT DOB value and accuracy of the Premier Platinum HpSA Plus test

To investigate whether DOB value (as a surrogate for bacterial load) affects the sensitivity of the HpSA Plus test, DOB values of UBT positive patients were categorised into low (<16‰), intermediate (16-35‰), and high (>35‰) (pre-treatment) groups as previously reported (79, 82, 86). The sensitivity of the HpSA test was then compared between the low and high groups by Fisher's exact test. Additionally, to investigate whether absorbance values of the Premier Platinum HpSA Test and UBT DOB values were correlated, the Pearson correlation coefficient was calculated.

2.2.3.4 UBT/ Premier Platinum HpSA Plus test results and response to *H. pylori* eradication

To investigate whether the UBT results can predict response to first line eradication of *H. pylori* infection, positive patients were categorised into low (<16‰), intermediate (16-35‰), and high (>35‰) (pre-treatment) DOB groups as described above. The eradication rate in each group was then compared to determine whether a significant difference existed. A subset of positive

patients was given clarithromycin-based triple therapy which consisted of 20 mg omeprazole, 500 mg clarithromycin, 1 g amoxicillin twice daily for 7 days. The treatment given to the remaining patients was unknown as their treatment was prescribed by their general practitioner. A follow-up breath test was performed at least 8 weeks post-treatment to confirm eradication. Additionally, a receiver operator curve analysis was carried out to determine whether the DOB value could predict treatment outcome with adequate sensitivity and specificity. To do this, patient's treatment outcome was plotted against their respective pre-treatment UBT DOB value. To investigate whether HpSA test results can predict response to first line eradication of *H. pylori* infection, positive patients were categorised into low (≤1.6) and high (>1.6) absorbance values and eradication between the groups was compared.

2.2.4 Invasive detection of *H. pylori*

2.2.4.1 Rapid urease test

H. pylori positive patients were identified by a positive RUT. During endoscopy, gastric biopsy specimens were taken and placed into the RUT well (TRI-MED Distributors, PTY LTD, Washington, USA). The test was examined after 30 min. If *H. pylori* was present in the biopsy, its enzyme urease converted urea present in the test well to ammonia which resulted in a colour change from yellow to red, which was interpreted as a positive result.

During the course of this research project, following a study in our centre (123) and studies conducted elsewhere (106, 125, 126), standard RUT protocol was changed from the use of single antral to combined antral and corpus biopsy samples. Endoscopy reports of patients recruited to this study were retrospectively reviewed to determine whether a single or combined biopsy RUT protocol was used. The accuracy of the two methods compared to histological testing as gold standard was then evaluated.

2.2.4.2 Histology

Results of the patients attending TUH were recorded from the microscopy notes on the histology report. These reports were made available on the hospital information system around 10 days after samples were taken at endoscopy. The specimens were examined by a consultant pathologist for the presence of *H. pylori* and the degree of inflammatory cell infiltration and intestinal metaplasia was recorded. During endoscopy, 2 biopsy specimens (1 from the lesser and 1 from the greater curvature) of the antrum and 2 specimens (1 from the lesser and 1 from the greater curvature) of the corpus were obtained for histological examination. Biopsies were taken with a standard forceps (requires 2.8 mm biopsy channel). The biopsies were formalin

fixed immediately following collection and paraffin embedded upon arrival in the pathology laboratory. The Giemsa stain was used for histological detection of *H. pylori*.

2.2.4.3 Bacterial culture

Biopsy samples from patients who had a positive RUT were processed for bacterial culture. In order to maximise *H. pylori* yield during culture, the use of combined antral and corpus biopsies compared to single antral biopsy samples was evaluated. First, single antral biopsies were collected from patients undergoing endoscopy. Subsequently, combined antral and corpus biopsies were collected from another group of patients undergoing endoscopy. Both combined antral and corpus biopsies collected for bacterial culture were stored within the same transport tube and inoculated onto the same plate. Gastric biopsy samples were processed for culture as soon as possible following endoscopy, ideally within 6 hours. If processing was delayed, samples were refrigerated until processed. The tissue samples were streaked onto Columbia Blood Agar (CBA) plates (Fannin Ltd, Dublin, Ireland) containing 5% laked horse blood and incubated under microaerophilic conditions (86% N_2, 4% O_2, 5% CO_2 and 5% H_2) using CampyGen™ 2.5 L Atmosphere Generation Systems (Oxoid, Basingstoke, UK) at 37°C for 7-10 days. Following incubation, plates were examined for the presence of *H. pylori* colonies and for contamination. Culture was positive if the presence of small, circular smooth colonies could be visualised on the CBA plates. A bacterial stock was prepared and stored in a Microbank Cyrovial (Pro-Lab Diagnostics, ON, Canada) at -80 °C.

2.2.4.4 Comparison of transport media for successful culture of *H. pylori*

Standard transport medium (nutrient broth), Dent's Transport Medium (Appendix 2) and Portagerm pylori (Biomerieux) were assessed according to their ability to promote successful *H. pylori* culture. To compare standard transport medium and Dent's transport medium, two single antral gastric biopsies were obtained (as was standard protocol at the time), with one biopsy stored and cultured from standard culture transport medium and one from Dent's transport medium. To compare Dent's transport medium and Portagerm pylori, two sets of combined antral and corpus biopsies were obtained, with one set stored and cultured from Dent's transport medium and the other stored and cultured from Portagerm pylori. Successful culture was defined as presence of *H. pylori* colonies within 7-10 days. Culture yield and contamination was compared on a visual analogue scale.

2.2.5 Statistical analysis

Statistical analysis was carried out using GraphPad Prism (GraphPad Software Inc., CA, USA). Continuous variables are presented as arithmetic mean and SD. Groups were compared using the two-tailed independent t-test (for comparison of two groups). P values less than 0.05 were considered to indicate statistically significant differences between groups. Categorical variables are presented as percentages and 95% confidence intervals (95% CIs). *P* values for categorical variables were calculated using the Fisher's exact test/ Pearson $\chi 2$-test.

To evaluate the accuracy of diagnostic tests, the following values (and 95% CIs) were calculated using GraphPad Prism. Sensitivity was calculated to determine the proportion of those with *H. pylori* infection correctly identified as positive by the test. Specificity was calculated to determine the proportion of those without *H. pylori* infection correctly identified as negative by the test. Positive predictive value was calculated to determine the proportion of patients with positive tests that have *H. pylori* infection. Negative predictive value was calculated to determine the proportion of patients with negative tests that do not have *H. pylori* infection. The kappa coefficient was calculated to describe the strength of agreement between two qualitative measures (315). Accuracy was calculated to determine the tests ability to differentiate between positive and negative cases correctly. The diagnostic accuracy of a test is expressed as the proportion of patients correctly classified as having the infection (true positive + true negative) among all patients (true positive + true negative + false positive +false negative) (316). The likelihood ratio was calculated to describe how many times more likely a particular test result is in patients with the infection than in those without the infection. It is expressed as the ratio of expected test result in patients with the infection to the subjects without the infection (67). A Receiver Operating Characteristic (ROC) curve analysis was carried out using GraphPad Prism to determine whether a DOB value could predict treatment outcome in our cohort with acceptable sensitivity and specificity. ROC curves determine a diagnostic test's performance over the range of thresholds for sensitivity and specificity (317, 318). The area under the curve (AUC) was determined, which measured how well the DOB value could distinguish between those who had successful treatment outcome and those whose treatment failed. An AUC of 1 is indicative of a very accurate test whereas an AUC of 0.5 indicates a completely uninformative test (318).

2.3 Results

2.3.1 Patient inclusion

Following informed consent, patient's data and their biological specimens described in this chapter were used in the subsequent chapters of this thesis. A schematic of the total amount of patients recruited and their inclusion in each study is presented in Figure 2.1.

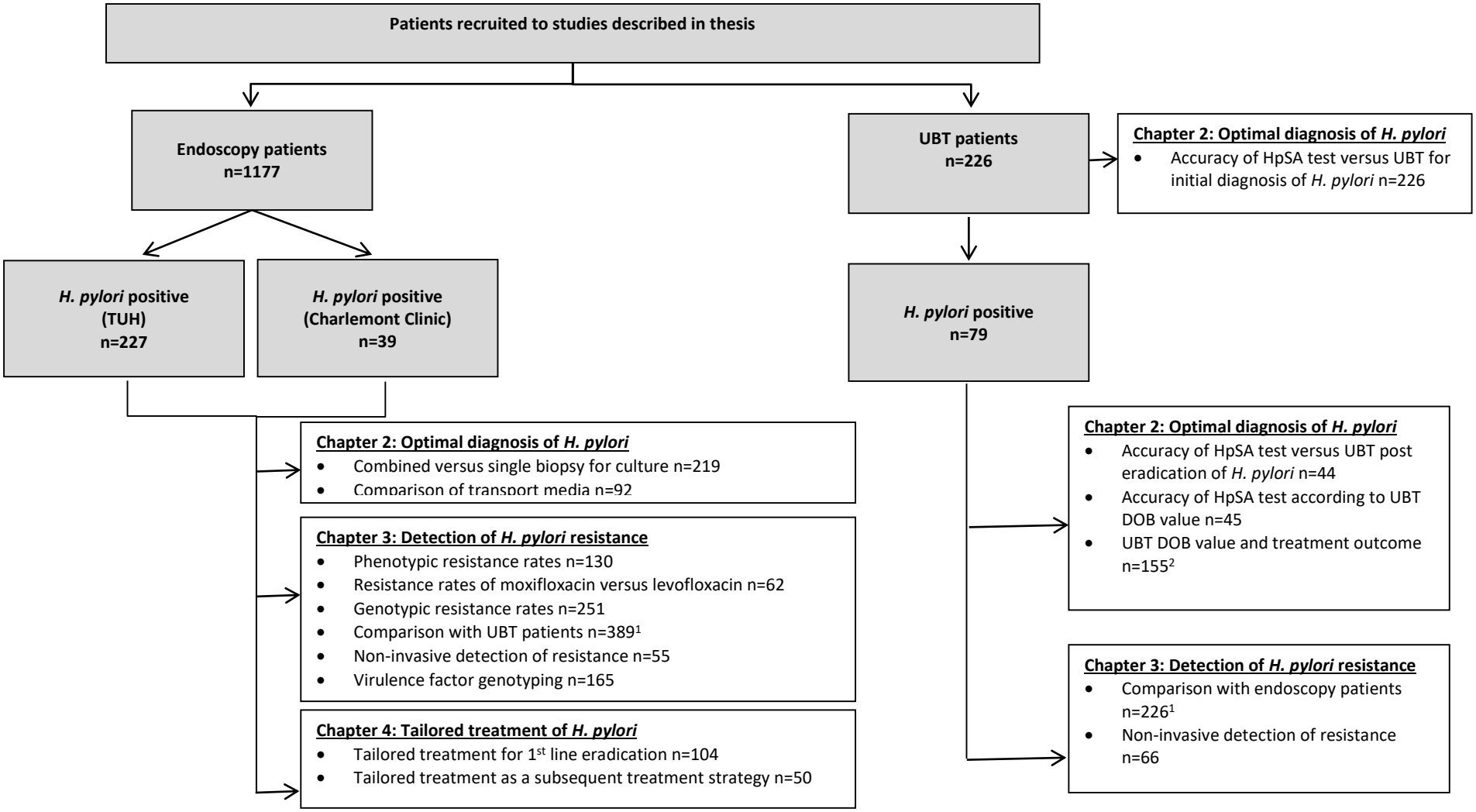


Figure 2.1: Flow chart of patient inclusion in all studies described in this thesis. [1]RUT/UBT positive and negative included; [2]Data on the DOB value and treatment outcome of these patients were collected as part of a clinical audit. No biological samples from these patients were used.

2.3.2 UBT patients

In all, 226 patients referred for UBT were recruited from August 2014 to March 2016. The average age was 39.8±12.8 years and 30.5% (69/226) were male. *H. pylori* infection was detected in 35% (79/226) of patients by a positive UBT. The average age of infected patients was 41.3±12.9 years and 38% (30/79) were male (Table 2.3). Although not significant, the rate of infection in those under 30 was lower than in those over 55 years (25.5% (n=14/55) vs 45.7% (n=16/35); p=0.07).

	Total	**UBT positive**	**UBT negative**	**p value**
	n (%)	**n (%)**	**n (%)**	
No. of patients	226	79 (35%)	147 (65%)	
Gender				
Male	69 (30.5%)	30 (38%)	39 (26.5%)	0.1[1]
Female	157 (69.5%)	49 (62%)	108 (73.5%)	
Age (years)				
Average (SD)	39.8±12.8	41.3±12.9	39.2±12.7	0.2[2]

Table 2.3: Demographics of all patients referred for a UBT who were included in the study. [1]Fisher's exact test, [2] unpaired t-test.

2.3.3 Optimal strategies for the diagnosis of *H. pylori* infection: Non-invasive tests

2.3.3.1 Accuracy of the Premier Platinum HpSA Plus test in initial diagnosis of *H. pylori* infection

The accuracy of the Premier Platinum HpSA Plus test for the diagnosis of *H. pylori* infection was evaluated by comparing results to those obtained from the UBT. Stool samples were collected from a total of 226 patients attending the UBT clinic (Table 2.3). The diagnostic test evaluation for the Premier Platinum HpSA Plus test compared to the UBT is outlined in Table 2.4. Factors such as age and gender did not significantly affect the accuracy of the test (Tables 2.5 & 2.6).

Statistic	Diagnosis of *H. pylori* infection n=226	Eradication of *H. pylori* infection n=44
Infection rate	**35.0%** (79/226; 95% CI 28.8 - 41.6)	N/A
Sensitivity	**58.2%** (46/79; 95% CI 46.6 - 69.2)	**41.7%** (5/12; 95% CI 15.2 - 72.3)
Specificity	**89.8%** (132/147; 95% CI 83.7 - 94.2)	**96.9%** (31/32; 95% CI 83.8 - 99.9)
PPV	**75.4%** (46/61; 95% CI 64.7 - 83.7)	**83.3%** (5/6; 95% CI 39.4 - 97.5)
NPV	**80.0%** (132/165; 95% CI 75.4 - 83.9)	**81.6%** (31/38; 95% CI 73.2 - 87.8)
Likelihood ratio	**5.7** (3.4 - 10.0)	**13.3** (1.7 – 102.8)
Accuracy	**78.8** (178/226; 95% CI 72.9 - 83.9)	**81.8%** (36/44; 95% CI 67.3 - 91.8)
Kappa[a]	**0.5** (0.4 - 0.6)[a]	**0.5** (0.2 – 0.8)[a]

Table 2.4: Accuracy of the Premier Platinum HpSA Plus test versus UBT for the diagnosis of *H. pylori* infection and for determining eradication of *H. pylori* infection. [a]The strength of agreement is considered to be 'moderate'.

Gender	Male	Female	p value
Sensitivity	**60.0%** (18/30)	**57.1%** (28/49)	0.8[1]
Specificity	**89.7%** (35/39)	**89.8%** (97/108)	1.0[1]

Table 2.5: Accuracy of the Premier Platinum HpSA Plus test according to gender. [1]Fisher's exact test

Age	Under 45 years	Over 45 years	p value
Sensitivity	**55.4%**	**65.2%**	0.5[1]
	(31/56)	(15/23)	
Specificity	**89.5%**	**90.5%**	1.0[1]
	(94/105)	(38/42)	

Table 2.6: Accuracy of the Premier Platinum HpSA Plus test according to age. Age under/over 45 years was chosen as per guidelines on the management *of H. pylori* infection (64, 65). [1]Fisher's exact test

2.3.3.2 Accuracy of the Premier Platinum HpSA Plus test post-eradication therapy

A subset of patients (n=44, 56%) who returned for post-eradication UBT agreed to provide a stool sample to evaluate the accuracy of the Premier Platinum HpSA Plus test post-eradication therapy. The diagnostic test evaluation is outlined in Table 2.4.

2.3.3.3 Is the accuracy of the Premier Platinum HpSA Plus test impacted by UBT DOB value?

Next, the correlation between UBT DOB values and the sensitivity of the Premier Platinum HpSA Plus test was investigated. DOB values of UBT positive patients which were available at the time (n=45, 57%) were grouped into low (<16), intermediate (16-35) or high (>35) groups according to their pre-treatment DOB value. The sensitivity of the test was then compared between low and high DOB groups by Fisher's exact test. The sensitivity of The Premier Platinum HpSA Plus significantly increased as DOB increased (45.5%, vs 90.9% in low vs high DOB groups; p=0.02; Table 2.7). However no correlation was found between absorbance values of the Premier Platinum HpSA Test and UBT DOB values (r=0.4, 95% CI 0.1- 0.6).

	Sensitivity	p value
Low DOB (<16)	**45.5%**	0.02[1]
	(10/22; 95% CI 26.9-65.3)	
Medium DOB (16-35)	**75%**	
	(9/12; 95% CI 46.8-91.1)	
High DOB (>35)	**90.9%**	
	(10/11; 95% CI 62.3-99.5)	

Table 2.7: Sensitivity of the Premier Platinum HpSA Plus test in low, medium and high DOB groups. [1]Fisher's exact test comparing sensitivity of low DOB to high DOB.

2.3.3.4 Can the UBT test result predict *H. pylori* eradication?

To investigate whether UBT results can predict response to first-line eradication of *H. pylori* infection, the association between DOB value and treatment outcome was examined. Pre- and post-treatment UBT results from 155 *H. pylori*-positive UBT patients who had undergone eradication therapy were available for analysis. Patients were sub-grouped into low (<16), intermediate (16-35) or high (>35) groups according to their pre-treatment DOB value. There was no significant difference in age and gender between the three groups (Table 2.8).

	UBT result (‰)			p value
	Low (<16)	**Intermediate (16-35)**	**High (>35)**	
	n=70	**n=52**	**n=33**	
Gender				
Male	31	18	12	1.0[1]
Female	39	34	21	
Age (years)				
Mean ±SD	43.2±14	42.5±14.8	49.5±16.4	0.1[2]

Table 2.8: Demographics of patients with low, intermediate or high DOB values. [1]Fisher's exact test, [2]unpaired t-test.

Regardless of DOB values, the overall eradication rate was low at 61.3% (n=95, 95%CI 53.4-68.6, per-protocol (PP) analysis). Factors such as age and gender did not affect eradication rates (p=0.2 and p=0.9 respectively). Table 2.9 shows PP eradication rates according to low, intermediate or high DOB value. There was no significant difference in eradication rate between low, intermediate and high DOB value groups. Treatment regimen information was available for 40 (25.8%) patients, who were given clarithromycin-based triple therapy (STT) for 7 days. When this subset of patients was categorised into low, intermediate and high DOB groups, there was no significant difference in eradication rates (Table 2.9).

UBT result (‰)	Overall eradication rate (n=155)	STT eradication rate (n=40)
Low (<16)	**65.7%** (46/70; 95% CI 54.0-75.8)	**77.8%** (14/18; 95% CI 54.8-91.0)
Intermediate (16-35)	**57.7%** (30/52; 95% CI 44.2-70.1)	**72.7%** (8/11; 95% CI 43.4-72.7)
Low + Intermediate	**62.3%** (76/122; 95% CI 53.4-70.4)	**75.9%** (22/29; 95% CI 57.9-87.8)
High (>35)	**57.6%** (19/33; 95% CI 40.8-72.8)[1,2]	**72.7%** (8/11; 95% CI 43.4-72.7)[3,4]

Table 2.9: Correlation between UBT results and PP eradication of *H. pylori* infection. [1]comparison of eradication rate between low, intermediate and high DOB groups; p=0.6; [2] comparison of eradication rate between low +intermediate and high DOB groups (2 groups), p=0.7; [3]comparison of eradication rate between low, intermediate and high DOB groups; p=1.0; [4]comparison of eradication group between low +intermediate and high groups (2 groups), p=1.0.

Patients were then categorised according to eradication status, with no significant difference observed (Table 2.10).

	Eradication success	Eradication failure	p value
Overall mean DOB value	22.7 ‰	25.8 ‰	0.3[1]
STT subset mean DOB value	23.3 ‰	27.5 ‰	0.6[1]

Table 2.10: Mean DOB in patients whose eradication of *H. pylori* was successful and unsuccessful. [1]unpaired t-test.

Finally, a receiver operator curve analysis was carried out to determine whether the DOB value could predict treatment outcome with adequate sensitivity and specificity in our cohort (Figure 2.2). The area under the ROC curve for DOB value predicting treatment outcome was low at 0.6 (95% CI 0.5-0.6, p=0.3), indicating that the DOB value is not an accurate predictor of treatment outcome.

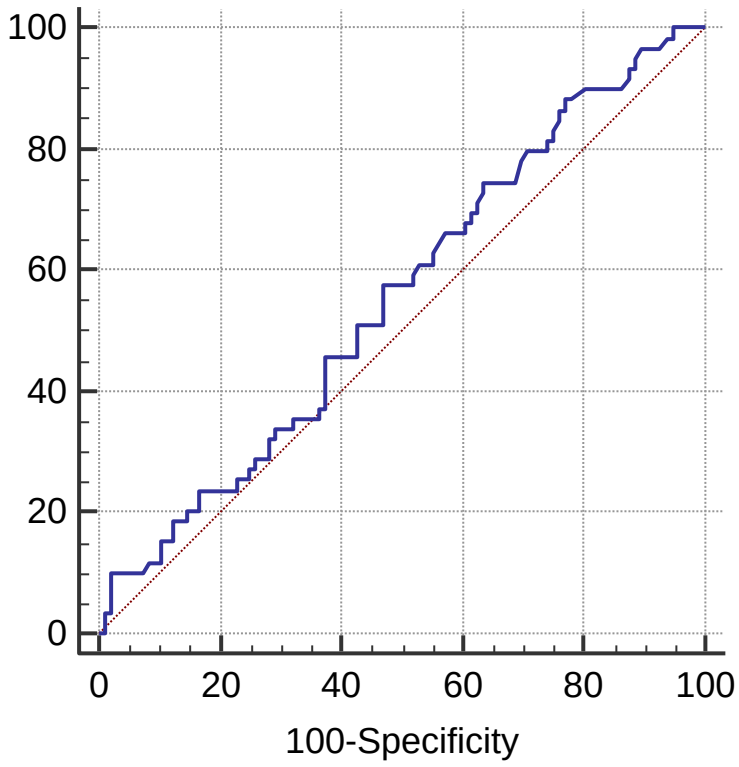


Figure 2.2: Receiver operator curve of DOB value for prediction of treatment outcome

2.3.3.5 Can the Premier Platinum HpSA Plus test result predict *H. pylori* eradication?

Similarly, the absorbance values of The Premier Platinum HpSA Test did not predict treatment outcome. There was no significant difference in eradication rate between low (≤1.6) and high (>1.6) absorbance values and no difference in the A450 value in eradicated and non-eradicated subjects.

In summary, in this facility, the UBT remains the gold standard non-invasive diagnostic test for *H. pylori* infection. The Premier Platinum HpSA exhibited poor sensitivity and positive predictive values for both initial diagnosis and confirmation of eradication in *H. pylori* infection. The sensitivity of The Premier Platinum HpSA Plus was affected by the DOB value of the UBT. As DOB value increased, the sensitivity of the Premier Platinum HpSA Plus test also significantly increased. Finally, neither UBT DOB values nor the Premier Platinum HpSA Plus test absorbance values were associated with *H. pylori* treatment outcome.

2.3.4 Endoscopy patients

In all, 1177 patients were included, with 1138 (96.7%) from TUH and 39 (3.3%) from Charlemont Clinic (Table 2.11). The average age was 52.5±16.2 years and 55.8% (n=657/1177) were male.

A total of 266 patients (22.6%) were identified as *H. pylori* positive by RUT. Histology results were available for the patients who attended TUH and are detailed in Table 2.11. The most common histological finding in RUT positive patients was chronic active gastritis at 53.7% (n=122/227). Normal mucosa was reported in 3% (n=7/227) of RUT positive patients. As chronic gastritis is universal in *H. pylori* infection, these histology results are most likely due to sampling error. In RUT negative patients, 9.1% (n=83/911) had chronic active gastritis and 56.6% (n=47/83) of these were in fact positive by histology. Of the RUT negative patients, 7.9% (n=72/911) had intestinal metaplasia and 9.7% (n=7/72) of these were positive by histology. A comparison of overall histology and RUT results is presented in Table 2.12. With histology as gold standard, the false negative rate of the RUT was 24.8% (n=59/238).

While RUT positive patients were significantly younger than RUT negative patients, (49.1 vs 53.5 years; p=0.0001, 95% CI -6.7 to -2.3), the age difference was just 4.5 years and these patients were selected from an endoscopy cohort in whom the majority of patients are over 45 years. To investigate this further, the possibility that there may have been false negative results in older patients due to the presence of intestinal metaplasia was explored. Overall, the sensitivity of the RUT test (compared to histology) was 70.2% in those over 60 years compared to 75.9% in those under 60 years (p=0.4). Those reported to have intestinal metaplasia (n=104/1138, 9.1%) were significantly older than patients with other histological findings (gastritis, normal mucosa etc.) (59 vs 51.8 years; p=0.0001, 95% CI -10.4 to -4). Of the patients with intestinal metaplasia who had a false negative RUT result (compared to histology; n=4), all 4 patients were over 60 years. Of those who had a false negative histology result (compared to RUT; n=8), 7/8 were over 60 years.

	Total n (%)	RUT positive n (%)	RUT negative n (%)	p value
No. of patients	1177	266 (22.6)	911 (77.4)	
Gender				
Male	657 (55.8)	135 (50.8)	522 (57.3)	0.07[1]
Female	520 (44.2)	131 (49.2)	389 (42.7)	
Age				
Mean ±SD	52.5 ± 16.2	49.1 ± 15.7	53.5 ±16.2	0.0001[2]
Histology findings	**n=1138**	**n=227**	**n=911**	
Normal mucosa	383 (33.7)	7 (3.1)	376 (41.3)	0.0001[1]
Chronic inactive gastritis	421 (37.0)	60 (26.4)	361 (39.6)	0.0002[1]
Chronic active gastritis	205 (18.0)	122 (53.7)	83 (9.1)	0.0001[1]
Chronic gastritis with atrophy	0	0	0	_
Chronic gastritis with intestinal metaplasia	104 (9.1)	32 (14.1)	72 (7.9)	0.006[1]
No data available	25 (2.2)	6 (2.6)	19 (2.1)	NA

Table 2.11: Demographics of all patients included in this study and histology findings of the patients who attended Tallaght University Hospital. [1]Fisher's exact test, [2]unpaired t-test.

		Histology HLO present	No HLO	Total
RUT	**RUT positive**	179	48	227
	RUT negative	59	852	911
	Total	238	900	1138

Table 2.12: Comparison of Histology and RUT results in patients undergoing endoscopy at TUH.
HLO: Helicobacter like organisms

The standard RUT protocol was changed from the use of single antral to combined dual antral and corpus biopsy samples during this study. Endoscopy reports of patients recruited to this study were reviewed to determine whether a single or combined biopsy RUT protocol was used. The accuracy of the two methods compared to histological testing as gold standard was then

evaluated. The RUT protocol was noted in 896 of the 1138 reports (78.7%); 271 (30.2%) noted the use of a single antral RUT and 625 (69.8%) noted that 2 biopsies were taken. No significant difference in the accuracy of the protocols was found (Table 2.13). However as this was a retrospective analysis of endoscopy reports, it is unknown whether a precise description of the actual RUT protocol in terms of number of biopsies and biopsy location was noted.

	Single biopsy (n=271)	**2x biopsy (n=625)**	**p value**
Sensitivity	**73.8%** (45/61; 95% CI 60.9- 84.2)	**75.8%** (100/132; 95% CI 67.5- 82.8)	0.9[1]
Specificity	**93.8%** (197/210; 95% CI 89.7- 96.7)	**92.9%** (32/493; 95% CI 90.3- 95.0)	0.7[1]
PPV	**77.6%** (45/58 95% CI 66.7- 85.7)	**74.1%** (100/135; 95% CI 67.2- 80.0)	0.7[1]
NPV	**92.5%** (197/213; 95% CI 89.0- 94.9)	**93.5%** (458/490; 95% CI 91.4- 95.1)	0.6[1]
Accuracy	**89.3%** (242/271; 95% CI 85.0- 92.7)	**89.3%** (558/625; 95% CI 86.6-91.6)	1.0[1]

Table 2.13: Accuracy of single versus 2x biopsy use in the RUT compared to histological assessment in the diagnosis of *H. pylori* infection. [1]Sensitivity, Specificity, PPV, NPV and accuracy of single versus combined biopsies were compared using Fisher's exact test.

2.3.5 Optimal strategies for the diagnosis of *H. pylori* infection: Invasive tests

2.3.5.1 Bacterial culture in the detection of *H. pylori* infection

In all, samples from 219 RUT positive patients were inoculated for *H. pylori* culture. The bacterial culture success rate was 59.4% (n=130/219).

2.3.5.2 Use of single versus combined biopsies in detection of *H. pylori* by bacterial culture

In an effort to improve our culture rate during the course of the study, the yield of *H. pylori* when using a single antral biopsy versus combined antral and corpus biopsies in bacterial culture was evaluated (Table 2.14). Results from 73 bacterial cultures performed using a single antral biopsy and 146 bacterial cultures performed using combined corpus and antral biopsies were included. The yield of *H. pylori* was significantly higher when combined biopsies were used compared to when single biopsies were used for bacterial culture (64.4% vs 49.3%; p=0.04).

	Culture positivity rate	p value
Single biopsy	**49.3%** (36/73; 95% CI 38.2- 60.5)	0.04[1]
Combined biopsy	**64.4%** (94/146; 95% CI 56.3- 71.7)	

Table 2.14: Culture rate of *H. pylori* using single antral biopsies versus combined biopsies. [1]Fisher's exact test

2.3.5.3 Comparison of transport media for successful culture of *H. pylori*

The efficacies of 3 types of transport media were assessed according to their ability to promote successful *H. pylori* culture. Specifically, the yield of *H. pylori* and level of contamination following incubation was investigated. Firstly, efficacy of standard transport media and Dent transport media was compared (Table 2.15). Single antral biopsy samples from 77 RUT positive patients were included in this study. No statistically significant difference in culture positivity was found between samples stored in Dent's transport medium and in standard transport medium. Next, the ability of each media to prevent contamination with other microorganisms was assessed. Significantly less contamination was observed in *H. pylori* cultures that had been stored in Dent's transport medium compared to contamination observed in samples stored in standard transport medium (32.5% vs 48.1%; p=0.048).

	Standard transport media	Dent's transport media	p value
Culture positive	**51.9%** (40/77; 95% CI 41.0- 62.7)	**57.1%** (44/77; 95% CI 46.0- 67.6)	0.6[1]
Contamination	**48.1%** (37/77; 95% CI 37.3- 59.0)	**32.5%** (25/77; 95% CI 22.7- 43.0)	0.048[1]

Table 2.15: Culture rate and contamination level for standard transport media versus Dent's transport media. [1]Fishers exact test

Subsequently, the efficacy of Dent's transport medium and Portagerm pylori in their ability to promote successful *H. pylori* culture was assessed (Table 2.16). Combined antral and corpus biopsy samples were obtained from 15 *H. pylori* positive patients. No difference in culture positivity or contamination was observed between samples stored in Dent media and Portagerm pylori.

	Dent's transport media	Portagerm pylori	p value
Culture positive	**66.6%**	**73.3%**	1.0[1]
	(10/15; 95% CI 41.7- 84.8)	(11/15; 95% CI 48.1- 89.1)	
Contamination	**73.3%**	**80%**	1.0[1]
	(11/15; 95% CI 48.1- 89.1)	(12/15; 95% CI 54.8- 93.0)	

Table 2.16: Culture rate and contamination level for Dent's transport media versus Portagerm pylori. [1]Fisher's exact test.

In summary, the positive culture rate culture of *H. pylori* in our centre was 59.4%. The use of combined antral and corpus biopsies for culture resulted in a significantly higher yield of *H. pylori*. By significantly decreasing contamination levels in culture of *H. pylori*, Dent's transport medium was found to be better at promoting culture of *H. pylori* compared to standard transport media.

2.4 Discussion

2.4.1 Non-invasive detection of *H. pylori*

2.4.1.1 UBT and The Premier Platinum HpSA Plus Test

In this study, the Premier Platinum HpSA Plus test was compared to the UBT, which was used as the gold standard for non-invasive diagnosis of *H. pylori* in this study. This is because it is the most investigated and best recommended non-invasive test, with high sensitivities of 90-96% and specificities of 88-98% reported (89-94). Vaira and Vakil reviewed studies on the performance of the three types of non-invasive tests and reported that the UBT had higher overall accuracy (95% specificity and sensitivity) than serology tests (85% sensitivity and 79% specificity) and stool antigen testing (93.1% sensitivity and 92.8% specificity) (91). A systematic review which consisted of 30 studies with 3,415 patients compared the UBT to serology and stool antigen testing (with biopsy-based tests as gold-standard). Sensitivity and specificity higher than 90% was found in 84% of the studies for the UBT, whereas sensitivity and specificity higher than 90% were found in only 62% of the studies for the stool antigen test and approximately 56% of studies for serology tests (95). Additionally, the UBT is cost-effective and easy to perform (93, 95). Nocon et al. reviewed heath economic evaluations, which compared cost-effectiveness of a test-and-treat approach based on the UBT to other management strategies for dyspepsia. The study concluded that test-and treat using the UBT was more cost-effective than a serology based strategy and empirical anti-secretory therapy (95).

Since the first description of the UBT in 1987 (319), many studies have been carried out with the aim to improve the accuracy and ease of use of the test, which have yielded controversial results (70, 92). For example, whether a patient needs to fast before the test (320-323), whether a test meal should be given (324, 325) and the type of test meal (326, 327) have been reported on, with conflicting conclusions. The optimum DOB cut-off to use in clinical practice has also been a source of much controversy. The DOB is dependent on patient factors such as fasting (321) and gender (328) as well as protocol related factors such as the dose of urea administered (72), or whether the UBT is carried put pre- or post-treatment (329). A cut-off value of 5.0‰ was originally recommended and was the most widely used (92). Recently, following further studies using ROC curves, it has been shown that cut-offs between 2-5‰ have equal accuracy (92, 96). True positive UBT results usually have a much higher DOB value than the cut-off point and true negative UBT results are much lower, usually very close to zero (92). Additionally, only about 5% of these samples fall into the 2-5‰ range (96). It has been shown that when using a reduced

urea dose and shortened measurement duration (75 mg and 30 min respectively, as was protocol in this thesis), reducing the DOB cut-off from 5.0‰ to 3.5‰ significantly improved accuracy of the UBT (72). A critical review conducted on studies dealing with the several aspects of the UBT protocol found that most of the protocols administering 75 mg of urea select a cut-off point lower than 5.0‰, usually between 3.5- 5.0‰. This study also stated that a change in cut-off value within the 3.5- 5.0‰ range would minimally affect clinical accuracy (92). Although it has been recommended that clinical laboratories optimise their DOB threshold values for UBT (330), adequate resources were not available in this study to perform this. Based on manufacturers suggestions (313), and on validation tests performed at regular intervals by the physiologist based in the GI laboratory, a UBT DOB value of >4.0 ‰ was chosen as the cut-off to determine *H. pylori* positivity.

Stool antigen testing is an alternative to UBT (91), and as the most recent study investigating the accuracy of stool antigen testing in Ireland was published approximately 12 years ago (27), the diagnostic accuracy of the Premier Platinum HpSA Plus was evaluated in our patient cohort. The sensitivity, specificity, PPV and NPV of the HpSA versus the UBT for initial diagnosis of *H. pylori* infection were 58.2%, 89.8%, 75.4% and 80.0%, respectively. Following eradication, the sensitivity, specificity, PPV and NPV of the test were 41.7%, 96.9%, 83.3% and 81.6%, respectively. In our population, the Premier Platinum HpSA Plus was found to be less accurate than other studies, both for initial diagnosis (98, 99, 331) and post-treatment testing (332). Based on the low sensitivity and specificity of the Premier Platinum HpSA Plus observed herein as well as the studies outlined above, the UBT remains the gold-standard non-invasive diagnostic tool for both diagnosis and assessing eradication of *H. pylori* infection.

The low sensitivity of the Premier Platinum HpSA Test may reflect specific collection and storage requirements, which are a common problem for many faecal tests. In this study, stool samples were collected the day before the patient attended the hospital. The manufacturer's instructions noted that samples should be tested as soon as possible, but could be held up to 72 hours after collection (314). To ensure minimal degradation of the sample and potentially improve sensitivity, the sample collection protocol could be refined to reduce the time between sample collection and testing. Additionally, the use of proton pump inhibitors (PPI) and/or the degree of *H. pylori* colonisation in the stomach influence the concentration of *H. pylori* antigens in stool samples (98, 311). Variations in the level of *H. pylori* colonisation in our population may account for the observed differences in the sensitivity of the assay. Indeed, this study found that the

sensitivity of The Premier Platinum HpSA Plus was affected by the UBT DOB value, which may be a surrogate marker for bacterial load. When a subset of UBT positive patients were grouped into low (<16), intermediate (16-35) or high (>35) DOB value, the sensitivity of the test was 45.5%, 75% and 90.9% respectively (p=0.02). Additionally, when patients were sub-divided based on DOB values, the greatest number of patients had DOB values potentially indicative of low bacterial load (45% with low DOB value compared to 34% with intermediate DOB and 21% with a high DOB), which may indicate that a high proportion of patients had recently taken PPI's.

It has been reported elsewhere that non-invasive diagnostic tests can be affected by age and gender of a patient (308, 328). For example, stool antigen testing may be less accurate in older patients as they frequently suffer from constipation, which slows down GI transit and may lead to degradation of *H. pylori* antigens (308). In this study, age and gender did not significantly affect the accuracy of the Premier Platinum HpSA Plus test.

It is well established that *H. pylori* infection can cause GI diseases such as gastritis, gastric and duodenal ulcers, gastric adenocarcinoma and MALT lymphoma (4, 18, 333, 334). Interestingly, an association between gender and different types of GI diseases has been found. Men are significantly more likely to develop peptic ulcers (335) and gastric cancer (336, 337). On the other hand, the prevalence of functional dyspepsia is significantly higher in women (338). In patients referred for endoscopy, a roughly equal amount of males and females were recruited (55.8% male). However of the patients recruited who were referred for UBT, 69.5% were women, which is unusual. The author was aware of this during patient recruitment for this study. Although efforts were made to recruit an equal amount of males and females, women were more likely to agree to participate and to provide a stool sample on the day of their scheduled UBT. It has also been reported that females are more likely to attend GPs (339, 340). Additionally, although the final diagnosis of the patients in this study is not known, a possible contributory factor is that more women are likely to be referred for UBT. This may be due to prevalence of functional dyspepsia being significantly higher in women (338, 341). Despite the higher proportion of women included in the study, there was no significant difference in the accuracy of the Premier platinum HpSA Plus test according to gender.

A total of 65 patients over the age of 45 years (28.8%) were included in the study to determine the accuracy of Premier Platinum HpSA Test. Based on their age, these patients should have undergone endoscopy and not UBT. However UBT may have been refused or contraindicated in these patients. As no significant difference was observed in the accuracy of the Premier Platinum HpSA Test according to age, and based on the low number of patients recruited to the study,

these patients were included in the analysis to evaluate the accuracy of the Premier Platinum HpSA Test.

2.4.1.2 Neither UBT nor the Premier Platinum HpSA Plus test results predict treatment outcome of *H. pylori*

Many studies compared UBT DOB results with histological and endoscopic findings and found significant associations (71, 72, 74-78). Kobayashi et al. compared UBT DOB results to PCR (to estimate *H. pylori* genome copies) and found a significant correlation between the mean density of *H. pylori* genomes and UBT DOB value. The authors also compared the DOB value to histology and suggested that the severity of *H. pylori* infection can be estimated by the UBT (73). On the other hand, other studies do not support this theory (309, 310). Tummala et al. compared UBT DOB values and bacterial counts in biopsies and found little correlation. However, this study was carried out on a total of 19 samples from *H. pylori* positive patients, so the generalisability of this is unclear (310). Studies have evaluated whether the UBT DOB value can predict treatment outcome (79-86). Similarly to above however, some support this (79-84), and others disagree (85, 86). Studies on stool antigen test result values and their association with treatment outcome is limited. For these reasons, this study sought to determine whether these values were associated with treatment outcome in our patient cohort.

No association was found between DOB values and eradication rate in our study. As previously mentioned, the sensitivity of The Premier Platinum HpSA Plus observed in this study is lower than others. The low, intermediate and high DOB cut-off values used to investigate whether there was an association between DOB values and eradication rate were based on previous studies (79, 82, 86) and may not be appropriate for our patient cohort. Because of this, a receiver operator curve analysis was carried out to determine whether a DOB value could predict treatment outcome in our patient cohort. However based on the low AUC value observed, the DOB value is not an accurate predictor of treatment outcome.

In this study the UBT DOB value was used as a surrogate marker for bacterial load, despite not all of the literature in the field supporting this theory. Additionally, there was no evidence to establish that the UBT DOB value can be used as a gold standard for bacterial load in our patient cohort. A preliminary study could have been done by comparing the bacterial load determined by histology to the respective DOB value of a patient. A very limited number of participating patients had undergone endoscopy in addition to UBT. In those who had undergone endoscopy as well as UBT, a considerable amount of time had elapsed between the two procedures and this would have implications for the validity of such a study, as the patient may have received

treatment for *H. pylori* infection during that time. Therefore to establish this, those who had undergone UBT would need to be invited back to undergo endoscopy. Resource constraints within our institution department would not allow this.

There are a number of confounding variables that could not be accounted for in this study. For example, as mentioned above, UBT DOB values can depend on patient and UBT protocol related factors (92) and treatment success can depend on patient compliance (247). As a number of patients included in this study were treated by their GPs, the exact regimen received was unknown. However a number of patients were treated in our centre and their treatment was known. As one such confounding variable was removed from this group of patients, they were analysed separately. However no association was found between UBT DOB values and eradication rate in this group.

Zullo et al. suggested that the use of duplicate UBTs post-treatment to confirm eradication (86), may improve the quality of non-invasive tests. However a later study was conducted by the same group to determine the incremental cost and accuracy of doing two UBT post treatment in clinical practice. They concluded that a single UBT, 4 weeks after treatment is as effective as two serial breath tests in confirming *H. pylori* eradication. Also, the incremental cost of the second breath test was high with no incremental clinical benefit (342). Therefore in this study, this approach was not considered as an option to optimise non-invasive diagnostic tests.

2.4.2 Invasive detection of *H. pylori*

2.4.2.1 RUT and histology

Of the 1177 patients who underwent an endoscopy, 266 were identified as *H. pylori* positive by the RUT (22.6%). As detailed in Section 1.2, *H. pylori* prevalence has decreased in many countries in the developed world (29, 33) and this is reflected in the low rate of *H. pylori* infection detected in our centre. The RUT was used to identify *H. pylori* positive patients during endoscopy, as this test allowed diagnosis of infection on the same day the procedure is carried out and facilitated the identification of *H. pylori* positive patients whose samples could be rapidly processed for culture. Infection was later confirmed by histology, culture or the GenoType HelicoDR assay (detailed in Chapter 3).

Not surprisingly, in those with a positive RUT, the most common histological finding was chronic active gastritis. In RUT negative patients, 9.1% had chronic active gastritis and 7.9% had intestinal metaplasia. As *H. pylori* infection is a causal factor in both, these findings would be uncommon in an uninfected patient and these are potentially false negative RUT results. With

histology as gold standard, the false negative rate of the RUT in this study was 24.8% (59/238). The incidence of false negative RUT is common. Prince et al. surveyed the use of RUTs in the UK and reported that the majority of endoscopy units read RUTs earlier than recommended, which lead to a decrease in sensitivity (122). The presence of atrophy and intestinal metaplasia can cause patchy distribution of bacterial colonization in the stomach (104, 106, 343). *H. pylori* infection may well be present in these patients but may not be detected by the RUT or histology. False-negative RUTs can also occur with GI bleeding (105). Although guidelines recommend that PPIs should be stopped at 14 days before a patient undergoes testing for *H. pylori* (64, 65), oftentimes patients do not stop their use of PPIs. PPIs increase gastric pH which in turn causes a decrease in the bacterial load of *H. pylori* (96, 157). The RUT, histology, culture, UBT and stool antigen testing all depend on the presence of a sufficient amount of bacteria and false negative tests can occur in these tests with PPI use (69, 155-158).

RUT positive patients were significantly younger than those who had a negative RUT result (49.1 vs 53.5 years; p=0.0001). It should be noted however this is an age difference of just 4.5 years and that these patients were selected from an endoscopy cohort in whom the majority of patients are over 45 years. As most epidemiological studies are carried out across populations comprising a wider age range, this finding likely does not accurately reflect the true prevalence of *H. pylori* infection in our population. It's possible that there may have been false negative results in older patients, due to the presence of intestinal metaplasia as described above (104). Indeed those reported to have intestinal metaplasia were significantly older than patients with other histological findings (59 vs 51.8 years; p=0.0001). The majority of patients who had either a false negative RUT or histology result were over 60 years.

For these reasons, histological testing alongside RUT for *H. pylori* is important to ensure minimal incidence of false negative diagnosis. False negative histology results can also occur however, due to PPI use and the presence of atrophy and intestinal metaplasia as outlined above (106). IHC is recommended in cases where no bacteria can be detected but chronic active gastritis is present (115-117).

Previous studies have shown that using combined biopsy samples from the antrum and corpus for the RUT improves its accuracy (106, 123, 125, 126). It is thought that PPI use increases colonisation in the corpus but decreases colonisation in the antrum (344) and this is why a combined antral and corpus approach increases accuracy. During this study, the RUT protocol in our centre was changed from the use of single antral to combined antral and corpus biopsy

samples. To compare the accuracy of a single versus combined biopsy RUT protocol (using histology as gold standard), endoscopy reports were retrospectively reviewed to determine whether a single or combined biopsy RUT protocol was used. No significant difference in the accuracy of the RUT protocol was found. As reports were retrospectively analysed, we cannot say for certain that a combined antral and corpus biopsy protocol was used because endoscopists may not have reported a precise description of specific sampling location for the RUT. Therefore the data generated from this study is limited.

2.4.2.2 Bacterial culture: The use of combined antral and corpus biopsies and optimising transport of samples can improve culture of *H. pylori*

Bacterial culture of *H. pylori* is necessary in order to perform susceptibility testing when a patient has had one or more failed eradication attempts. Regular resistance surveillance is also recommended in regions where antibiotic resistance rates are high (64, 65, 87, 88). With ever increasing resistance to commonly prescribed antibiotics being reported, it may become standard practice to perform susceptibility testing prior to first-line treatment of *H. pylori* infection to halt this worrying trend. It is therefore important to endeavour to improve culture rates. This study aimed to do so by investigating whether the use of combined antral and corpus biopsies and various transport media improved culture rate of *H. pylori*. In this study, culture success of *H. pylori* was 59.4% (n=130/219). This rate is reflective of the fastidious nature of the bacteria and other studies have reported a similar culture rate (182, 345). As mentioned in Section 1.4.2.3, a number of factors can affect the rate of culture of *H. pylori,* for example biopsy site, transport media, time from sampling to processing, culture media and incubation conditions (69). We found that the use of combined antral and corpus biopsies in bacterial culture significantly improved culture yield by 15% when compared to the use of a single biopsy in culture. It is logical that the more biopsy specimens used for culture- the higher the chance of recovery of *H. pylori* and this practice has been recommended elsewhere (346). Additionally, collecting biopsies from both sites may account for patchy distribution of the bacterium (347). Furthermore, in terms of using bacterial culture to perform antimicrobial susceptibility testing, it is important to collect biopsies from both sites. It has been found that differences exist in the antimicrobial susceptibility to clarithromycin, metronidazole, levofloxacin and rifabutin between strains isolated from the corpus and those from the antrum in the same patient (348). Another study suggested that heteroresistance is widely present in antibiotic-resistant *H. pylori* infection- they reported heteroresistance between antrum and corpus samples in 38% of patients. The authors recommended that a single biopsy site cannot be considered representative of an individual's antibiotic susceptibility profile (349). Resistance to these antibiotics could be missed

or underestimated if only one biopsy is taken, which may negatively affect treatment outcome. Although not investigated in this study, it would be interesting to determine whether differences in susceptibility occur in our patient cohort. In light of these findings and the studies detailed above, the use of a combined biopsy protocol is recommended to improve diagnostic accuracy and potentially improve the detection of antibiotic resistance.

A key factor in the success of *H. pylori* culture is the conditions in which the biopsy is kept following endoscopy and prior to processing. If it is necessary to store a gastric biopsy for more than 1 day, the effectiveness of transport media becomes very important in culture. Biopsy samples must be prevented from contact with air, oxygen and room temperature. In order to optimise transport and storage of biopsy samples, the efficacies of different transport media were investigated. Both Dent's transport media and Portagerm pylori improved bacterial yield. When compared to rates in standard transport media, Dent's transport media improved bacterial yield by 5.2% ($p=0.6$) and significantly decreased contamination by 15.6% ($p<0.05$). Dent's transport media is a selective liquid media containing nutrients and antibiotics for selective growth of *H. pylori* and its use has been recommended elsewhere (132, 134). In investigating the efficacy of Portagerm pylori (a semi-solid media), although sample size was small, bacterial yield was improved by 6.7% when compared to Dent's transport media ($p=1.0$). It has shown promise in other studies, with reports of a culture rate of approximately 94% after transport in Portagerm pylori (129-131). Several other transport media have been described, for example GESA medium, a semi-solid medium which allowed the recovery of *H. pylori* from biopsy samples up to 10 days after collection, when stored at 4°C (133). In light of this, for future storage and transport of biopsy samples, the use of *H. pylori*-specific transport media is recommended. This study found Dent's transport medium to be effective, while further evaluation of the efficacy and cost-effectiveness of Portagerm pylori is required.

2.4.3 Summary

In our facility, the Premier Platinum HpSA Plus test was found to be inferior and the UBT remains the gold-standard non-invasive tool for both diagnosis and assessing eradication of *H. pylori* infection. The DOB value in the UBT and absorbance in the Premier Platinum HpSA Plus test did not predict treatment outcome.

In an effort to improve culture success, this study found that using combined antral and corpus biopsies significantly improved culture yield and the use of Dent's transport media significantly reduced contamination. As such we recommend the use of combined biopsies for culture as well as the use of Dent's transport media. Further investigation into the efficacy and cost-

effectiveness of Portagerm pylori is warranted. As bacterial culture of *H. pylori* is still generally difficult, time consuming and expensive, molecular testing for *H. pylori* infection is an attractive option which is explored in Chapter 3.

Chapter 3 Detection of antibiotic resistance in *Helicobacter pylori* infection

3.1 Introduction

It is well known that the success rates of a number of *H. pylori* treatments have fallen below the recommended 80% intention-to-treat eradication rate in recent years (64, 162, 282, 350). Potential contributing factors to *H. pylori* treatment failure include low gastric pH, impaired mucosal immunity, dormant forms of *H. pylori*, and high bacterial load (162). The main contributory factors are considered to be poor patient compliance (247), and the presence of antimicrobial resistant strains of *H. pylori* (162, 163, 169, 171, 172).

As discussed in detail Section 1.5, *H. pylori* antibiotic resistance has risen in Ireland, the UK and Europe from the 1990's through to 2018. The most recent publications on resistance rates worldwide are also detailed in Section 1.5. A meta-analysis published in 2018 on the prevalence of antibiotic resistance in World Health Organisation (WHO) regions reported that primary resistance rates for clarithromycin, metronidazole and levofloxacin were 15% or higher in nearly all WHO regions investigated. Resistance rates in the European region were 18%, 32% and 11% for clarithromycin, metronidazole and levofloxacin respectively (169). The most recent published study which assessed resistance rates locally in Ireland was a decade ago (2007-2009). It found a primary resistance rate to clarithromycin of 9.3%; a significant increase on the 3.9% resistance rate found in a study done in 1997 (175, 178). Primary metronidazole resistance was 29.1% (175), while primary levofloxacin resistance was 12% (179). No resistance to tetracycline or rifabutin was found and amoxicillin resistance was not investigated (179). As antibiotic resistance is continuously evolving and resistance rates vary between geographical regions (as shown in Section 1.5), guidelines recommend local surveillance of existing and emerging antibiotic resistance in order to guide clinicians in the most appropriate therapy for a given population (64, 65, 88, 173, 174). The aim of this chapter was to investigate antibiotic resistance in our cohort and to evaluate methods of detection of resistance. Firstly, resistance in our patient cohort was investigated by culture and subsequent susceptibility testing.

As detailed in Chapter 2, *H. pylori* is a fastidious bacterium and culture can be difficult. The success rate of culture of *H. pylori* from gastric biopsy samples has been reported to be as low as 55% (128). Indeed, in Chapter 2, we reported a yield of *H. pylori* following bacterial culture of 59.4%. Results for phenotypic testing can take 7-10 days to obtain, which may affect patients who need to be treated promptly. Due to these difficulties, culture-based susceptibility testing is mostly used only after treatment failure and at that is not routinely used in all hospital laboratories in Ireland. Molecular tests are an attractive alternative to standard culture and antimicrobial susceptibility testing, a number of which are described in Section 1.6.2. Molecular

testing has been recommended to detect *H. pylori* and both clarithromycin and fluoroquinolone resistance when standard culture and sensitivity testing are not available (64, 174). Gastric biopsy samples can be rapidly analysed, with sensitivity results obtained 1-2 days following endoscopy. It is also possible for these tools to detect heteroresistance; the co-existence of *H. pylori* strains susceptible and resistant to the same antibiotic within the same patient (218). Currently molecular tests are available only for detection of resistance to clarithromycin and fluoroquinolones. The GenoType HelicoDR assay (Hain Lifescience, Nehren, Germany) is a commercially available multiplex PCR-based assay which enables the determination of resistance to clarithromycin and fluoroquinolones in DNA isolated from *H. pylori* and/or gastric biopsies. Compared to the gold standard of culture and susceptibility testing, other studies have reported sensitivity and specificity for detecting clarithromycin resistance in the range of 55-100% and 80-100% respectively; while for detecting quinolone resistance, 74-89% and 70-98.5% respectively (138, 140, 151, 222). The second aim of this chapter was to evaluate the performance of the GenoType HelicoDR assay, to provide insight into the clinical validity of molecular testing in our centre.

Currently, *H. pylori* antibiotic resistance surveillance is based primarily on patients undergoing invasive testing by means of endoscopy. However, most patients are diagnosed by non-invasive methods such as the UBT. *H. pylori* DNA has been detected in a number of clinical specimens including blood, stool samples and oral cavity specimens (146, 215, 351-353). Analysis of stool samples has shown the most promise for the molecular detection of clarithromycin resistance-mediating mutations to date (145, 215, 223-227). Studies have demonstrated sensitivity and specificity values of 83-98% and 98-100%, respectively, for the detection of clarithromycin resistance using the *H. pylori* ClariRes Assay (Ingenetix) to analyse stool samples (145, 223-225). Data on the use of molecular methods, in particular the GenoType HelicoDR assay, for the analysis of stool samples is lacking. Therefore the third aim of this chapter was to evaluate the GenoType HelicoDR assay for the detection of clarithromycin and fluoroquinolone resistance using DNA isolated from stool samples compared to biopsy samples.

H. pylori is a highly heterogeneous bacterium and its virulence varies geographically. Virulence factors such as cytotoxin associated gene A (*cagA*) and the vacuolating associated gene A (*vacA*) (which are described in Section 1.3) are also strongly associated with GI diseases such as gastric inflammation, peptic ulcer, atrophy, intestinal metaplasia, dysplasia, and malignancy (38-44, 54-56). A study conducted in 2009 in our centre determined the prevalence of virulence-factor genotypes and reported on an association between resistance to metronidazole and the absence

of *cagA* virulence factor (50). The fourth aim of this chapter was to investigate the current prevalence of virulence factor genotypes in our patient cohort. To gain further insight into the virulence of the *H. pylori* strains in our patient population, the impact of virulence factors on antibiotic resistance was investigated.

3.1.1 Aims of the study

1. To assess the prevalence of phenotypic *H. pylori* antibiotic resistance by standard culture and Etest
    a) *H. pylori* resistance to the antibiotics clarithromycin, metronidazole, levofloxacin, moxifloxacin, amoxicillin, tetracycline and rifampicin was assessed in treatment-naïve patients (primary resistance) and those previously treated (secondary resistance).

2. To assess the performance of the GenoType HelicoDR assay in the detection of *H. pylori* antibiotic resistance
    a) The GenoType HelicoDR assay was used to detect mutations associated with resistance to clarithromycin and fluoroquinolones in biopsy samples obtained from *H. pylori*-positive treatment naïve patients and those previously treated
    b) Agreement between the GenoType HelicoDR assay and culture and Etest was investigated
    c) The diagnostic accuracy of the GenoType HelicoDR assay in detecting resistance compared to culture-based methods was assessed.

3. To evaluate the potential use of the GenoType HelicoDR assay for the non-invasive detection of *H. pylori* and resistance to clarithromycin and fluoroquinolones
    a) The GenoType HelicoDR assay was used to detect *H. pylori* and genotypic resistance to clarithromycin and fluoroquinolones in stool samples
    b) Agreement between the GenoType HelicoDR results from stool samples and results from biopsy samples was investigated by directly comparing a stool DNA sample with that of a biopsy DNA sample isolated from individual patients
    c) The diagnostic accuracy of the GenoType HelicoDR assay in detecting resistance in stool samples compared to culture-based methods using biopsy samples was assessed.

4. To evaluate the impact of virulence factors on the prevalence of genotypic antibiotic resistance.
    a) The frequency/distribution of the *cagA* and *vacA* genotype in treatment naïve and previously treated *H. pylori* positive patients was assessed
    b) The potential association of virulence factor genotype and clinical phenotype via endoscopy and histology findings was assessed
    c) The relationship between virulence factor genotype and genotypic clarithromycin and fluoroquinolone resistance in treatment naïve and previously treated patients was assessed.

3.2 Methods

3.2.1 Study design

The study was carried out in a tertiary referral teaching hospital (Tallaght University Hospital, formerly the Adelaide and Meath Hospital, incorporating the National Children's Hospital, Dublin) affiliated with Trinity College Dublin. Figure 3.1 illustrates how results from each method discussed in the following sections contributed to each aim described in this chapter.

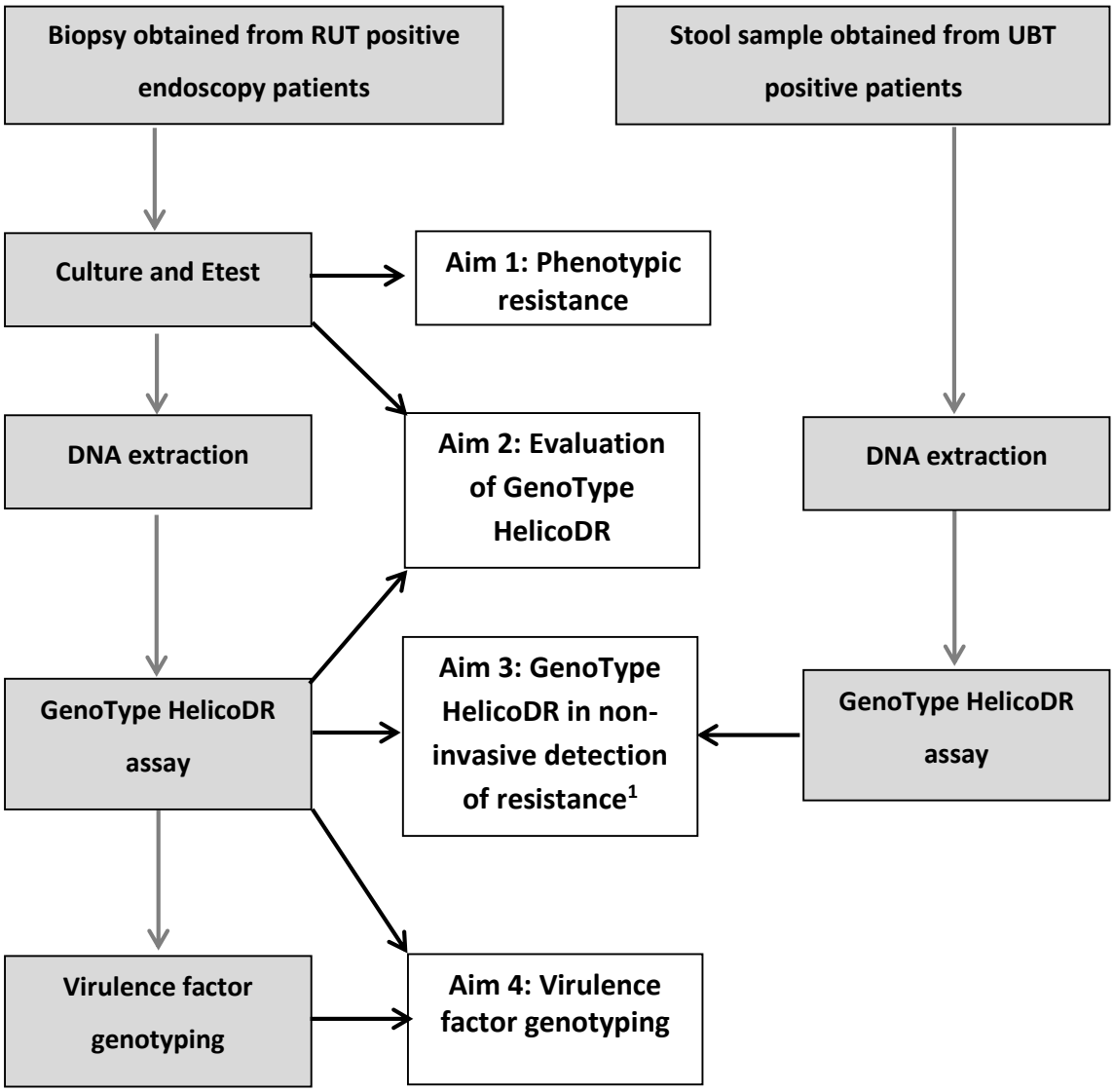


Figure 3.1: Schematic illustrating how results from each method described in this chapter contributed to aims 1-4.[1]For aim 3, stool samples were also obtained from a subset of endoscopy patients.

3.2.2 Study population

Recruited patients described in Chapter 2 were included in this study. Inclusion and exclusion criteria were as described in Chapter 2, Section 2.2.2.

3.2.3 Sample Collection

3.2.3.1 Biopsy sample collection

Biopsy samples from RUT positive patients described in Chapter 2 were included.

3.2.3.2 Stool sample collection

Stool samples from a subset of the positive UBT positive patients described in Chapter 2 were included. Additionally, a subset of *H. pylori* positive patients who had provided a biopsy sample were asked to provide a stool sample. The stool sample was taken on the morning of or the day before the patient was due to attend their hospital appointment and storage at 4 °C was recommended until transportation to the laboratory.

3.2.4 Culture of *H. pylori*

As a result of the study detailed in Section 2.3.5, the protocol for biopsy collection for culture was changed to the use of combined antral and corpus biopsies instead of a single antral biopsy. Both antral and corpus biopsies collected for bacterial culture were stored within the same transport tube and inoculated onto the same plate. Gastric biopsy samples were processed for culture as soon as possible following endoscopy, ideally within 6 hours. Culture of *H. pylori* was performed as detailed in Section 2.2.4.3. Once biopsies were processed for culture, they were stored at -20°C until DNA extraction was performed. Culture was positive if the presence of small, circular smooth colonies could be visualised on the CBA plates.

3.2.5 Phenotypic susceptibility testing

Phenotypic susceptibility to clarithromycin, metronidazole, levofloxacin, amoxicillin, tetracycline, rifampicin and moxifloxacin was tested using Etest strips (Biomerieux, Marcy l'Etoile, France). A bacterial inoculum of *H. pylori* was prepared in Maximum Recovery Diluent (MRD) (Oxoid, Basingstoke, UK) from the positive bacterial cultures. CBA plates (Fisher Scientific Ireland Ltd, Dublin, Ireland) were inoculated with the bacterial suspension using a cotton swab to produce a lawn of growth. The MICs of two reference strains were tested at the beginning of the study and once during the study (at 18 months) to validate the process (*H. pylori* NCTC11637 and NCTC11638 (ATCC43504)). Etest strips for each antibiotic to be tested were applied to a separate

plate using a sterile tweezers and plates were incubated for 72 hours at 37°C under microaerophilic conditions. MIC values were read at the point on the Etest strip where the inhibition ellipse intersects the strip, and resistance to each antimicrobial agent was determined according to the EUCAST MIC breakpoints for each agent (Table 3.1, Version 8.0 (354)).

Antibiotic	Susceptible (mg/L)	Resistant (mg/L)
Amoxicillin	≤0.125	>0.125
Clarithromycin	≤0.25	>0.5
Metronidazole	≤8	>8
Levofloxacin	≤1	>1
Rifampicin*	≤1	>1
Tetracycline	≤1	>1
Moxifloxacin	≤1	>1

Table 3.1: Proposed clinical antimicrobial MIC breakpoints for *H. pylori*. *Although rifabutin is used clinically, rifabutin Etests are not available routinely and rifampicin is used to screen for rifabutin resistance (354).

3.2.6 DNA extraction

3.2.6.1 Biopsy samples

Genomic DNA was extracted from gastric biopsies using the QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany). To lyse the tissue, the sample was placed in a 1.5 ml microcentrifuge tube, 180 µl of Buffer ATL and 20 µl of proteinase K was added. The sample was mixed using a vortexer (Heidolph Reax Top, Essex, UK) and incubated at 56 °C using a Thermomixer (Eppendorf, Hamburg, Germany) until the tissue was completely lysed. 200 µl Buffer AL was added to the sample, mixed for 15 seconds (s), and incubated at 70 °C for 10 min. 200 µl of ethanol (96-100 %) was then added and the sample was mixed. This mixture was carefully applied to the QIAamp Mini spin column. The column was then centrifuged at 6000 x g (8000 rpm) for 1 min using an Eppendorf Centrifuge 5415 (Eppendorf, Hamburg, Germany). The column was then placed in a clean 2 ml collection tube and the tube containing the filtrate was discarded. To wash the column, 500 µl Buffer AW1 was added. The tube was centrifuged at 6000 x g (8000 rpm) for 1 min. The column was again placed in a clean collection tube and the tube containing the filtrate discarded. 500 µl Buffer AW2 was added to the column. It was centrifuged at full speed (20,000 x g; 14,000 rpm) for 3 min. The column was placed in a new collection tube and centrifuged again at full speed for 1 min. To elute the extracted DNA, the spin column was

placed in a clean 1.5 ml microcentrifuge tube. 200 µl Buffer AE was added and incubated for 5 min. The tube was centrifuged at 6,000 x g (8,000 rpm) for 1 min. To further increase DNA yield, this final step was repeated. All isolated DNA was stored -20 °C until genotyping was performed.

3.2.6.2 Stool samples

The PSP® Spin Stool DNA Plus kit (STRATEC Molecular GmbH, Berlin, Germany) was used to extract DNA from stool specimens from *H. pylori* positive patients. The stool sample was transferred to a Stool Collection Tube which contained Stool DNA Stabilizer. The tube was mixed for a short time to ensure homogenization of the stool sample. 1.4 ml of stool sample was transferred into a 2.0 ml Safe-Lock Tube. For enrichment of bacterial DNA, the sample was incubated for 10 min at 95 °C at 900 rpm. 5 Zirconia Beads II were added to the solution and mixed for 2 min at room temperature (RT). The sample was centrifuged at 13,400 x g (12,000 rpm) for 1 min to pellet solid stool particles and beads. To remove PCR inhibitors, the supernatant was transferred into an InviAdsorb-Tube and vortexed vigorously for 15 sec. The suspension was incubated for 1 min at RT and centrifuged at full speed for 3 min. To ensure all PCR inhibitors were removed, the supernatant was transferred completely into a new 1.5 ml Receiver Tube and the pellet discarded. The sample was centrifuged again at full speed for 3 min. 25 µl Proteinase K was added to a new 1.5 ml Receiver Tube and 800 µl of the supernatant from the previous step added. This was mixed shortly and the sample was incubated for 10 min at 70 °C at 900 rpm. 400 µl of Binding Buffer was added to the lysate and mixed shortly. The mixture was transferred completely onto the membrane of the RTA Spin Filter, incubated for 1 min at RT and centrifuged at 9,300 x g (10,000 rpm) for 2 min. The filtrate and RTA receiver tube was discarded. To wash the membrane, the RTA Spin Filter was placed in a new RTA Receiver Tube. 700 µl of Wash buffer I was added to the membrane of the RTA Spin Filter and centrifuged at 9,300 x g (10,000 rpm) for 1 min. The filtrate and RTA receiver tube were discarded. For the second wash step, the RTA Spin Filter was placed in a new RTA Receiver Tube. 700 µl of Wash Buffer II was added to the membrane of the RTA Spin Filter and centrifuged at 9,300 x g (10,000 rpm) for 1 min. The filtrate was discarded and the RTA Spin Filter was placed back in the same Receiver Tube. To remove all traces of ethanol, the sample was centrifuged again for 3 min at maximum speed and the RTA Receiver Tube discarded. Finally to elute DNA, the RTA Spin Filter was placed into a new 1.5 ml Receiver tube and 150 µl preheated (70 °C) Elution Buffer was added. This was incubated for 5 min and centrifuged at 9,300 x g (10,000 rpm) for 1 min to elute the DNA. The RTA Spin Filter was discarded. The eluted DNA was stored long-term at -20 °C.

3.2.7 GenoType HelicoDR Assay

The GenoType HelicoDR assay procedure involved multiplex amplification with biotinylated primers, reverse hybridization and interpretation of results. Prior to performing the assay, all work areas and equipment were treated with DNA-Zap (Ambion, Winsford, UK) to degrade any potential contaminating DNA. In addition, designated work-areas, pipettes, filter tips and consumables were used for each step of the assay, with the PCR reaction mix prepared in a designated DNA-free area. Similar to the phenotypic AST, the GenoType HelicoDR assay was used to analyse known reference strains of *H. pylori* (*H. pylori* NCTC11637 and NCTC11638 (ATCC43504) at intervals throughout the project.

3.2.7.1 Multiplex amplification of DNA

Multiplex amplification of DNA regions of interest was performed using biotinylated primers supplied in the GenoType HelicoDR kit and the Hotstart Taq DNA polymerase kit (Qiagen). The number of samples to be amplified was determined and a master mix containing all reagents was prepared. Per reaction, 17.5 µL Primer Nucleotide Mix (PNM) (Hain Lifescience), 2.5 µL 10x polymerase incubation buffer, 1 µL 25mM MgCl2 solution, 0.1 µL HotStar Taq DNA polymerase and 1.5 µl H_2O was added to a 1.5 ml microcentrifuge tube. A 22.6 µl aliquot of master mix and 2.5 µl DNA solution was added to a 0.2 ml PCR tube, leading to a final volume of 25.1 µl. The samples were then placed in a PTC-200 Peltier Thermal Cycler (Biosciences, Dublin, Ireland) at the following amplification profile: 15 min at 95 °C for 1 cycle, 30 sec at 95 °C, 2 min at 58 °C for 10 cycles, 25 sec at 95 °C, 40 sec at 53 °C, 40 sec at 70 °C for 25 cycles, 8 min at 70 °C for 1 cycle.

3.2.7.2 Reverse hybridisation

PCR products were reverse hybridised to DNA strips (supplied in the GenoType HelicoDR kit) containing probes for gene regions of interest. A TwinCubator ® (Hain Lifescience) was pre-warmed to 45 °C. All reagents were pre-warmed to room temperature (RT) before use, with exception of Conjugate Concentrate (CON-C) and Substrate Concentrate (SUB-C). CON-C and SUB-C were diluted 1:100 with their respective buffers (CON-C with CON-D, SUB-C with SUB-D). 20 µl Denaturation solution (DEN) was dispensed into a corner of each of the wells used. 20 µl of amplification solution was added, mixed well and incubated at RT for 5 min. 1 ml of pre-warmed Hybridization Buffer (HYB) was added to each well. The tray was gently shaken until a homogenous colour was obtained. A Hybridization strip was placed in each well. The tray was placed in the TwinCubator® and incubated for 30 min at 45 °C. The HYB was completely aspirated. 1 ml of Stringent Wash Solution (STR) was added to each well and incubated for 15

min at 45 °C. The STR was completely removed. Each strip was washed once with Rinse Solution (RIN) for 1 min. The RIN was completely removed and 1 ml of diluted Conjugate was added to each strip and incubated for 30 min. The solution was removed and each strip was washed twice for 1 min with 1 ml of RIN and once for 1 min with 1 ml of distilled water. All traces of water were removed from each well. 1 ml of diluted substrate was added to each strip and incubated protected from light for 3-20 min. The reaction was stopped by rinsing twice with distilled water. The strips were removed from the wells and dried.

3.2.7.3 Interpretation of results

All strips were analysed for the presence of a conjugate control band (to indicate successful conjugate binding and substrate reaction), an amplification control band (to indicate a successful amplification reaction), a *H. pylori* control band (to document the presence of a *H. pylori* strain) and gene locus control bands for gyrA and 23S (to indicate successful detection of the gene regions of interest). If *H. pylori* DNA was detected in the negative control, any results from that experiment were considered invalid and the samples re-analysed. In addition, the strips were analysed for the presence of wild type and/or mutation bands. An infection was considered clarithromycin sensitive when the 23S wild-type probe stained positive and clarithromycin resistant if one of the 23S mutation probes stained positive. As per manufacturers' instructions, results of both positions of the gyrA gene were combined to draw conclusions about fluoroquinolone resistance. Thus, an infection was only considered fluoroquinolone sensitive when one of the wild-type probes for codon 87 of the gyrA gene stained positive together with a positive wild-type probe for codon 91. Fluoroquinolone resistance was indicated if either the wild-type probes for codon 87 or the wild-type probe for codon 91 stained negative, or if one of the mutant codon 87 or 91 probes stained positive. For all mutations probes, only bands whose intensities were equal to or stronger than the amplification control were considered positive. Heteroresistance for either clarithromycin or fluoroquinolone resistance was indicated if both a wild-type and mutant probe stained positive.

3.2.8 Virulence factor genotyping

To evaluate the impact of virulence factors on the prevalence of antibiotic resistance, PCR-based virulence factor genotyping was performed on DNA extracted from biopsies obtained from *H. pylori* positive patients. To investigate the impact of virulence factor genotype on clinical phenotype, histology and endoscopy findings from patients included in this study were reviewed. Previously described specific primer sets (50) (Invitrogen, Biosciences, Dublin, Ireland) were used and are presented in Table 3.2. CAGA forward (CAGA-F) and CAGA reverse (CAGA-R)

primers were used to amplify a 349-bp product from the middle of *cagA.* VA1 forward (VA1-F) and VA1 reverse (VA1-R) primers were used to amplify a 259-product for s1 and a 286-product for s2 from the signal region of *vacA*, and VAG forward (VAG-F) and VAG reverse (VAG-R) were used to amplify a 567-bp product from m1 and a 642-bp product for m2 from the middle of *vacA*.

Primer	Primer sequence[1]	Gene	Product size (bp)[2]
CAGA-F	5'-GATAACAGGCAAGCTTTTGATG-3'	*cagA*	349
CAGA-R	5'-CTGCAAAAGATTGTTTGGCAGA-3'		
VA1-F	5'- ATGGAAATACAACAACAAACACAC-3'	*vacA* signal region	259/286 (s1/s2)
VA1-R	5' – CTGCTTGAATGCGCCAAAC-3'		
VAG-F	5' – CAATCTGTCCAATCAAGCGAG-3'	*vacA* middle region	567/642 (m1/m2)
VAG-R	5'- GCTTCAAAATAATTCCAAGG-3'		

Table 3.2: Polymerase chain reaction primers used in this study. [1]A- adenine; T- thymine; G- guanine; C- cytosine; [2]bp- base pairs.

In addition to the primers, a PCR kit (Roche PCR Kit Plus, Roche Diagnostics Limited, West Sussex, United Kingdom) was used which contained the necessary components to carry out the PCR reaction. All kit components were thawed on ice, vortexed and centrifuged before use. A master-mix for each gene of interest was prepared in a DNA-free area and stored on ice. The components, amounts and their respective final concentrations in the master-mix are detailed in Table 3.3. To prevent contaminating carryover from previous PCR reactions, Uracil-N-glycosylase (UNG) was included in the master-mix.

Reagents	Volume (adjusted to 50µl)	Final Concentration
Water (H_2O)	32.75 µl	
dNTP[1] mix	1 µl	200 µM dATP
		200 µM dCTP
		200 µM dGTP
		600 µM dUTP
Forward primer	2.5 µl	0.5 µM
Reverse primer	2.5 µl	0.5 µM
Taq DNA polymerase (5U/µl)	0.25 µl	2.5U
UNG[2], heat-labile (1U/ µl)	1 µl	2U
PCR buffer with	5 µl	2.5 mM $MgCl_2$
25 mM $MgCl_2$, 10 x conc.		
DNA Template	5 µl	

Table 3.3: Components, amounts and final concentrations of the PCR master mix for each gene of interest. [1]dNTP- nucleoside triphosphate; [2]UNG- Uracil-N-glycosylase.

The DNA template was added to the master-mix in a separate area, mixed well and centrifuged. The master-mix was incubated at room temperature for 2 mins to allow the UNG reaction to occur. The samples were placed in a PTC-200 Peltier Thermal Cycler (Biosciences, Dublin, Ireland) at the amplification profile detailed in Table 3.4. The final cycle included a seven minute extension step to ensure full extension of the PCR product.

	Temperature	Time	Cycles
UNG inactivation	95°C	2 min	1 x
Denaturation	95°C	10 s	
Annealing	53°C	30 s	30 x
Elongation	72°C	60 s	
Elongation time	72°C	7 min	1 x
Held at	4°C	*no longer than 8 h*	

Table 3.4: Amplification profile of PCR reaction

3.2.8.1 Agarose Gel electrophoresis

PCR products were then separated by gel electrophoresis using 1% agarose gel. The gel apparatus was assembled for gel casting. A 1% agarose gel was prepared by dissolving 1 g agarose (Fisher Scientific Ireland, Dublin, Ireland) per 100 ml 1X TAE (Fisher Scientific Ireland, Dublin, Ireland) by heat. The mixture was allowed to cool a little. Ethidium Bromide (Fisher Scientific Ireland, Dublin, Ireland) was added to achieve a final concentration of 0.5 µg/ ml. The gel was poured and allowed to set. The PCR samples were prepared for gel loading by adding 4 µl 6X gel loading dye (Fisher Scientific Ireland, Dublin, Ireland) to 20 µl PCR product. Once the gel had set, 1X TAE was poured into the apparatus to cover the gel and the comb was removed. To enable accurate identification of PCR products, 10 µl of 100 base pair ladder (Fisher Scientific Ireland, Dublin, Ireland) was added to the first well of the gel. Next, 15 µl of each PCR sample was added to the remaining wells.

The gel was run at 100V on a Biometra standard power pack P25 (Analytikjena, Germany) for approximately 40 min or until the lower bands could be seen 3/4 way down the gel. The gels were visualised under UV light (Omega Lum G, Aplegen, CA, USA). The presence or absence of the *cagA* gene and *vacA* genotype was evaluated by comparing the bands of the separated PCR products to the base-pair ladder. A strain was deemed to be *cagA* positive if the presence of a band at 349 base pairs was observed. The signal region of the *vacA* gene was deemed to be either s1 or s2 type when a band of either 259 or 286 base pairs was present respectively. The middle region of the *vacA* gene was deemed to be either m1 or m2 type when a band of either 567 or 642 base pairs was present respectively.

3.2.9 Statistical analysis

Statistical analysis was carried out using GraphPad Prism (GraphPad Software Inc., CA, USA). Continuous variables are presented as arithmetic mean and SD. Groups were compared using the two-tailed independent t-test (for comparison of two groups). P values less than 0.05 were considered to indicate statistically significant differences between groups. Categorical variables are presented as percentages and 95% confidence intervals (95% CIs). *P* values for categorical variables were calculated using the Fisher's exact test/ Pearson $\chi 2$-test. To evaluate the accuracy of the GenoType HelicoDR assay, the sensitivity, specificity, PPV and NPV (and 95% CIs) were calculated using GraphPad Prism.

3.3 Results

3.3.1 Patient inclusion

Biopsy samples from the RUT positive patients described in Section 2.3.4 were included in this study.

3.3.2 Phenotypic *H. pylori* antibiotic resistance

As detailed in Section 2.3.5, samples from 219 RUT positive patients were inoculated for *H. pylori* culture. The bacterial culture success rate was 59.4% (n=130/219). Patient demographics of the culture-positive patients are included in Supplementary Table 3.1 (Appendix 3). The number of patients who were treatment naïve was 72 (55.4%, mean age 51.3±5.8 years, 72.2% male), while 58 (44.6%, mean age 47±13.8, 36.2% male) had received treatment for *H. pylori* previously (Table 3.5).

Overall, primary and secondary resistance rates are presented in Table 3.5. To obtain primary and secondary rates of resistance, *H. pylori* strains were separated based on whether the patient had received treatment for *H. pylori* infection previously. Primary resistance of *H. pylori* (in strains isolated from previously untreated patients) was 36.1% (95% CI 26.0-47.6) for clarithromycin, 56.9% (95% CI 45.4-67.7) for metronidazole, 16.7% (95% CI 9.8-26.9) for levofloxacin, 22.2% (95% CI 14.2-33.1) for amoxicillin, 18.1% (95% CI 10.9-28.5) for tetracycline and 11.1% (95% CI 5.7-20.4) for rifampicin (Table 3.5). Clarithromycin resistance was significantly higher in those previously treated for *H. pylori* than in treatment naïve patients (70.7% vs 36.1% respectively; p=0.0001).

Antibiotic	Number of resistant strains n (%)			p value[1]
	All patients	**Treatment Naïve**	**Previously treated**	
	130 (100)	**72 (55.4)**	**58 (44.6)**	
	95% CI	**95% CI**	**95% CI**	
Clarithromycin	67 (51.5) (43.0–60.0)	26 (36.1) (26.0-47.6)	41 (70.7) (58.0-80.8)	<0.05[2]
Metronidazole	78 (60.0) (51.4-68.0)	41 (56.9) (45.4-67.7)	37 (63.8) (50.9-74.9)	0.5
Levofloxacin	22 (16.9) (11.4-24.3)	12 (16.7) (9.8-26.9)	10 (17.2) (9.6-28.9)	1.0
Amoxicillin	25 (19.2) (13.4-26.8)	16 (22.2) (14.2-33.1)	9 (15.5) (8.4-26.9)	0.4
Tetracycline	19 (14.6) (9.6-21.7)	13 (18.1) (10.9-28.5)	6 (10.3) (4.8-20.8)	0.3
Rifampicin	13 (10.0) (5.9-16.4)	8 (11.1) (5.7-20.4)	5 (8.6) (3.7-18.6)	0.8

Table 3.5: Phenotypic resistance rates of isolated *H. pylori* strains to commonly used antibiotics. [1]comparison of resistance rates in treatment naïve versus previously treated patients by Fisher's exact test; [2]p=0.0001.

3.3.2.1 The pattern of phenotypic drug resistance

The pattern of drug resistance in *H. pylori* strains successfully cultured from gastric biopsy specimens is shown in Table 3.6. Combined resistance to clarithromycin and metronidazole was present in 39.2% of strains (95% CI 31.3- 47.8) and combined resistance to clarithromycin and levofloxacin was present in 12.3% of strains (95% CI 7.7- 19.1). The rate of combined resistance

to clarithromycin and metronidazole was significantly higher in those treated previously than in treatment naïve patients (50% vs 30.5% respectively; p=0.03).

	Number of strains n (%)			p value[1]
	All patients	**Treatment Naïve**	**Previously treated**	
	130 (100)	**72 (55.4)**	**58 (44.6)**	
	95% CI	**95% CI**	**95% CI**	
Susceptible to all	**24 (18.5)**	**18 (25.0)**	**6 (10.3)**	0.04
	(12.7- 26.0)	**(16.4- 36.1)**	**(4.8- 20.8)**	
Mono-resistance	**43 (33.1)**	**25 (34.7)**	**18 (31.0)**	0.7
	(25.6- 41.5)	**(24.8- 46.2)**	**(20.6- 43.8)**	
Clarithromycin	13 (30.2)	4 (16.0)	9 (50.0)	0.02
	(18.6-45.1)	(6.4- 34.7)	(29.0- 71.0)	
Metronidazole	20 (46.5)	13 (52.0)	7 (38.9)	0.5
	(32.5- 61.1)	(33.5- 70.0)	(20.3- 61.4)	
Levofloxacin	2 (4.7)	1 (4.0)	1 (5.6)	1.0
	(0.8- 15.5)	(0.2- 19.5)	(0.3- 25.8)	
Amoxicillin	2 (4.7)	2 (8.0)	0 (0)	0.5
	(0.8- 15.5)	(1.4- 25.0)	(0- 17.6)	
Tetracycline	0 (0)	0 (0)	0 (0)	NA
	(0-8.2)	(0- 13.3)	(0- 17.6)	
Rifampicin	6 (14.0)	5 (20.0)	1 (5.6)	0.4
	(16.6- 27.3)	(8.9- 39.1)	(0.3- 25.8)	
Dual resistance	**67 (51.5)**	**30 (41.7)**	**37 (63.8)**	
	(43.0- 60.0)	**(31.0- 53.2)**	**(50.9- 74.9)**	
Clarithromycin & metronidazole	51 (39.2)	22 (30.6)	29 (50.0)	0.03
	(31.2- 47.8)	(21.1- 42.0)	(37.5- 62.5)	
Clarithromycin & levofloxacin	16 (12.3)	8 (11.1)	8 (13.8)	0.8
	(7.7- 19.1)	(5.7- 20.4)	(7.2- 24.9)	
Multiple resistance	**63 (48.5)**	**29 (40.3)**	**34 (58.6)**	0.05
	(40.0- 57.0)	**(29.7- 51.8)**	**(45.8- 70.4)**	
R[2] to 2 antibiotics	36 (57.1)	14 (48.3)	22 (64.7)	0.2
	(44.9- 68.6)	(31.4 – 65.6)	(47.9- 78.5)	
R to 3 antibiotics	9 (14.3)	4 (13.8)	5 (14.7)	1.0
	(7.7- 25.0)	(5.5- 30.6)	(6.4- 30.1)	
R to 4 antibiotics	9 (14.3)	5 (17.2)	4 (11.8)	0.7
	(7.7- 25.0)	(7.6- 34.5)	(4.7- 26.6)	
R to 5 antibiotics	8 (12.7)	5 (17.2)	3 (8.8)	0.5
	(6.6- 23.1)	(7.6- 34.5)	(3.0- 23.0)	
R to 6 antibiotics	1 (1.6)	1 (3.4)	0 (0)	0.5
	(0.1- 8.5)	(0.2- 17.2)	(0- 10.2)	

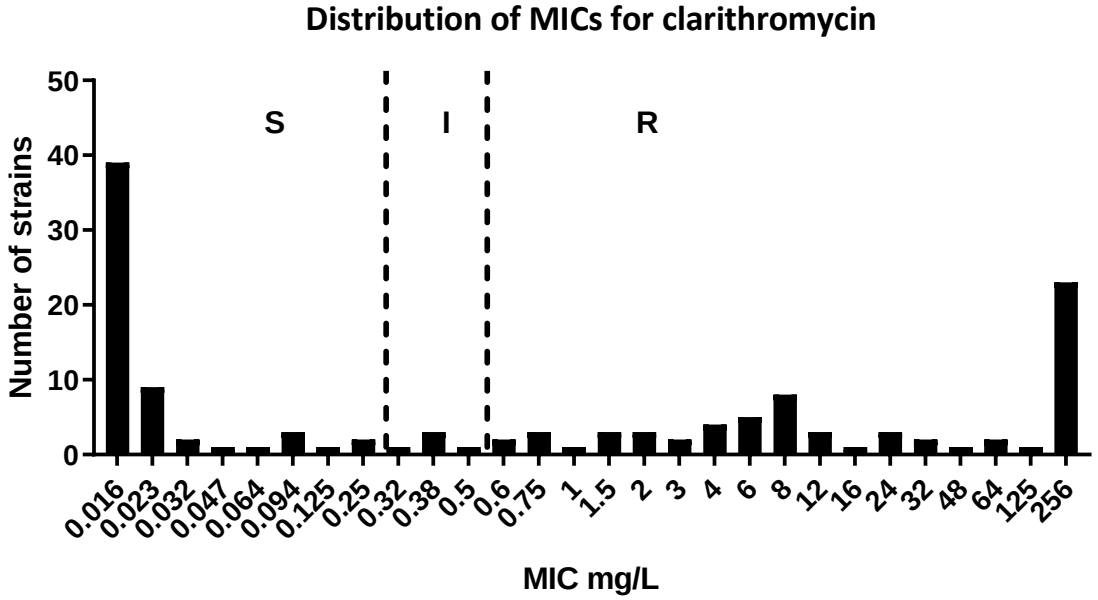
Table 3.6: Phenotypic resistance pattern in *H. pylori* strains. [1]comparison of resistance rates in treatment naïve versus previously treated patients by Fisher's exact test; [2]R= Resistant

3.3.2.2 Distribution of MICs for each antibiotic

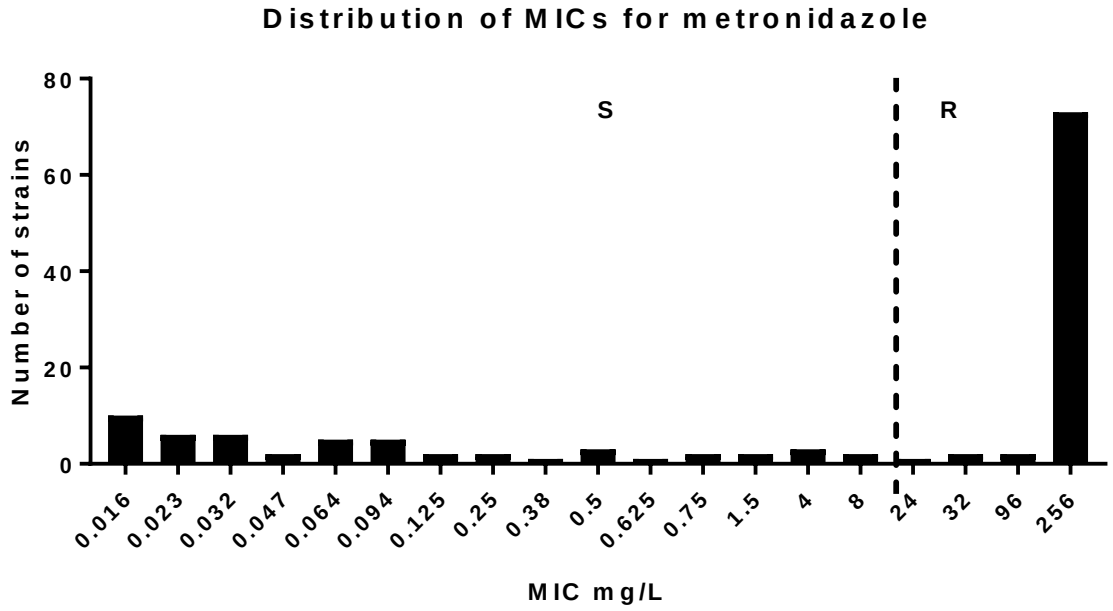
The distribution of all MIC values for each antibiotic is shown in Figure 3.2 (a-f). Of the 63 strains susceptible to clarithromycin, 61.9% had an MIC of ≤0.016 mg/L (n=39/63). Of the 67 strains that were resistant to clarithromycin, 46.3% (n=31/67) had low MIC values of >0.5 to ≤8 mg/L. For metronidazole, 93.6% resistant strains exhibited high level resistance of ≥256 mg/L (n=73/78). For amoxicillin, 64% of resistant strains (n= 16/25) had low MIC values of 0.16-0.5 mg/L. MIC values to levofloxacin, rifampicin and tetracycline ranged widely: the most common concentrations were 0.032 mg/L (15.4%, n=20/130), 0.5 mg/L (15.4%, n=20/130) and 0.016 mg/L (29.2%, n=38/130) respectively.

The average MIC of clarithromycin in treatment naïve patients was significantly lower that of previously treated patients (24.5 vs 81.8 mg/L; p=0.007). Average MIC's of all other antibiotics tested did not vary according to treatment status.

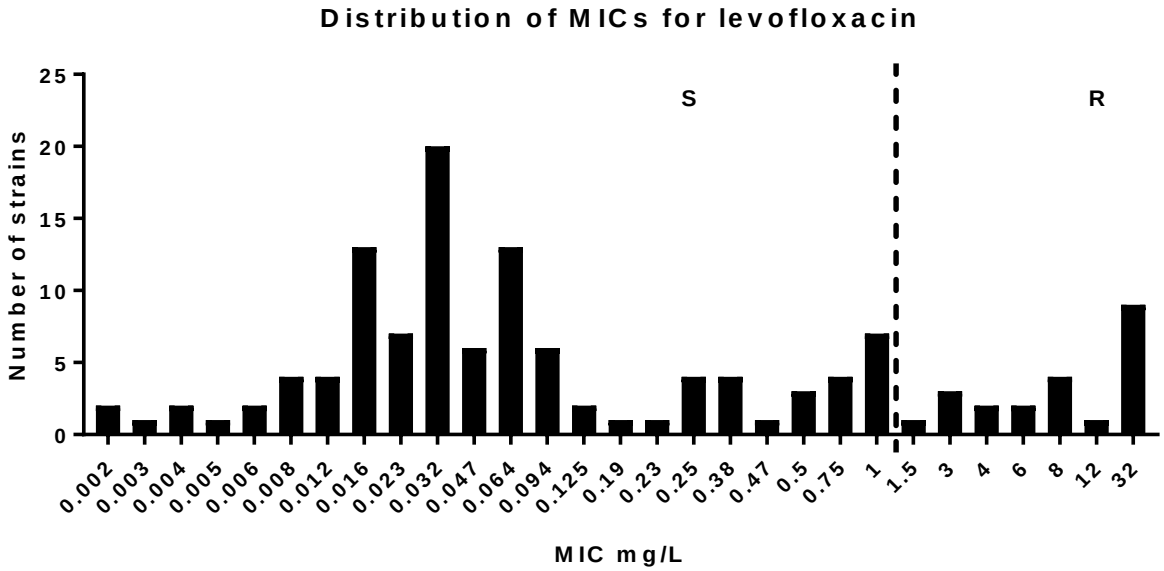
3.2(a)


Distribution of MICs for clarithromycin
Number of strains
50
40
30
20
10
0
S
I
R
0.016
0.023
0.032
0.047
0.064
0.094
0.125
0.25
0.32
0.38
0.5
0.6
0.75
1
1.5
2
3
4
6
8
12
16
24
32
48
64
125
256
MIC mg/L

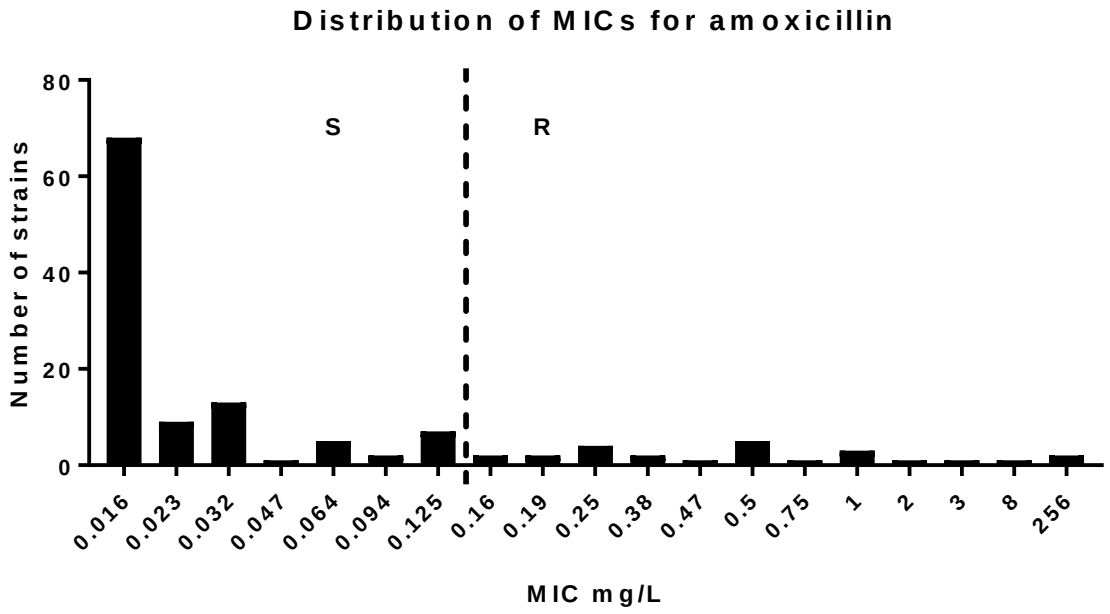
3.2(b)


Distribution of MICs for metronidazole
Number of strains
80
60
40
20
0
S
R
0.016
0.023
0.032
0.047
0.064
0.094
0.125
0.25
0.38
0.5
0.625
0.75
1.5
4
8
24
32
96
256
MIC mg/L

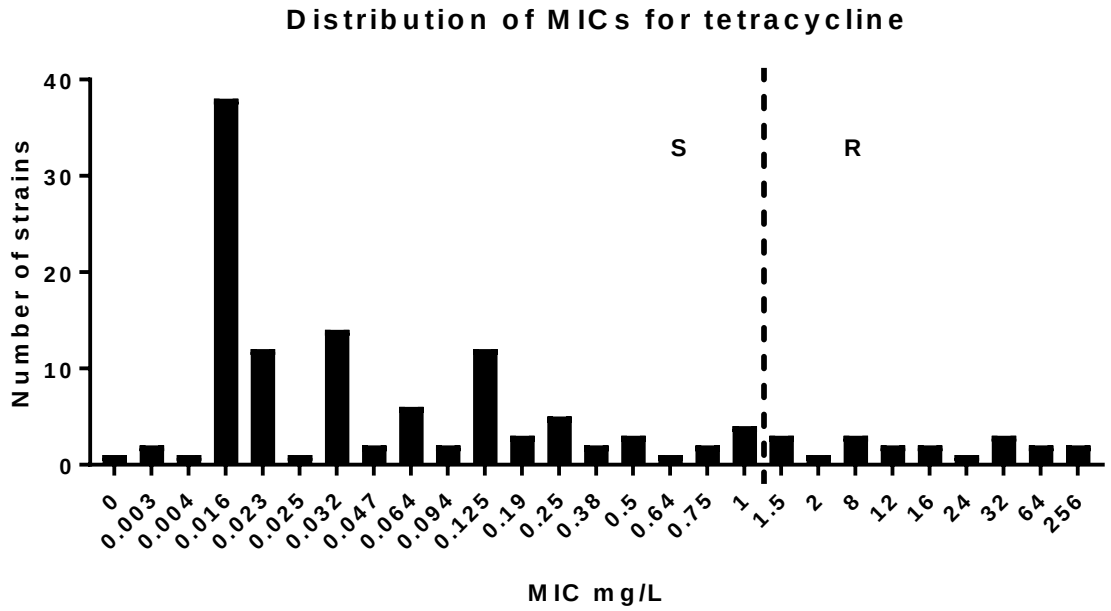
3.2(c)


Distribution of MICs for levofloxacin
Number of strains
25
20
15
10
5
0
S
R
0.002
0.003
0.004
0.005
0.006
0.008
0.012
0.016
0.023
0.032
0.047
0.064
0.094
0.125
0.19
0.23
0.25
0.38
0.47
0.5
0.75
1
1.5
3
4
6
8
12
32
MIC mg/L

3.2(d)


Distribution of MICs for amoxicillin
Number of strains
80
60
40
20
0
S
R
0.016
0.023
0.032
0.047
0.064
0.094
0.125
0.16
0.19
0.25
0.38
0.47
0.5
0.75
1
2
3
8
256
MIC mg/L

3.2(e)



3.2(f)

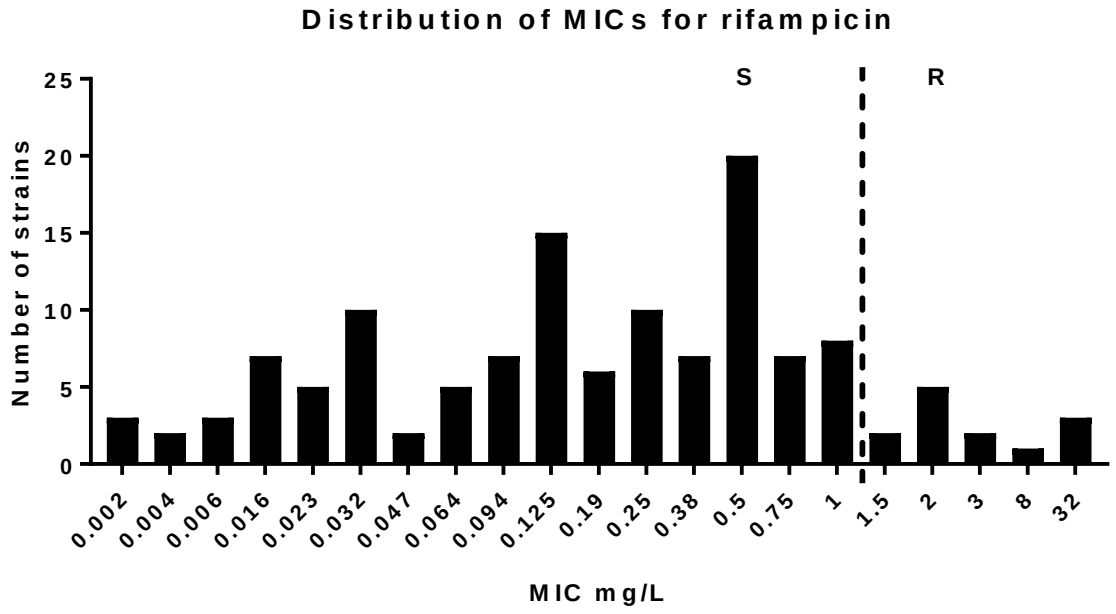


Figure 3.2: Distribution of minimum inhibitory concentrations of (a) clarithromycin, (b) metronidazole, (c) levofloxacin, (d) amoxicillin, (e) tetracycline and (f) rifampicin. Dashed lines depict the EUCAST clinical breakpoints for *H. pylori* proposed in 2018 (354).

3.3.2.3 The association between gender and age and presence of phenotypic resistance

The association between gender and age on overall, primary and secondary phenotypic resistance was investigated (Tables 3.7, 3.8 and 3.9 respectively). According to current guidelines, patients under 45 years are managed by the "test and treat" strategy and patients over 45 years (who present with alarm symptoms) are managed by "endoscope and treat". Therefore to investigate the impact of age on resistance, patients were divided into those under 45 years and over 45 years. Regarding overall resistance, females were significantly more likely to be infected with clarithromycin resistant strains of *H. pylori* than males (64.9% vs 41.1%; p=0.008, Table 3.7). There was no association between gender or age and overall resistance to all other antibiotics tested (Table 3.7). Regarding both primary and secondary phenotypic resistance, there was no association between gender or age and resistance to all antibiotics tested (Tables 3.8 and 3.9 respectively).

	n	CLAR-R	p	MET-R	p	LEVO-R	p	AMOX-R	p	TET-R	p	RIF-R	p
	130		value[1]		value[1]		value[1]		value[1]		value[1]		value[1]
Gender													
Male	73	30 (41.1%)	0.008	40 (54.8%)	0.2	10 (13.7%)	0.3	16 (21.9%)	0.7	12 (16.4%)	0.6	7 (9.6%)	1.0
Female	57	37 (64.9%)		38 (66.6%)		12 (21.1%)		9 (15.8%)		7 (12.3%)		6 (10.5%)	
Age													
>45 years	74	38 (51.4%)	1.0	46 (62.2%)	0.7	11 (14.9%)	0.5	13 (17.6%)	0.5	10 (13.5%)	0.8	8 (10.8%)	1.0
<45 years	56	29 (52.7%)		32 (58.2%)		11 (20%)		12 (21.4%)		9 (16.4%)		5 (9.1%)	

Table 3.7: Association of gender and age (>45 or <45 years) with overall phenotypic resistance to commonly prescribed antibiotics in Ireland. CLAR-R: Clarithromycin resistant, MET-R: Metronidazole resistant, LEVO-R: Levofloxacin resistant, AMOX-R: Amoxicillin resistant, TET-R: Tetracycline resistant, RIF-R: Rifampicin resistant. [1]Fisher's exact test.

	n	CLAR-R	p value[1]	MET-R	p value[1]	LEVO-R	p value[1]	AMOX-R	p value[1]	TET-R	p value[1]	RIF-R	p value[1]
	72												
Gender													
Male	52	16 (30.8%)	0.2	29 (55.8%)	0.8	8 (15.4%)	0.7	13 (25%)	0.5	9 (17.3%)	0.7	6 (11.5%)	1.0
Female	20	10 (50%)		12 (60%)		4 (20%)		3 (15%)		4 (20%)		2 (10%)	
Age													
>45 years	42	13 (31%)	0.3	24 (57.1%)	1.0	5 (11.9%)	0.2	7 (16.6%)	0.3	8 (19%)	1.0	5 (11.9%)	1.0
<45 years	30	13 (43.3%)		17 (56.6%)		7 (23.3%)		9 (30%)		5 (16.6%)		3 (10%)	

Table 3.8: Association of gender and age (>45 or < 45 years) with primary phenotypic resistance to commonly prescribed antibiotics in Ireland. CLAR-R: Clarithromycin resistant, MET-R: Metronidazole resistant, LEVO-R: Levofloxacin resistant, AMOX-R: Amoxicillin resistant, TET-R: Tetracycline resistant, RIF-R: Rifampicin resistant. [1]Fisher's exact test.

	n	CLAR-R	p value[1]	MET-R	p value[1]	LEVO-R	p value[1]	AMOX-R	p value[1]	TET-R	p value[1]	RIF-R	p value[1]
	58												
Gender													
Male	21	14 (66.6%)	0.8	11 (52.4%)	0.3	2 (9.5%)	0.3	3 (14.3%)	1.0	3 (14.3%)	0.7	1 (4.8%)	0.6
Female	37	27 (73%)		26 (70.3%)		8 (21.6%)		6 (16.2%)		3 (8.1%)		4 (10.8%)	
Age													
>45 years	32	25 (78.1%)	0.2	22 (68.8%)	0.4	6 (18.8%)	1.0	6 (18.8%)	0.5	2 (6.3%)	0.4	3 (9.4%)	1.0
<45 years	26	16 (61.5%)		15 (57.7%)		4 (15.4%)		3 (11.5%)		4 (15.4%)		2 (7.7%)	

Table 3.9: Association of gender and age (>45 or < 45 years) with secondary phenotypic resistance to commonly prescribed antibiotics in Ireland. CLAR-R: Clarithromycin resistant, MET-R: Metronidazole resistant, LEVO-R: Levofloxacin resistant, AMOX-R: Amoxicillin resistant, TET-R: Tetracycline resistant, RIF-R: Rifampicin resistant. [1]Fisher's exact test.

3.3.2.4 The resistance rates of moxifloxacin versus levofloxacin

The efficacy of moxifloxacin in inhibiting growth of *H. pylori* compared to levofloxacin was explored by comparing the MICs of levofloxacin and moxifloxacin. Antimicrobial susceptibility testing for moxifloxacin was performed on a subset of samples (n=62; mean age 48.3±14.5 years, 58.1% male) and results were compared to levofloxacin results from the same patients. Levofloxacin and moxifloxacin resistance rates in treatment naïve and previously treated patients are presented in Table 3.10. The mean MICs of levofloxacin and moxifloxacin in all strains and in susceptible strains are presented in Table 3.11.

	Levofloxacin n (%)	**Moxifloxacin n (%)**	**p value[1]**
All patients n=62 (100)	15 (24.2)	10 (16.1)	0.7
Treatment Naïve n=34 (54.8)	7 (20.5)	5 (14.7)	0.8
Previously treated n=28 (44.2)	8 (28.6)	5 (17.9)	0.5

Table 3.10: Levofloxacin and moxifloxacin resistance rates in treatment naïve and previously treated patients. [1]Comparison of resistance to levofloxacin and moxifloxacin in treatment naïve and previously treated patients by Fisher's exact test.

	Mean levofloxacin MIC (mg/l)	**Mean moxifloxacin MIC (mg/l)**	**p value[1]**
All strains (mg/l)	4.5	3.0	0.4
Susceptible strains only (mg/l)	0.2	0.1	0.1

Table 3.11: Mean MICs of levofloxacin and moxifloxacin in all strains and in susceptible strains. [1]unpaired t-test

In summary, following phenotypic susceptibility testing, primary resistance rates of 36.1%, 59.9%, 16.7%, 22.2%, 18.1% and 11.1% were found for the antibiotics clarithromycin, metronidazole, levofloxacin, amoxicillin, tetracycline and rifampicin, respectively. A higher proportion of strains isolated from previously treated patients displayed multiple resistance than in treatment-naïve patients (58.6% vs 40.3%, p=0.05). Regarding distributions of MICs; 46.3% of clarithromycin resistant strains and 64% of amoxicillin resistant strains had low MIC

values, whereas 93.6% metronidazole resistant strains had a high MIC value. MIC values to levofloxacin, rifampicin and tetracycline ranged widely. Overall, females were significantly more likely to be infected with clarithromycin resistant strains of *H. pylori* than males (64.9% vs 41.1%; p=0.008). Average MICs and both primary and secondary resistance to moxifloxacin was lower than that of levofloxacin, although not significant at the number tested.

3.3.3 Performance of the GenoType HelicoDR assay in the detection of *H. pylori* antibiotic resistance

3.3.3.1 Genotypic resistance to clarithromycin and fluoroquinolones

Biopsy samples from 251 RUT positive patients were analysed using the GenoType HelicoDR assay. *H. pylori* DNA was detected in 241 (96%) samples, which is significantly higher than the detection of *H. pylori* by culture (96%, n=241/251 vs 59.4%, n=130/219; p=0.0001). The number of patients who had not been treated previously was 138 (57.3%, mean age 51.1 ±16.5 years, 63.0% male), while 103 (47.2%, mean age 49.2 ±15.8, 35% male) had received treatment for *H. pylori* previously. Genotypic resistance rates for clarithromycin and fluoroquinolones are presented in Tables 3.12 and 3.13 respectively.

Primary resistance rates to clarithromycin and fluoroquinolones were 58.0% (95% CI 49.6- 65.9) and 15.2% (95% CI 10.2- 22.2) respectively. In those previously treated for *H. pylori* infection, the resistance rates for both clarithromycin and fluoroquinolones were higher at 85.4% (95% CI 77.2- 91.1) and 24.3% (95% CI 17- 33.4), respectively. Among patients infected with a clarithromycin-resistant strain, the most common point mutation was A2147G, at 70.8% (95% CI 63.5- 77.2; Table 3.12). The most common point mutation conferring resistance to fluoroquinolones in resistant patients was gyr91 D91Y, at 54.3% (95% CI 40.2- 67.9; Table 3.13).

The pattern of genotypic resistance is presented in Table 3.14. Although not significant, dual resistance for clarithromycin and fluoroquinolones was higher in those previously treated compared to treatment-naïve patients (22.3% vs 13.0%; p=0.08).

Genotype	Number of gastric biopsy specimens n (%)			p value[1]
	All patients	**Treatment Naïve**	**Previously treated**	
	241 (100)	**138 (57.3)**	**103 (42.7)**	
Susceptible to clarithromycin (WT)				
	73 (30.3)	**58 (42.0)**	**15 (14.6)**	**<0.01[2]**
Resistant to clarithromycin				
	168 (69.7)	**80 (58.0)**	**88 (85.4)**	
Point mutations				
A2147G	119 (70.8)	57 (71.3)	62 (70.5)	
A2146G	10 (6.0)	3 (3.8)	7 (8.0)	
A2146C	13 (7.7)	7 (8.8)	6 (6.8)	
A2146C + A2147G	16 (9.5)	11 (13.8)	5 (5.7)	
A2146G + A2147G	8 (4.8)	2 (2.5)	6 (6.8)	
A2146G + A2146C	1 (0.6)	0 (0.0)	1 (1.1)	
A2146G + A2146C +A2147G	1 (0.6)	0 (0.0)	1 (1.1)	

Table 3.12: The rates of genotypic clarithromycin resistance and the distribution of resistance-mediating mutations among patient samples. [1]comparison of resistance rates in treatment naïve versus previously treated patients by Fisher's exact test, [2]p=0.000003.

Genotype	Number of gastric biopsy specimens n (%)			p value[1]
	All patients	**Treatment**	**Previously**	
	241	**Naïve**	**treated**	
	(100)	**138 (57.3)**	**103 (42.7)**	
Susceptible to fluoroquinolones (WT)				
	195 (80.9)	**117 (84.8)**	**78 (75.7)**	
Resistant to fluoroquinolones				**0.1**
	46 (19.1)	**21 (15.2)**	**25 (24.3)**	
Point mutations				
*gyr*91 D91Y	25 (54.3)	13 (61.9)	12 (48)	
*gyr*91 D91N	8 (17.4)	2 (9.5)	6 (24)	
*gyr*91 D91G	3 (6.5)	0 (0.0)	3 (12)	
gyr91 D91N +*gyr91* D91G	2 (4.3)	1 (4.8)	1 (4)	
gyr91 D91N +*gyr91* D91Y	3 (6.5)	2 (9.5)	1 (4)	
gyr87 N87K	2 (4.3)	2 (9.5)	0	
gyr87 N87K +*gyr91* D91N +*gyr91* D91G	1 (2.2)	0 (0.0)	1 (4)	
gyr87 N87K +*gyr91* D91N + *gyr91* D91G +*gyr91* D91Y	1 (2.2)	1 (4.8)	0	
gyr87 N87K +*gyr91* D91N	1 (2.2)	0 (0.0)	1 (4)	

Table 3.13: The rates of genotypic fluoroquinolone resistance and the distribution of resistance-mediating mutations among patient samples. [1]comparison of resistance rates in treatment naïve versus previously treated patients by Fisher's exact test.

Genotype	Number of gastric biopsy specimens n (%)			p value[1]
	All patients 241 (100)	**Treatment Naïve 138 (57.3)**	**Previously treated 103 (42.7)**	
Susceptible (to both)	68 (28.2)	55 (39.9)	13 (12.6)	<0.01[2]
Resistant (to at least one)	173 (71.8)	83 (60.1)	90 (87.4)	
Susceptible/resistant to one	200 (83)	120 (87.0)	80 (77.7)	0.08
Resistant to both	41 (17)	18 (13.0)	23 (22.3)	

Table 3.14: Genotypic antimicrobial susceptibility results for both clarithromycin and fluoroquinolone. [1]comparison of resistance rates in treatment naïve versus previously treated patients by Fisher's exact test; [2]p=0.000003

3.3.3.2 GenoType HelicoDR resistance rates versus phenotypic resistance rates

Table 3.15 illustrates the comparison of GenoType HelicoDR resistance rates versus phenotypic resistance rates. There was a significant difference in primary genotypic and phenotypic resistance to clarithromycin (58% vs 36.1%; p=0.004). A significant difference in clarithromycin resistance was also observed between the 2 methods in assessing secondary resistance rates (85.4% vs 70.7%; p=0.04). No difference was found in genotypic and phenotypic resistance to levofloxacin and moxifloxacin (by either primary or secondary resistance).

	GenoType HelicoDR % (n)	Culture & Etest % (n)	p value[1]
Clarithromycin			
Primary resistance	58% (80/138)	36.1% (26/72)	0.004
Secondary resistance	85.4% (88/103)	70.7% (41/58)	0.04
Levofloxacin			
Primary resistance	15.2% (21/138)	16.7% (12/72)	0.8
Secondary resistance	24.3% (25/103)	17.2% (10/58)	0.3
Moxifloxacin			
Primary resistance	15.2% (21/138)	14.7% (5/34)	1.0
Secondary resistance	24.3% (25/103)	17.9% (5/28)	0.6

Table 3.15: Comparison of GenoType HelicoDR resistance rates versus phenotypic resistance rates in both primary and secondary resistance. [1]comparison of resistance rates in treatment naïve versus previously treated patients by Fisher's exact test.

3.3.3.3 Correlation between GenoType HelicoDR and phenotypic results for clarithromycin, levofloxacin and moxifloxacin susceptibility

Correlation was assessed by measuring the number of cases in which culture and Etest and the GenoType HelicoDR results were in agreement. Of the 130 patient samples from which phenotypic resistance testing was successful, genotypic antimicrobial susceptibility test results were available for 119 (91.5%). Agreement between culture and the Genotype HelicoDR assay in detecting *H. pylori* was 100% (119 of 119).

Agreement between phenotypic and genotypic susceptibility test results to clarithromycin, levofloxacin and moxifloxacin are detailed in Tables 3.16, 3.17 and 3.18 respectively. For clarithromycin susceptibility testing, agreement between Etest and GenoType HelicoDR results was 73.1% (n=87/119; Table 3.16). For levofloxacin susceptibility test results, agreement between the two methods was 78.2% (n=93/119; Table 3.17), In the 62 samples that Etesting for

moxifloxacin susceptibility had been performed, agreement between Etest and HelicoDR results was 79.0% (n=49 /62; Table 3.18)

n=119		Culture (n)		Agreement
		Clarithromycin resistant	Clarithromycin sensitive	% (n)
GenoType HelicoDR	Clarithromycin resistant	54	25	73.1% (87)
	Clarithromycin sensitive	7	33	

Table 3.16: Correlation between culture and the GenoType HelicoDR for clarithromycin susceptibility testing

n=119		Culture (n)		Agreement
		Levofloxacin resistant	Levofloxacin sensitive	% (n)
GenoType HelicoDR	Fluoroquinolone resistant	9	14	78.2% (93)
	Fluoroquinolone sensitive	12	84	

Table 3.17: Correlation between culture and the GenoType HelicoDR for levofloxacin susceptibility testing.

n=62		Culture (n)		Agreement
		Moxifloxacin resistant	Moxifloxacin sensitive	% (n)
GenoType HelicoDR	Fluoroquinolone resistant	6	9	79.0% (49)
	Fluoroquinolone sensitive	4	43	

Table 3.18: Correlation between culture and the GenoType HelicoDR for moxifloxacin susceptibility testing.

The accuracy of the Genotype Helico DR compared to phenotypic testing (culture and Etest) as a gold standard in detecting clarithromycin, levofloxacin and moxifloxacin resistance is described in Table 3.19.

	Clarithromycin resistance	Levofloxacin resistance	Moxifloxacin resistance
Sensitivity	**88.5%**	**42.9%**	**60.0%**
	(54/61; 95% CI 78.2-94.3)	(9/12; 95% CI 24.5- 63.5)	3/5; 95% CI 31.3-83.2
Specificity	**56.9%**	**85.7%**	**82.7%**
	(33/58; 95% CI 44.1-68.8)	(6/7; 95% 77.4-91.3)	(43/52; 95% CI 70.3-90.6)
PPV	**68.4%**	**39.1%**	**40.0%**
	(54/79; 95% CI 57.5-77.6)	(9/23; 95% CI 22.2-59.2)	(2/5; 95% CI 19.8-64.3)
NPV	**82.5%**	**87.5%**	**91.5%**
	(33/40; 95% CI 68.1-91.3)	(7/8; 95% CI 79.4-92.7)	(43/47; 95% CI 80.1-96.6)

Table 3.19: Evaluation of the accuracy of the GenoType HelicoDR assay compared to culture and Etesting in the detection of antibiotic resistant *H. pylori* infection.

3.3.3.4 Does the presence of heteroresistance reflect phenotypic resistance?

In order to investigate possible reasons for differences in genotypic and phenotypic antimicrobial susceptibility test results, the prevalence of heterogeneous resistance (the presence of both a wild-type and resistant genotype in a sample) and its association with phenotypic resistance was next assessed.

Figure 3.3 shows the agreement between standard culture and Etest and the GenoType HelicoDR assay in detecting resistance to clarithromycin. Overall, a heteroresistant genotype was found in 60 of the 79 clarithromycin resistant strains (75.9%) by the GenoType HelicoDR assay. Of the 25 discordant cases that were sensitive by culture but resistant by the GenoType HelicoDR assay, 84% (n=21/25) had a heteroresistant genotype. In the samples that were deemed resistant by culture and by the GenoType HelicoDR assay, 72.2% (n=39/54) were heteroresistant.

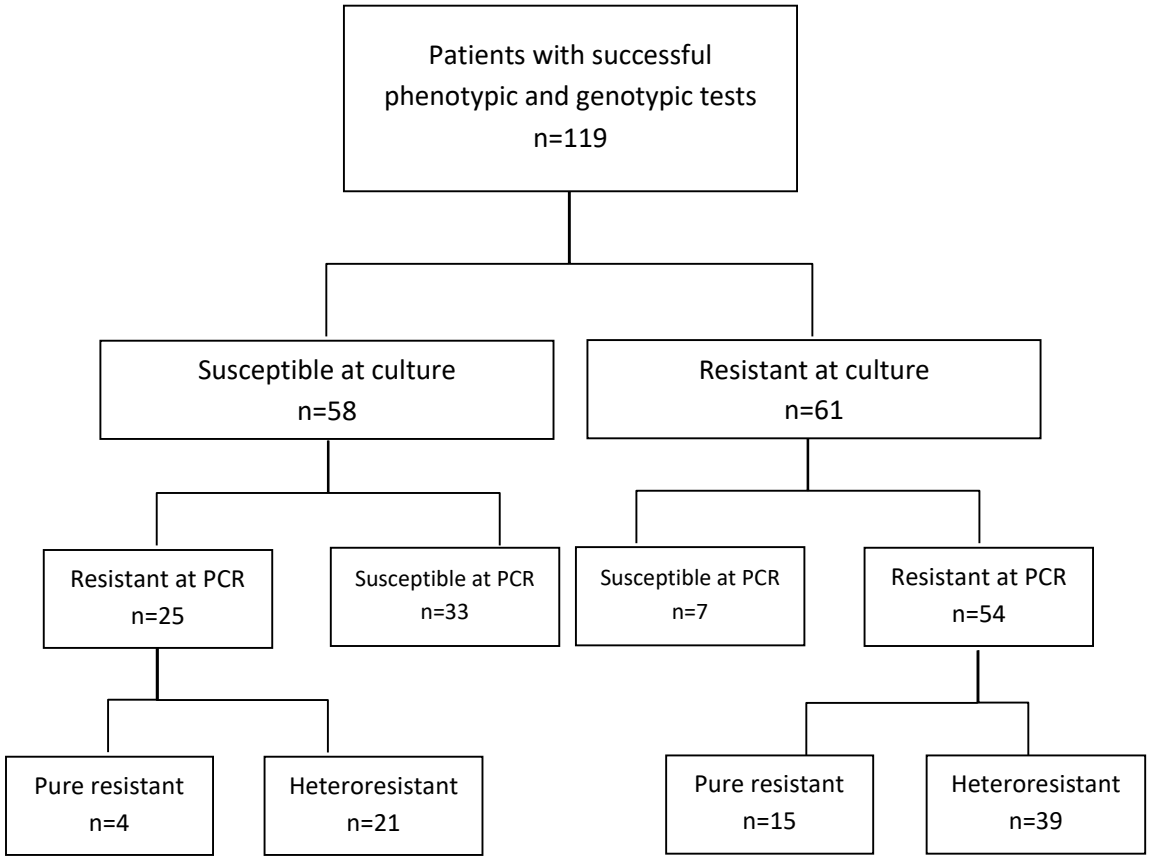


Figure 3.3: *H. pylori* clarithromycin resistance according to culture and the GenoType HelicoDR assay.

Figure 3.4 shows the agreement of culture and the GenoType HelicoDR assay in detecting resistance to levofloxacin. Using the GenoType HelicoDR assay, a heteroresistant genotype was found in 95.7% (n=22/23) of levofloxacin resistant strains. In this case, all of the samples deemed resistant by the GenoType HelicoDR assay but susceptible by culture were heteroresistant (100%, n=14/14), while 88.9% (n=8/9) of the samples deemed resistant by culture were heteroresistant by the GenoType HelicoDR assay.

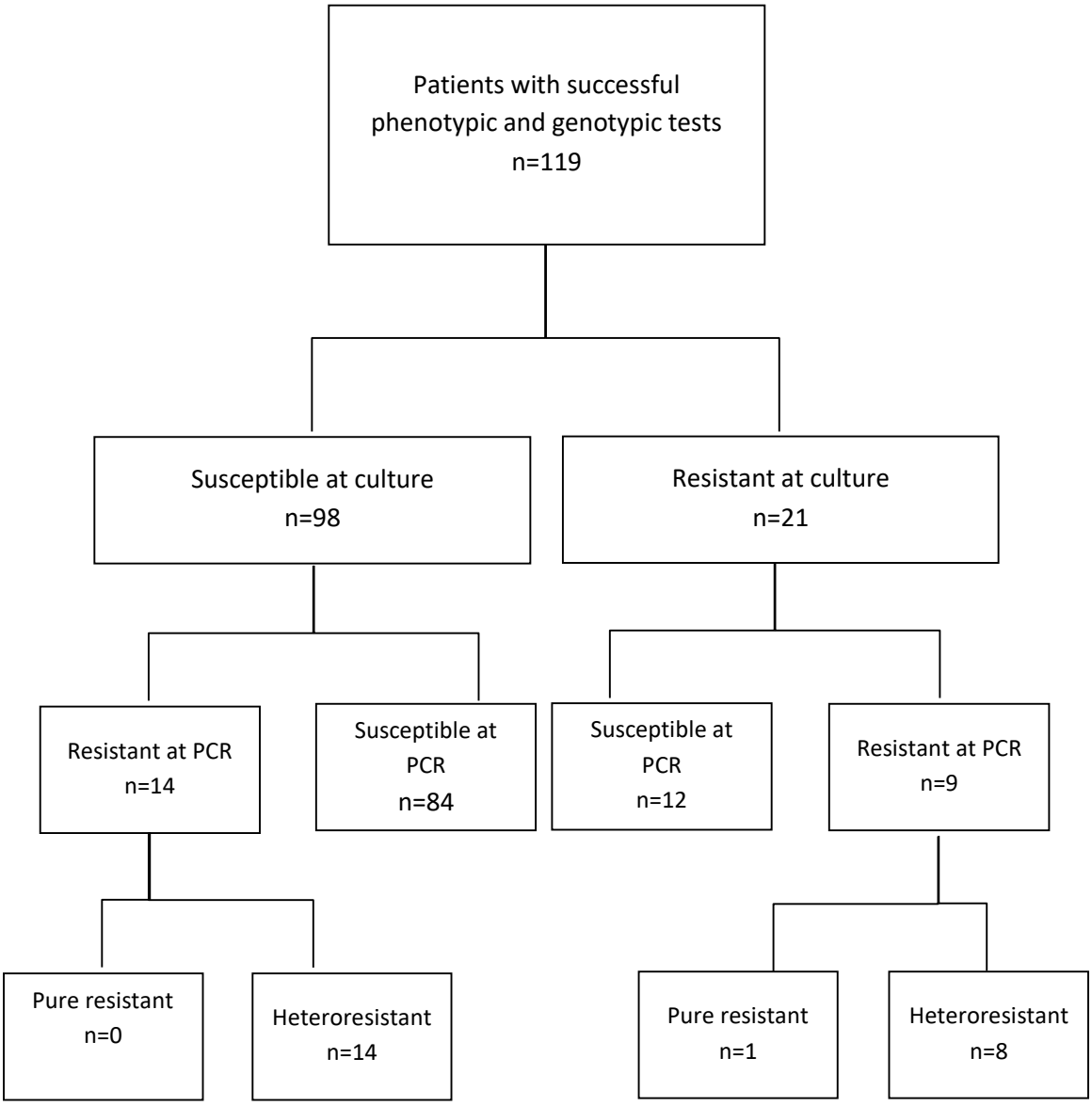


Figure 3.4: *H. pylori* levofloxacin resistance according to culture and the GenoType HelicoDR assay.

A similar trend was found examining the agreement of culture and the GenoType HelicoDR assay in detecting resistance to moxifloxacin (Figure 3.5), with the majority of strains deemed moxifloxacin resistant by culture displaying heteroresistance upon genotyping (93.3%, n=14/15).

As the presence of heteroresistance was detected in the majority of samples deemed resistant by the GenoType HelicoDR assay for clarithromycin, levofloxacin and moxifloxacin, regardless of phenotypic susceptibility result, no clear impact of heteroresistance on phenotypic resistance was found.

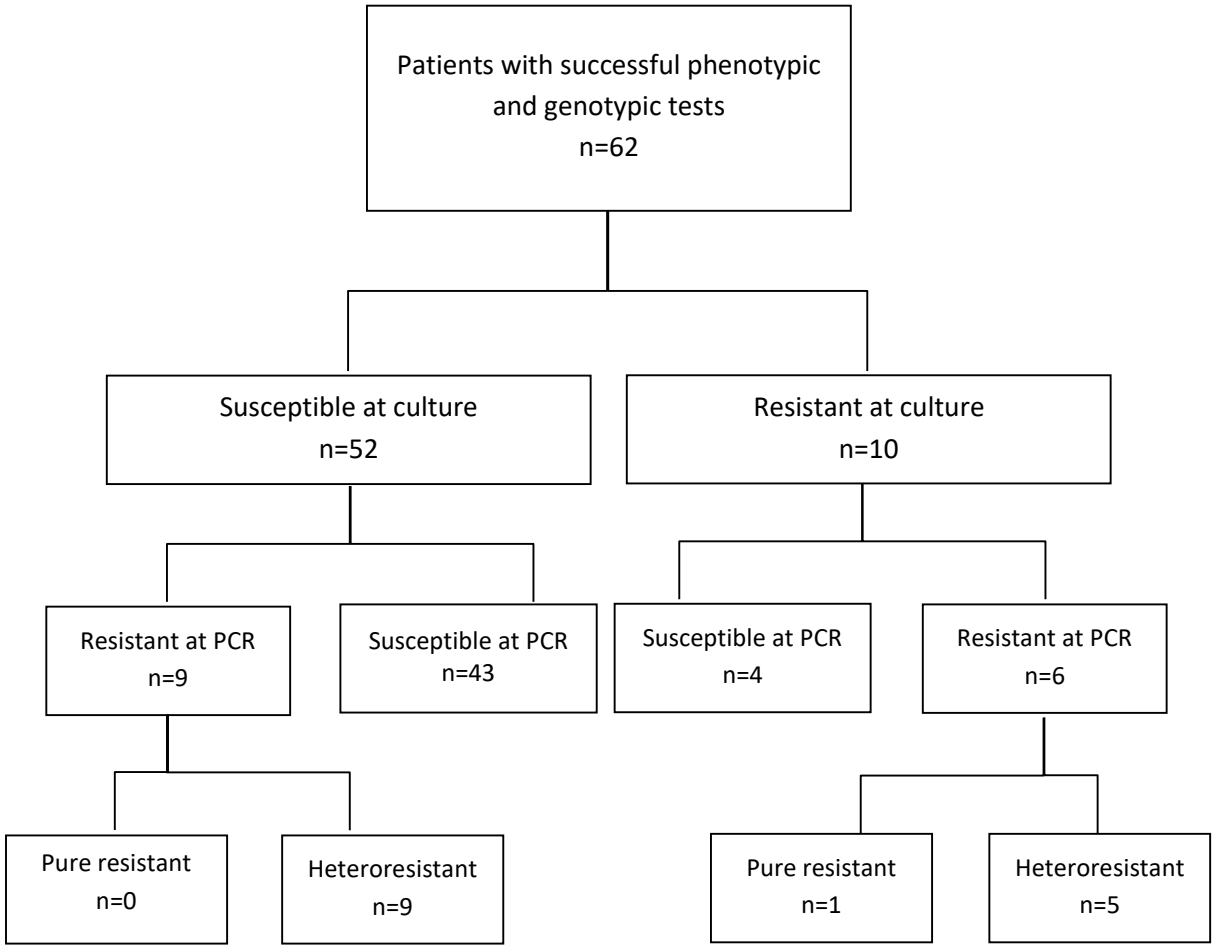


Figure 3.5: *H. pylori* moxifloxacin resistance according to culture and the GenoType HelicoDR assay.

3.3.3.5 Association of resistance mediating mutations with MICs

The presence of mutations conferring resistance to clarithromycin, levofloxacin and moxifloxacin and their association with MIC values was investigated. As illustrated in Table 3.5, Section 3.3.2, 67 *H. pylori* isolates were deemed resistant to clarithromycin by culture-based methods (MIC >0.5 mg/L). Genotypic susceptibility was available for 61 of these (Table 3.20). In those with genotypic resistance (n=54), the A2147G mutation (either alone or in conjunction with another mutation) was significantly more likely to occur than any other mutation (88.8% (n=48/54) vs 11.1%, n=6/54, $p<0.01$). In the isolates deemed resistant by GenoType HelicoDR (n=79), those containing the A2147G mutation had an average MIC of 52 mg/L.

	Clarithromycin MIC >0.5 mg/L n=61
Point mutations	
A2147G	41 (67.2)
A2146G	5 (8.2)
A2146C	1 (1.6)
A2146G + A2147G	3 (4.9)
A2146C +A2147G	3 (4.9)
A2147G+ A2146G+ A2146C	1 (1.6)
Genotypically sensitive	7 (11.5)

Table 3.20: Point mutations in the *rrl* gene associated with a clarithromycin MIC >0.5 mg/L

With respect to levofloxacin, 22 *H. pylori* isolates were deemed resistant by culture-based methods (MIC >1.0 mg/L, Table 3.5). Genotypic susceptibility was available for 21 of these (Table 3.21). In those with genotypic resistance (n=9), the *gyr91* D91N and *gyr91* D91Y were most likely to occur (44.4%, n=4/9), either alone or in conjunction with another mutation. In the isolates deemed resistant to fluoroquinolones by GenoType HelicoDR (n=23), those containing *gyr91* D91N had an average MIC of 8.9 mg/L and those containing *gyr91* D91Y had an average MIC of 3.6 mg/L. Regarding moxifloxacin, 10 *H. pylori* isolates were deemed resistant by culture-based methods (MIC >1.0 mg/L, Table 3.10). The most commonly occurring mutation was *gyr91* D91N (66.6%, n=4/6). In the isolates deemed resistant to fluoroquinolones by GenoType HelicoDR (n=23), those containing *gyr91* D91N had an average MIC of 10.3 mg/L.

	Levofloxacin MIC >1 mg/L n=21	Moxifloxacin MIC >1 mg/L n=10
Point mutations		
gyr87 N87K	1 (4.8)	1 (10)
gyr91 D91Y	3 (14.3)	1 (10)
gyr91 D91N	2 (9.5)	2 (20)
gyr91 D91G	1 (4.8)	-
gyr87 N87K+ *gyr91* D91N	1 (4.8)	1 (10)
gyr87 N87K+ *gyr91* D91N, D91G, D91Y	1 (4.8)	1 (10)
Genotypically sensitive	12 (57.1)	4 (40.0)

Table 3.21: Point mutations in the gyrA gene associated with a levofloxacin and moxifloxacin MIC >1 mg/L

In summary, the GenoType HelicoDR assay detected primary and secondary resistance rates to clarithromycin of 58.0% and 85.2% respectively. Genotypic primary and secondary resistance to fluoroquinolones was 15.2% and 25.9%. Detection rates of primary and secondary clarithromycin resistance significantly varied between the Genotype HelicoDR assay and culture and Etest. Agreement between the GenoType HelicoDR assay and Etesting results for clarithromycin, levofloxacin and moxifloxacin was 73.1%, 78.2% and 79.0% respectively. The GenoType HelicoDR assay displayed suboptimal sensitivity and specificity in detecting levofloxacin and clarithromycin resistance, respectively, when compared to culture-based results. The high false positive rate for detection of clarithromycin resistance (43.1%) suggests that the GenoType HelicoDR assay may overestimate resistance. The presence of heteroresistance was detected in the majority of samples deemed resistant by the GenoType HelicoDR assay and no clear impact of heteroresistance on phenotypic resistance was found. In strains with an MIC >0.5 mg/L for clarithromycin, the A2147G mutation is significantly more likely to occur than any other mutation (88.8%). In strains with an MIC >1.0 mg/L for levofloxacin and moxifloxacin, the mutations *gyr91* D91N and *gyr91* D91Y were most prevalent (44.4% and 66.6% respectively).

3.3.4 Potential use of the GenoType HelicoDR assay for the non-invasive detection of *H. pylori* antibiotic resistance

3.3.4.1 Comparison of endoscopy versus UBT patients

Patient demographics were compared between those referred for endoscopy and for UBT. A subset of endoscopy patients described in Section 2.3.4 (n=389) and all UBT patients described in Section 2.3.2 were included. In keeping with guidelines recommending endoscopy for symptomatic patients (with alarm symptoms) over 45 years, *H. pylori*-positive patients in the endoscopy cohort were significantly older than those in the UBT cohort (48.4 vs 41.4 years; p= 0.005; 95% CI 2.2-11.8). There was a greater number of *H. pylori*-positive men in the endoscopy cohort than the UBT cohort (52.6% vs 33.3%; p=0.03; 95% CI 1.2-36.3, Table 3.22). These findings indicate significant differences in *H. pylori* positive patients referred for endoscopy and those referred for the UBT.

	n (%)		
	Endoscopy	**UBT**	***p* value**
	389 (100)	**227 (100)**	
Mean age (SD)	52.3 ±16.4	39.6 ±12.6	<0.0001[1]
Male	164 (42.2)	69 (30.4)	0.004[2]
H. pylori infection	57 (14.7)	75 (33.0)	
Mean age of positives (SD)	48.4 ±14.9	41.4 ±13.0	0.005[1]
Number of *H. pylori* positive males	30 (52.6)	25 (33.3)	0.03[2]

Table 3.22: Demographics of endoscopy and UBT patients. [1]unpaired t-test, [2]Fisher's exact test.

3.3.4.2 Detection of *H. pylori* and resistance-mediating mutations using the GenoType HelicoDR on stool versus biopsy samples

Figure 3.6 illustrates the study design, analysis and results.

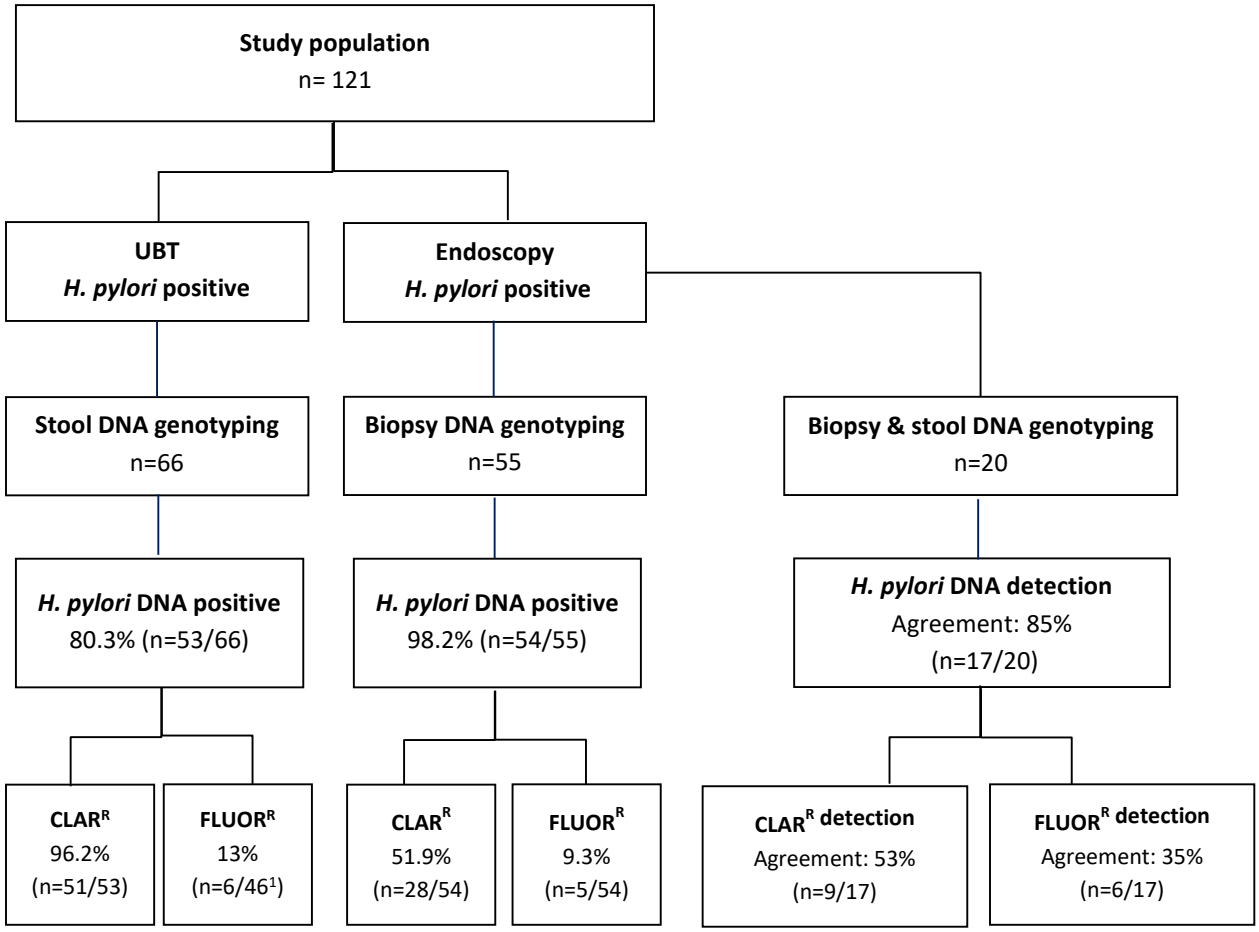


Figure 3.6: Flow chart of study and analysis. [1]Only samples that were positive for the control gene locus for the fluoroquinolone resistance gene were included. CLAR^R: Clarithromycin resistant; FLUOR^R: Fluoroquinolone resistant.

In order to evaluate the GenoType HelicoDR assay for the non-invasive detection of *H. pylori* using stool samples, we first set out to compare the detection rate of *H. pylori* DNA using stool samples from *H. pylori*-positive UBT patients with that obtained using biopsy samples from *H. pylori*-positive endoscopy patients. Initial control experiments showed that the assay did not detect *H. pylori* DNA in stool samples from 2 uninfected UBT-negative patients. In *H. pylori*-infected patients, the GenoType HelicoDR assay was more accurate at detecting *H. pylori* infection using biopsy samples than stool samples (98.2%, vs 80.3%; p= 0.002; 95% CI 6.1-29.7; Figure 3.6)

3.3.4.3 Antibiotic resistance-mediating mutations detection using the GenoType HelicoDR assay in stool versus biopsy samples

Next, the detection of antibiotic resistance-mediating mutations was compared using stool samples from *H. pylori*-positive UBT patients and biopsy samples from *H. pylori*-positive endoscopy patients. Using the GenoType HelicoDR assay, the *23S* gene locus control was positive in all of *H. pylori*-positive DNA samples isolated from either biopsy tissue (100%, n=54/54) or stool specimens (100%, n=53/53). A significantly higher level of clarithromycin resistance-mediating mutations was detected using stool samples from UBT-positive patients than biopsy samples from *H. pylori*-positive patients (96.2% vs 51.9%; p< 0.001, 95% CI: 27.7-58.7; Figure 3.6)

In terms of *gyrA* genotyping, the *gyrA* locus control probe was positive in all DNA samples isolated from biopsy tissue (100%, n=54/54), but only 86.8% (n=46/53) of *H. pylori*-positive DNA samples isolated from stool. Fluoroquinolone resistance-mediating mutations were detected in 9.3% of biopsy samples from *H. pylori*-positive patients compared to 13% of stool samples from UBT-positive patients (p=0.6, 95% CI -10.0-18.3; Figure 3.6). For both endoscopy and UBT patients, all samples that were positive for fluoroquinolone resistance mutations were positive for clarithromycin resistance mutations.

3.3.4.4 The accuracy of the GenoType HelicoDR assay in detection of resistance-mediating mutations by comparing stool and biopsy analyses from individual patients

Given the high rate of clarithromycin resistance detected using stool specimens (96.2%) and the lack of published data on the use of the GenoType HelicoDR assay for stool sample analysis, we next set out to investigate it's sensitivity and specificity in detecting antibiotic resistance-mediating mutations by directly comparing a stool DNA sample with that of a biopsy DNA sample isolated from individual *H. pylori*-positive endoscopy patients. In all, stool and biopsy samples from 20 *H. pylori*-positive endoscopy patients were analysed (mean age 46.8 ±15.8 years, 50% male). *H. pylori* DNA was detected in 95% (n=19/20) of biopsy samples and 90% (n=18/20) of stool samples. Agreement between results from biopsy and stool samples of individual patients for the detection of *H. pylori* DNA, clarithromycin resistance and fluoroquinolone resistance is detailed in Figure 3.6.

The sensitivity and specificity of the GenoType HelicoDR assay in detecting genotypic clarithromycin and fluoroquinolone resistance in stool samples compared to biopsy samples is detailed in Table 3.23.

	Sensitivity	Specificity
Clarithromycin resistance	**100%**	**0**
	(9/9; 95% CI 66.4-100.0)	(0/8; 95% CI 0.0-36.9)
Fluoroquinolone resistance	**25%**	**38.5%**
	(1/4; 95% CI 0.6-80.6)	(5/13; 95% CI 13.9-68.4)

Table 3.23: Accuracy of the GenoType HelicoDR assay for the detection of antibiotic resistance-mediating mutations in stool samples compared to biopsy samples.

In summary, these findings indicate that the GenoType HelicoDR assay is more accurate at detecting *H. pylori* DNA using biopsies from *H. pylori*-positive endoscopy patients than stool DNA isolated from UBT-positive patients. The assay detected a significantly higher rate of clarithromycin resistance using stool samples compared to biopsy samples. Agreement between biopsy and stool samples in the detection of *H. pylori* DNA was 85%, in detection of clarithromycin resistance 52.9% and in detection of fluoroquinolone resistance 35.3%. Higher rates of both clarithromycin and fluoroquinolone resistance was detected in stool samples compared to biopsy samples obtained from the same patient. This is reflected in the lack of/low specificity of the assay (0% for clarithromycin and 38.5% for fluoroquinolones) in the detection of genotypic resistance using stool samples.

3.3.5 Virulence factor and resistance

To evaluate the impact of virulence factors on the prevalence of antibiotic resistance, gastric biopsy samples from 165 of the *H. pylori* positive endoscopy patients described in Section 2.3.4 were included in this study. Of the patients included in the study, 63.6% (n=105) of patients had not been treated for *H. pylori* infection previously, while 36.4% (n=60) had undergone at least one eradication treatment regimen.

3.3.5.1 Distribution of *H. pylori* virulence-factor genotype

Firstly, the prevalence of virulence factor genotypes in our population was determined. Table 3.24 illustrates the distribution of *H. pylori* virulence factor genotype in infected patients. Interestingly, the frequency of the *vacA* S1 genotype (the more virulent S region genotype) was significantly lower in those previously treated than the treatment-naïve group (58.3% vs 74.3%; p=0.04). Additionally, the frequency of the S2/M2 genotype (the least virulent genotype) was

significantly higher in those patients who have been treated previously (36.7% vs 21.0%; p=0.04).

	(n) %			
Genotype	**Overall (n=165)**	**Treatment naïve (n=105)**	**Previous treatment (n=60)**	**p value[1]**
cagA status				
Positive	37 (22.4)	25 (23.8)	12 (20)	0.7
Negative	128 (77.6)	80 (76.2)	48 (80)	
vacA allele				
S1	113 (68.5)	78 (74.3)	35 (58.3)	
S2	52 (31.5)	27 (25.7)	25 (41.7)	0.04
M1	47 (28.5)	31 (29.5)	16 (26.7)	
M2	118 (71.5)	74 (70.5)	44 (73.3)	0.7
S1/M1	39 (23.6)	26 (24.8)	13 (21.7)	0.7
S1/M2	74 (44.8)	52 (49.5)	22 (36.7)	0.1
S2/M1	8 (4.8)	5 (4.8)	3 (5.0)	1.0
S2/M2	44 (26.7)	22 (21.0)	22 (36.7)	0.04

Table 3.24: Distribution of *H. pylori* virulence-factor genotypes among infected patients in Ireland [1]comparison of prevalence of genotype treatment naïve versus previously treated patients by Fisher's exact test

Table 3.25 details the distribution of *vacA* genotypes in *cagA* positive and negative strains. In those strains that were *cagA* positive, the frequency of the most virulent genotype S1/M1 was significantly higher than in strains that were *cagA* negative (37.8% vs 19.5%; p=0.03). Similarly, in those strains that were *cagA* negative, the frequency of the least virulent genotype S2/M2 was significantly higher in strains that were *cagA* positive (32% vs 8.1%; p=0.003).

	n (%)		
***vacA* allele**	***cagA* positive**	***cagA* negative**	**p value[1]**
	37 (22.4)	**128 (77.6)**	
S1	31 (83.8)	82 (64.1)	0.03
S2	6 (16.2)	46 (35.9)	
M1	17 (45.9)	30 (23.4)	0.01
M2	20 (54.1)	98 (76.6)	
S1/M1	14 (37.8)	25 (19.5)	0.03
S1/M2	17 (45.9)	57 (44.5)	0.04
S2/M1	3 (8.1)	5 (3.9)	0.4
S2/M2	3 (8.1)	41 (32.0)	0.003

Table 3.25: Distribution of *vacA* genotypes in *cagA* positive and negative strains. [1]comparison of prevalence of *vacA* genotype in *cagA* positive versus negative patients by Fisher's exact test.

3.3.5.2 Virulence factor genotype and clinical phenotype

The potential association of virulence factor genotype and clinical phenotype via endoscopy and histology findings was assessed. Histological findings were available for 154 (93.3%) of the samples analysed in this study. Table 3.26 illustrates the distribution of virulence factor genotypes according to the main histological findings. *CagA*-negative strains were most common in both groups of patients (77.7% and 69.6% in those with chronic gastritis and intestinal metaplasia respectively), with no difference in *cagA* status among the two main histological findings. The most common *vacA* genotype in both groups of patients was S1/M2 (44.6% and 43.5% for chronic gastritis and intestinal metaplasia respectively), with no difference in distribution of *vacA* genotype among the two main histological findings.

n=154[1]	n (%)		
	Chronic Gastritis	**Intestinal Metaplasia**	**p value**
	130 (84.4)	**23 (14.9)**	
cagA +	29 (22.3)	7 (30.4)	0.4[2]
cagA -	101 (77.7)	16 (69.6)	
***vacA* S1/M1**	28 (21.5)	7 (30.4)	
***vacA* S1/M2**	58 (44.6)	10 (43.5)	0.8[2]
***vacA* S2/M1**	7 (5.4)	1 (4.3)	
***vacA* S2/M2**	37 (28.5)	5 (21.7)	

Table 3.26: Distribution of virulence factor genotype according to histological findings. [1]1 patient reported to have normal mucosa; [2]Fisher's exact test

Of the 165 samples included in this study, endoscopic findings were available for 155 (93.9%). Table 3.27 illustrates the distribution of virulence factor genotypes according to the main endoscopic findings. *CagA*- negative strains were most common in all groups of patients (83.3% for gastritis; 64.7% for normal mucosa and 68.2% for those with PUD). There was no difference in *cagA* status among endoscopy findings. The most common *vacA* genotype in those with gastritis and a normal mucosa was S1/M2 (41.7% and 50% respectively). While both S1/M1 and S1/M2 genotypes were most common in those with PUD (40.9% for both). There was no difference in the distribution of *vacA* genotype among the three main endoscopy findings.

n=155[1]	n (%)			
	Gastritis	**Normal mucosa**	**PUD**	**p value**
	96 (58.2)	**34 (20.6)**	**22 (13.3)**	
cagA +	16 (16.7)	12 (35.3)	7 (31.8)	0.05[2]
cagA -	80 (83.3)	22 (64.7)	15 (68.2)	
***vacA* S1/M1**	18 (18.8)	9 (26.5)	9 (40.9)	
***vacA* S1/M2**	40 (41.7)	17 (50.0)	9 (40.9)	0.2[2]
***vacA* S2/M1**	6 (6.3)	2 (5.9)	0 (0.0)	
***vacA* S2/M2**	32 (33.3)	6 (17.6)	4 (18.2)	

Table 3.27: Distribution of virulence factor genotype according endoscopic findings. [1]other findings not relevant to this study were found in 2 patients (1.2%) and atrophic mucosa was found in 1 patient. [2]Pearson *χ2*-test

In summary, the most prevalent *cagA* genotype in *H. pylori* strains isolated from our cohort of patients was *cagA*-negative*,* at 77.6%. In terms of distribution of *vacA* genotype, the most prevalent was S1/M2 at 44.8%, followed by S2/M2, S1/M1 and S2/M1. The frequency of the more virulent S1 genotype was significantly higher in treatment naïve patients (74.3% vs 58.3%; p=0.04). The frequency of the least virulent *vacA* genotype S2/M2 was significantly higher in previously treated patients (36.7% vs 21.0%*;* p=0.04). *CagA*-positive strains of *H. pylori* were more likely to possess the most virulent *vacA* genotype S1/M1 (37.8% vs 19.5%; p=0.03). *CagA*-negative strains were more likely to possess the least virulent *vacA* genotype S2/M2 (32% vs 8.1%; p=0.003). *CagA* and *vaca* genotypes did not appear to affect clinical phenotype in our cohort.

3.3.5.3 The relationship between virulence factor genotype and genotypic antibiotic resistance

Next, the relationship between virulence factor genotype and genotypic antibiotic resistance was investigated. The GenoType HelicoDR result for each of the 165 patients from whom virulence factor genotyping was carried out was obtained (data was available from the study described in Section 3.3.3). Specifically, information on genotypic resistance to clarithromycin and fluoroquinolones was obtained (genotypic clarithromycin and fluoroquinolone resistance rates of this subset of patients are available in Supplementary Tables 3.2, 3.3 and 3.4). Analysis of all patients revealed that genotypic resistance to clarithromycin was significantly lower in *cagA*-positive strains than in *cagA*-negative strains (40.5% vs 66.4%; χ^2=8.04; p=0.004; Pearson *χ2*-test; Figure 3.7(a)). When patients were sub-grouped into treatment-naïve (Figure 3.7(b)) and those previously treated (Figure 3.7(c)), clarithromycin resistance was also lower in *cagA*-positive strains compared to *cagA*-negative strains, although this only reached statistical significance in the treatment-naïve cohort (32% vs 56.3%; χ^2=4.5; P=0.03; Pearson χ2-test; Figure 3.7(b)).

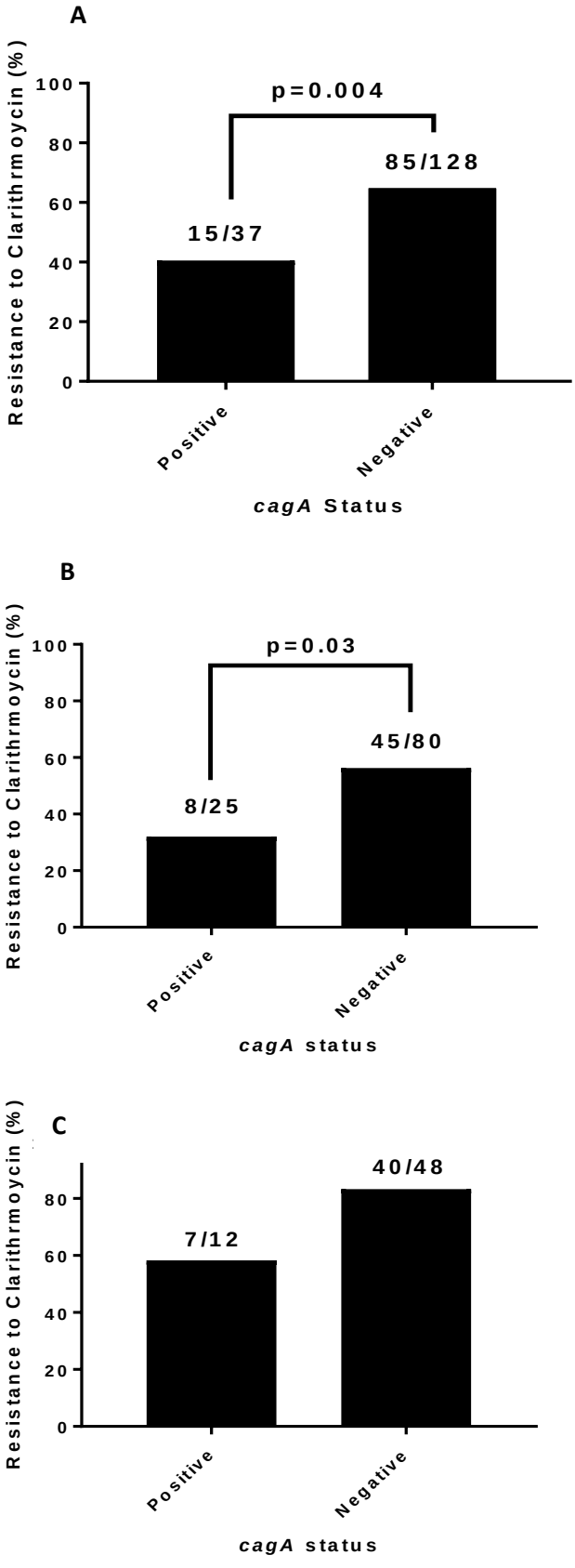


Figure 3.7: Prevalence of clarithromycin-resistance according to cagA genotype (A) all patients, (B) treatment naïve patients and (C) previously treated patients.

Similarly, in patients infected with more virulent *H. pylori* strains bearing the *vacA* s1 genotype, clarithromycin resistance was significantly lower than in those infected with less virulent strains bearing the *vacA* s2 genotype, when all patients were included (52.2% vs 78.8%; χ^2=10.6; p=0.001; Pearson χ2-test; Figure 3.8(a)) and in those that were treatment-naïve (41%, vs 77.8%; χ^2=10.8; p=0.0001; Pearson χ2-test; Figure 3.8(b)), but not in patients that were previously treated (77.1% vs 80.0%; χ^2=0.07; p=0.8; Figure 3.8(c)).

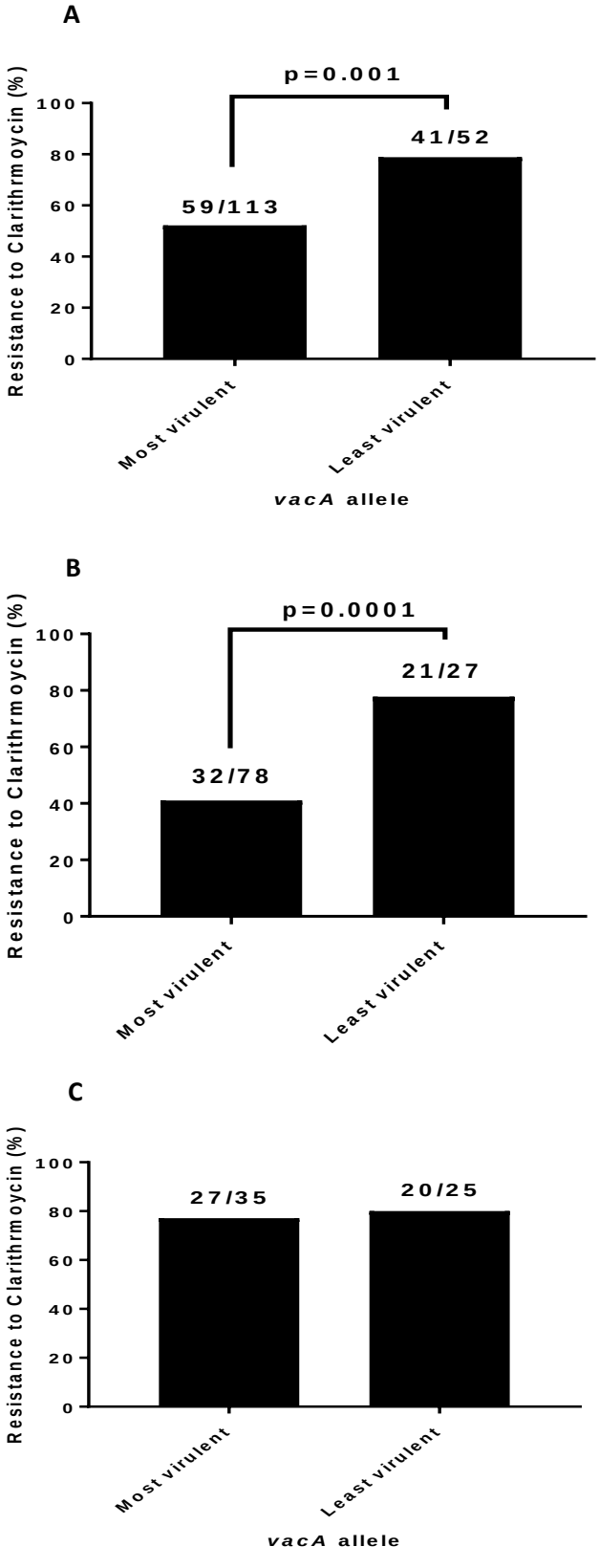


Figure 3.8: Prevalence of clarithromycin resistance according to vacA genotype (A) all patients, (B) treatment naïve patients and (C) previously treated patients. Most virulent: S1/M1, S1/M2; Least virulent: S2/M1; S2/M2.

The frequency of resistance to fluoroquinolones in each virulence factor genotype was also examined. *CagA* status was not significantly associated with fluoroquinolone resistance when all patients were analysed (13.5% vs 21.9%; χ^2=1.3; p=0.3; Pearson χ2-test ;Fig. 3.9(a)) or when the patients were sub-divided into those with primary infections (16.0% vs 15.0%; χ^2=0.01; p=0.9; Pearson χ2-test; Fig. 3.9(b)) and those previously treated (8.3% vs 15.0%; χ^2=3.0; p=0.09; Pearson χ2-test; Fig. 3.9(c)).

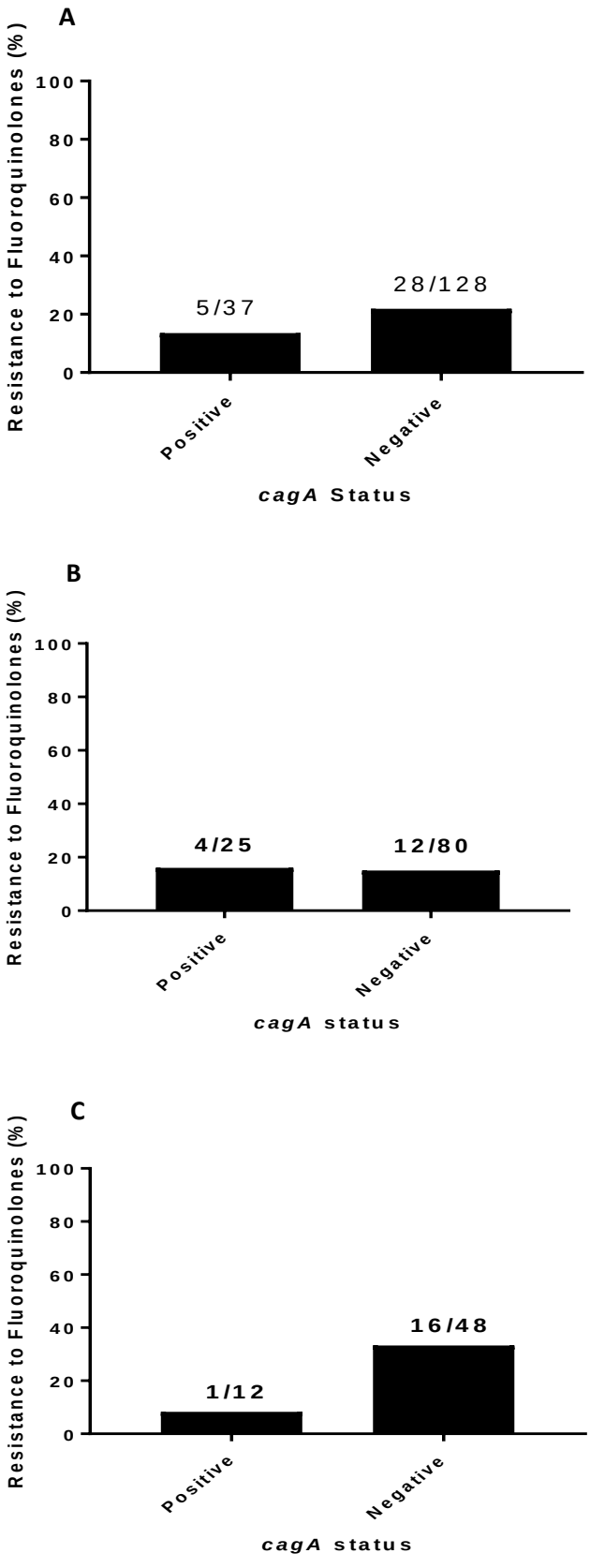
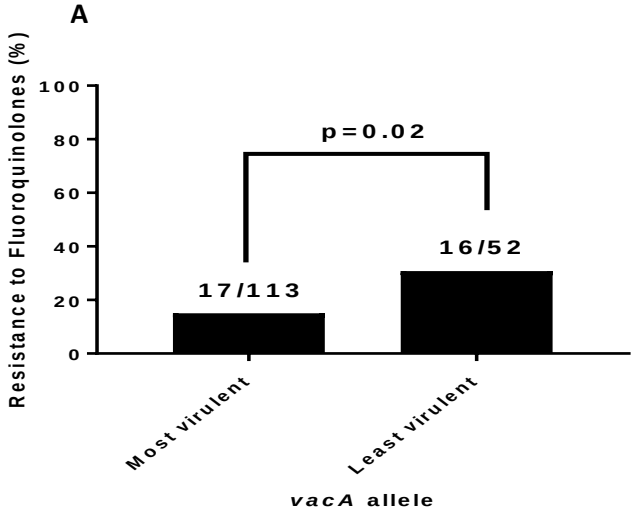
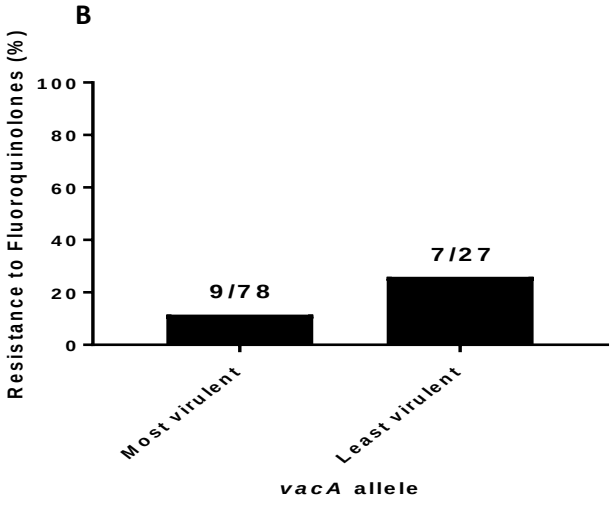


Figure 3.9: Prevalence of fluoroquinolone-resistance according to cagA genotype (A) all patients, (B) treatment naïve patients and (C) previously treated patients.

While there was a significant association between the less virulent *vacA* s2 genotype and fluoroquinolone resistance when all patients were included (15% vs 30.8%; χ^2=5.5; p=0.02; Pearson χ2-test; Fig. 3.10(a)), this did not reach statistical significance in treatment naïve patients (11.5% vs 25.9%; χ^2=3.2; p=0.07; Pearson χ2-test; Fig. 3.11(b)) or those previously treated (22.9%,vs 36.0%; χ^2=1.2; p=0.3; Fig. 3.12 (c)).





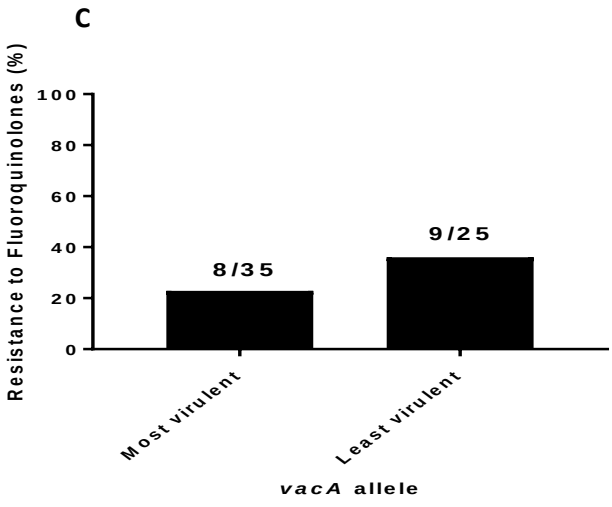


Figure 3.10: Prevalence of fluoroquinolone-resistance according to vacA genotype (A) all patients, (B) treatment naïve patients and (C) previously treated patients. Most virulent: S1/M1, S1/M2; Least virulent: S2/M1; S2/M2.

In summary, *cagA*-negative strains of *H. pylori* were more likely to be genotypically resistant to clarithromycin compared to *cagA*-positive strains, in all patients analysed (66.4% vs 40.5%; p=0.004) and in treatment naïve patients (56.3% vs 32%; p=0.03). Patients infected with the least virulent *vacA* strain of *H. pylori* (*vacA* s2) were more likely to be resistant to clarithromycin compared to the most virulent *vacA* strain (*vacA* s1) in all patients analysed (78.8% vs 52.2%; p=0.001) and in treatment naïve patients (77.8% vs 41%; p=0.0001). *CagA* status was not associated with the presence of fluoroquinolone resistance. *H. pylori* strains bearing the least virulent *vacA* s2 genotype were more likely to be resistant fluoroquinolones only when all patients were included in the analysis (30.8% vs 15%; p=0.02). Taken together, these findings indicate that the absence of *cagA* and the less virulent *vacA* genotypes (S2/M1 and S2/M2) may be predictors of primary genotypic clarithromycin resistance in treatment-naïve patients.

3.4 Discussion

3.4.1 Phenotypic antimicrobial resistance of *H. pylori*

This study found a sharp increase in *H. pylori* resistance to commonly prescribed antibiotics in our patient population. Firstly, in terms of primary clarithromycin and metronidazole resistance, this study reports rates of 36.1% and 59.9% respectively. Dual clarithromycin and metronidazole resistance was 30.6%. In Chapter 1, Table 1.1 illustrates previous Irish, UK and European studies on *H. pylori* resistance rates. To the best of our knowledge, the earliest study on *H. pylori* clarithromycin and metronidazole resistance in Ireland was in 1997, with primary rates of 3.4% and 35.6% reported (178). Resistance was subsequently investigated in 2007-2009 (175, 179) and the results presented herein show that primary clarithromycin and metronidazole resistance rates have increased significantly since then (36.1% at present vs 9.3% in 2007-2009, $p<0.01$ and 59.9% at present vs 29.1% in 2007-2009, $p<0.01$, respectively). These increases are a cause for concern and have implications on the choice of therapy as guidelines recommend that clarithromycin-based triple therapy should not be used as first line treatment when the primary resistance rate exceeds 15% (64, 87, 174). Eradication rates using clarithromycin-based triple therapy are investigated in Chapter 4, as well as the impact of clarithromycin resistance on treatment outcome in the cohort of patients included in this study.

In Chapter 1, Table 1.2 describes the most recent European and worldwide data on *H. pylori* resistance rates. The rate of primary clarithromycin resistance found in this study (36.1%) is high compared resistance reported in Lithuania, The Netherlands and Spain (8.2%, 18.1% and 22.4%, respectively) (181, 182, 184), but similar to rates reported in Italy, Poland and in the Eastern Mediterranean region (35.9%, 35.3-46%, and 33%, respectively) (169, 185, 191, 300), as well as further afield in Peru and Iran (35.5% and 43.1% respectively) (183, 192). Regarding primary metronidazole resistance in comparison to recent published data, the rate of 59.9% reported herein is higher than rates reported for Lithuania, The Netherlands and Spain (32.8%, 23.2% and 27%, respectively) (181, 182, 184), but similar to rates reported in Poland and in the Eastern Mediterranean region (56.7% and 56%, respectively) (169, 185, 300), as well as in South East Asia, China and Iran (51%, 63.8%, 73.8%) (169, 183, 188).

Primary resistance to levofloxacin has increased compared to rates reported in 2008-2009 in Ireland (16.7% at present vs 12.0% in 2008-09) (179). This rate is similar to those reported in The Netherlands (13%) (184) and in the Americas and Asia-Pacific regions (15% and 18% respectively) (169). On the other hand, our rate is lower than in Spain and Italy (38.7% and 29.3% respectively) (182, 191), but higher than reported for Poland (6%) (185, 300). The

increase in levofloxacin resistance in Ireland is also cause for concern; especially as levofloxacin-based triple therapy is one of the recommended second-line treatments for *H. pylori* infection in Ireland (65). With this in mind, resistance of a newer generation fluoroquinolone- moxifloxacin- was investigated. In the subset of samples analysed, although not statistically significant, primary resistance to moxifloxacin was lower than levofloxacin resistance (14.7% vs 20.5%, respectively, p=0.8), as were the average MICs required to inhibit growth of the bacteria (3.0 versus 4.5 mg/L, respectively; p=0.4). Unfortunately due to lack of availability of moxifloxacin Etest strips at the time of the study, it was only possible to perform susceptibility testing on a subset of strains (n=62). Studies have suggested that the emergence of bacterial resistance appears to be less common for moxifloxacin than for other fluoroquinolones and that moxifloxacin may be less affected by quinolone resistance than levofloxacin in *H. pylori* (292, 355). This may be because 2 mutations are required for moxifloxacin resistance to occur; one in the *gyrA* gene and one in the gene for topoisomerase IV (356). As a result, the findings presented herein provide a rationale for further investigations into the use of moxifloxacin as an alternative to levofloxacin in second-line and rescue therapy for *H. pylori*, should levofloxacin resistance continue to rise.

In this study, primary resistance to amoxicillin, tetracycline and rifampicin was 22.2%, 18.1% and 11.1%, respectively. This is a substantial increase compared to tetracycline and rifampicin rates previously reported in Ireland and Europe in 2007-2009, which were reported as ≤1% for all three antibiotics (163, 179). However, other recent data shows an emergence of amoxicillin resistance in The Netherlands and the Eastern Mediterranean region, with resistance rates of 10% (184) and 14% (169), respectively. Similarly tetracycline resistance has emerged in these areas (2.3% in the Netherlands (184); 10% in the Eastern Mediterranean region (169)). Finally, rifampicin resistance has also emerged at 7.5% in Lithuania (181) and at 13.5% in Germany (357) (both these studies involved children and adolescents). Even higher rates have been reported in The Netherlands and in Spain (44.2% and 33.3%, respectively) (182, 184).

In terms of secondary resistance, resistance to clarithromycin has also significantly increased when compared to rates reported in Ireland in 2007-2009 (70.7% vs 32.4%, p=0.003) (175). In this study, secondary resistance to clarithromycin was significantly higher than primary (70.7% vs 36.1%, p=0.02), as well as the rate of dual resistance to clarithromycin and metronidazole (50% vs 30.6%, p=0.03), emphasizing the importance of prescribing regimens that will eradicate the infection at the first attempt. This significantly higher rate of secondary clarithromycin resistance is likely due to induced resistance, reflecting the common use of clarithromycin-based

triple therapy as a first-line treatment for *H. pylori* in Ireland. Primary and secondary resistance rates for all other antibiotics tested did not differ significantly however. The rates of secondary resistance to clarithromycin and metronidazole found in this study (70.7% and 63.8%, respectively) are higher than those reported by Savoldi et al. in Europe (48% and 19%) whereas secondary resistance to levofloxacin is similar (17.2% versus 19% in Europe) (169). Similar to the rates in this study, a secondary clarithromycin resistance rate of 67% was found for the Western Pacific region and a secondary metronidazole resistance rate of 65% was found for the Eastern Mediterranean (169).

Both age and sex have been reported as risk factors for *H. pylori* antibiotic resistance. For example, age >50 years has been reported as a risk factor for levofloxacin resistance and being female has been associated with metronidazole resistance in the most recent pan-European study on antimicrobial resistance (163, 176, 180). Following univariate analysis, this study found that being female was significantly associated with the presence of phenotypic clarithromycin resistance (64.9% vs 41.1% for female and male respectively, p=0.008). This could be due to previous clarithromycin use for treatment of respiratory or urinary tract infections, especially as urinary tract infections are more common in women.

There are a number of explanations for the variations in antibiotic resistance in different regions. Firstly, some of the antibiotics used for *H. pylori* treatment are also commonly used in the treatment of community acquired infections. For example, clarithromycin is often the first-line macrolide prescribed for many infections and preferred over erythromycin in penicillin allergic patients (358). There is a strong link between primary *H. pylori* antibiotic resistance and previous antibiotic use (163, 358, 359). Analysis of antibiotic consumption in Europe revealed a significant association between the use of macrolides and *H. pylori* clarithromycin resistance, and between previous quinolone use and levofloxacin resistance (163). Studies from the UK and USA have also shown previous antibiotic use increases the risk of harbouring resistant strains of *H. pylori* (358, 359). Therefore, variations in antibiotic prescribing patterns are likely to influence the emergence of *H. pylori* antibiotic resistance in different regions.

Secondly, technical variations in the manner in which antimicrobial susceptibility testing is performed in different laboratories could contribute to variations in results.

As discussed in Section 2.4.2, differences exist between strains isolated from biopsies obtained from different sites in the stomach (348, 349). It's possible that not all centres have moved to a

combined biopsy protocol for culture and some are still using a single biopsy, which may not accurately represent an individual's antibiotic susceptibility profile.

In most studies, cultures are incubated for 72 hours prior to MICs being read (127, 175, 360), as was protocol in this study. A study which investigated MICs after 4 and 5 days incubation, using different culture media and under different atmospheric conditions (CO_2 incubator or gas jar) found that MICs varied according to different conditions (361). Importantly, there are variations in the MIC cut-offs used to deem a strain resistant or sensitive. Indeed, an earlier study from our centre in 2009 used a MIC cut-off of >1 mg/L for clarithromycin resistance (50), compared to the >0.5 mg/L cut-off used here, according to EUCAST recommendations. There are also variations in the reported MIC cut-offs used to determine susceptibility to amoxicillin and rifampicin. The cut-off for amoxicillin resistance recommended by EUCAST has been modified in the past and differs in the literature - it can range from >0.125 mg/L to >0.5 mg/L (50, 163, 354, 362, 363). The amoxicillin breakpoint proposed by EUCAST is based on the epidemiological cut-off value, however no clinical evidence exists to confirm whether strains with a low MIC of 0.125- 0.5 mg/L could be eliminated with a higher dose of amoxicillin (>1 mg/L) (360). In this study, the 64% of amoxicillin resistant strains had low MIC values of 0.16-0.5 mg/L (Section 3.3.3.2, Figure 3.2(d)). Regarding primary amoxicillin resistance, 62.5% had low MIC values. It is common practice to use rifampicin to determine rifabutin resistance, as rifabutin Etests are not available routinely. The cut-off to determine susceptibility or resistance of *H. pylori* isolates to rifampicin also differs according to European (EUCAST), French (CASFM/EUCAST) and American guidelines (The Clinical and Laboratory Standards Institute (CLSI)) at >1 mg/L, >4 mg/L and ≥4 mg/L respectively (357, 364). Hays et al. suggested that only strains with MICs > 4 mg/L be classified as being resistant to *H. pylori* (364).

The EUCAST steering committee have just recently decided to change the definitions of the susceptibility testing categories S, I and R (version 9.0) (365). Previously, EUCAST used the S, I, R definitions to categorise a bacteria as treatable or not treatable with the antibiotic in question- i.e. S= susceptible, I= intermediate and R= resistant would predict clinical outcome in a patient. In 2019, the term "intermediate" would be replaced by "susceptible, increased exposure". This is to be used when there is a high likelihood of therapeutic success because exposure to the antibiotic can be increased by adjusting the dosing regimen or mode of administration. Previously, it was common practice to combine susceptibility categories ´Resistant´ and ´Intermediate´ as non-susceptible when reporting antimicrobial resistance rates. EUCAST advised that from 2019, "S" and "I" categories should be reported together and "R" separately.

EUCAST specified clarithromycin as one of the antibiotics to be classified in this way, which has implications for determining clarithromycin resistance with regards to *H. pylori* infection.

While there are variations in resistance rates reported in different regions, the rates presented herein are high, albeit similar to some recent studies from countries such as Italy, Poland, Spain, Iran, Peru and China (183, 185, 188, 191, 192, 300, 360). While every effort was made to exclude previously treated patients from the analysis on primary *H. pylori* resistance, it is possible that patients provided incorrect information in relation to their treatment history. Additionally, as *H. pylori* antibiotic resistance has been shown to be influenced by previous antibiotic use (163, 358, 359), it is noteworthy that Ireland has a proportionately higher use of macrolides and amoxicillin compared to many other countries in the EU (366).

As this study was conducted in only two Dublin-based Hospitals, the generalizability of the resistance rate to Ireland as a whole is uncertain. Efforts were made during this research project to determine the rate of resistance in patients attending other hospitals in Ireland by attempting to obtain *H. pylori* strains from referral centres in Cork and from other Dublin-based hospitals. However taking into account the resources available to the project at the time, this was not logistically feasible. In order to accurately assess the national prevalence of antibiotic resistance, analysis of patient samples from additional hospital sites around the country is recommended going forward.

3.4.2 The performance of the GenoType HelicoDR assay in the detection of genotypic *H. pylori* antibiotic resistance

As standard culture and antimicrobial susceptibility testing can be time-consuming and difficult, molecular based assays which identify genetic material to detect the presence and susceptibility profile of *H. pylori* offer an attractive alternative to standard culture-based susceptibility testing. The aim of this study was to evaluate the qualitative PCR-based GenoType HelicoDR assay in detection of genotypic resistance to clarithromycin and fluoroquinolones. The assay detected *H. pylori* in 96% samples included in this study, significantly higher than the rate of detection by culture, whereby *H. pylori* was detected in 59.4% samples ($p<0.01$).

In this study, the GenoType HelicoDR assay detected overall, primary and secondary genotypic resistance to clarithromycin in 69.9%, 58.0% and 85.4% of samples respectively. Data on genotypic resistance rates from other centres in Ireland is lacking. Some studies which used the GenoType HelicoDR assay report levels of primary genotypic clarithromycin resistance in the range of 12.5%- 16.9% (151, 367-369). However other studies also report high genotypic

clarithromycin resistance rates, with overall rates of 66.7-68.3% in Israel (143, 370) and primary rates of 37.5% in Greece (307). In a conference paper presented in 2018, a research group based in Madrid, Spain reported 52.5% overall clarithromycin resistance using the Genotype HelicoDR assay (371). This group has reported consistently on high rates of clarithromycin resistance in their patient population since 2010 (360, 372, 373). Other studies in Austria and France used alternative molecular methods (by real-time PCR and the AmpliDiag® *H. pylori* + ClariR test), with rates of primary genotypic clarithromycin resistance reported as 21% and 36% respectively (children were included in French study) (187, 189). Among patients infected with a resistant strain presented herein, the most common point mutation conferring clarithromycin resistance was A2147G, in keeping with other studies (138, 140, 143, 151, 222, 368).

The GenoType HelicoDR assay detected overall, primary and secondary genotypic resistance to fluoroquinolones in 19.9%, 15.2% and 24.3% of samples respectively. These data are similar to other studies which used the GenoType HelicoDR assay to investigate levofloxacin resistance, with rates ranging from 10.3% to 21.2% (143, 151, 307, 367-370) and to the primary genotypic resistance rate of 13% reported in Austria by real-time PCR (187). The most common point mutation conferring resistance to fluoroquinolones was *gyr91* D91Y. This contrasts with other studies in which *gyr91* D91N and *gyr87* N87K mutations were reported with highest frequency (140, 143, 222, 368).

The validity of the GenoType HelicoDR assay was investigated by comparing results to those from phenotypic clarithromycin and fluoroquinolone susceptibility, as determined by culture and Etest. Firstly, primary and secondary genotypic and phenotypic resistance rates were compared. According to the GenoType HelicoDR assay, both primary and secondary clarithromycin resistance was significantly higher than phenotypic rates (58.0% vs 36.1%; p=0.004 for primary resistance and 85.4% vs 70.7%; p=0.04 for secondary resistance). This difference has been reported elsewhere (374). Regarding levofloxacin, there was no difference between genotypic and phenotypic rates in both primary and secondary resistance (15.2% vs 16.7%; p=0.8 and 24.3% vs 17.2%; p=0.3 respectively). Similarly with moxifloxacin, there was no difference between genotypic and phenotypic rates (15.2% vs 14.7%; p=1.0 for primary resistance and 24.3% 17.9%; p=0.6 for secondary resistance). This may be because a small number of strains were resistant to these antibiotics and there wasn't enough power to detect a difference in resistance detection between the two methods. Second, agreement between the two methods was measured by counting the number of cases in which culture and Etest and the GenoType HelicoDR results were in agreement. Agreement between the GenoType HelicoDR

assay and Etest in the detection of clarithromycin, levofloxacin and moxifloxacin susceptibility was 73.1%, 78.2% and 79.0% respectively. Other studies have reported on the correlation between GenoType HelicoDR results and culture and Etest, with agreement of 56.6%- 96% for detection of clarithromycin susceptibility and 75.5%- 94% for detection of fluoroquinolone susceptibility (138, 222, 370, 375). Finally, the accuracy of the Genotype HelicoDR assay in detecting clarithromycin, levofloxacin and moxifloxacin resistance was determined by calculating sensitivity and specificity (using culture and Etest results as gold standard). The sensitivity and specificity of the GenoType HelicoDR assay in detection of clarithromycin resistance was 88.5% and 56.9% respectively. Other studies have reported sensitivities and specificities in the range of 55%- 94% and 80-99% (138, 222). Regarding levofloxacin resistance in this study, sensitivity and specificity was 42.9% and 85.7% respectively. In detecting moxifloxacin resistance, sensitivity and specificity was 60.0% and 82.7%. Other studies have reported sensitivities and specificities in the range of 74.4%- 87% and 70-98.5% for detection of fluoroquinolone resistance (138, 222).

There are a few interesting explanations for the high rate of clarithromycin resistance detected by the GenoType HelicoDR assay and the significant difference in resistance rates reported by culture and Etest and by the GenoType HelicoDR assay. The difference in phenotypic and genotypic resistance may potentially be due to detected mutations not being translated into an actual change in the bacterial protein and so resistance does not occur phenotypically (201). In addition, the presence of heteroresistance could potentially be the reason for this difference. Heteroresistance is defined as the presence of both susceptible and resistant genotypes in the same sample. This may be due to heterozygous resistance-mediating mutation on the 23S rRNA gene or the presence of both susceptible and resistant strains of *H. pylori* being present in the stomach, due to selection of resistant mutants (138). Heteroresistance was common in our patient cohort, with clarithromycin heteroresistance detected in 75.9% resistant strains, higher than reported in other studies (138, 140). It is possible that the balance of resistant and susceptible organisms infecting a given patient influences the phenotypic susceptibility result, which could also explain why resistance is detected by genotypic testing but not phenotypically. However no clear link was found between the presence of genotypic heteroresistance and phenotypic antimicrobial susceptibility in this study. The impact of heteroresistance on treatment outcome will be explored in Chapter 4.

As this study was conducted in only two Dublin-based Hospitals and data on genetic resistance rates of *H. pylori* strains isolated from other centres around Ireland is limited, it is difficult to put

in context the high clarithromycin resistance observed in this study. To address this, potential future work for this research project would be to analyse samples both genotypically and phenotypically from centres around the country.

To ensure quality control, a negative control which consisted of H_2O instead of template bacterial DNA in the PCR mix was included in every GenoType HelicoDR assay run included in this study. However, due to the nature of this well-based hybridisation assay, the possibility of contamination causing false positive results has to be considered. It is possible that contamination of neighbouring wells could occur by spillage during addition of Hybridization Buffer. Additionally, as is the case with other molecular assays, a possible risk of contamination of the amplified DNA during PCR performance is present, which may yield false positive results. To further explore this possibility of the GenoType HelicoDR assay producing false positive results, future studies are planned to compare results from the GenoType HelicoDR assay with results from other genotypic tests such as the *H. pylori* ClariRes Assay (Ingenetix) and the RIDA®GENE Helicobacter pylori assay (R-Biopharm AG, Darmstadt, Germany). This will also allow further investigation into the accuracy of these genotypic methods.

Another limitation of this study is the lack of investigation into genotypic susceptibility of metronidazole and amoxicillin, two antibiotics used as commonly as clarithromycin in the treatment of *H. pylori*. As explained in Section 1.6.2, the mechanism of resistance to metronidazole is unclear and a definitive panel of resistance-mediating point mutations has not yet been described (204-207). Because of this, at present there are no commercially available molecular tests to determine metronidazole resistance. Metronidazole resistance can be overcome by increasing dose and duration of treatment (186, 220), whereas amoxicillin resistance is relatively rare in most regions at present (169). Therefore development of commercial tests for resistance to these antibiotics has not been a priority for developers of *in-vitro* diagnostic devices to date.

3.4.2.1 Should the GenoType HelicoDR assay replace standard culture and Etest for susceptibility testing?

The GenoType HelicoDR assay has its advantages. It is efficient in detecting *H. pylori*; in this study, it detected a significantly higher number of infections than culture. The assay can obtain susceptibility results in 6 hours and can be used directly on gastric biopsy specimens, eliminating the need for bacterial culture. It can detect the presence of infection with multiple strains harbouring different susceptibility profiles. However, there are also limitations to the assay. The

workflow in carrying out the Genotype HelicoDR assay requires necessary expertise and specialized equipment, as well as costing more than standard culture and Etest. Additionally, the kit does not detect the entire range of known mutations that confer resistance to clarithromycin, such as T2182C and C2611A (197), and to fluoroquinolones, such as N87H, N87I and N87Y (376). However this may not be clinically relevant in our centre as a relatively low rate of false negative genotypic resistance was found (17.5%, 12.5%, and 8.5% for clarithromycin, levofloxacin and moxifloxacin respectively). As with any molecular-based assay, it must be kept in mind that only the DNA sequence is analysed and not the amino acid sequence. Therefore, silent mutations may be present that are not translated phenotypically. In addition, the GenoType HelicoDR assay does not detect metronidazole or amoxicillin resistance, which are also used routinely in treatment of *H. pylori* infection. The probes used in the assay would need to be adapted to the local prevalence of mutations to effectively detect the majority of mutations in a particular population (140) and the impact of genetic resistance, including heteroresistance, on treatment outcome requires more in-depth evaluation in different populations.

In this study, the GenoType HelicoDR assay displayed suboptimal sensitivity and specificity in detecting levofloxacin and clarithromycin resistance respectively. Although culture-based susceptibility testing is challenging in terms of culture success rate and the time taken to obtain results (7-10 days), currently culture-based methods should remain the method of choice for resistance surveillance and tailoring therapy in our cohort, as these methods enable phenotypic testing of the complete range of antibiotics used to treat *H. pylori*.

3.4.3 The potential of the GenoType HelicoDR assay in non-invasive detection of *H. pylori* infection and antibiotic resistance

To determine whether *H. pylori*-infected endoscopy patients were representative of the wider *H. pylori*-infected population, patient demographics were compared between endoscopy and UBT patients and significant differences were found. As such, this study sought to evaluate the GenoType HelicoDR assay in the non-invasive detection of *H. pylori* resistance, as it could enable resistance data to be gathered from patients managed by the 'Test and Treat' strategy. More accurate recommendations on the correct treatment regimen for *H. pylori* could then be provided.

The GenoType HelicoDR assay detected *H. pylori* DNA in a significantly lower percentage of *H. pylori*-infected patients when stool rather than biopsy specimens were analysed (80.3% versus 98.2%; p=0.002). As *H. pylori* specifically colonizes the stomach and is not an intestinal

bacterium, it is present only in low numbers in the stool, a factor which may have impacted the sensitivity of *H. pylori* detection using stool samples in our study. Additionally, *H. pylori* DNA may be exposed to enzymatic or mechanical degradation during transit from the stomach through the intestines (146).

When results using biopsy samples from individual *H. pylori*-infected patients were directly compared with those obtained from their stool samples, agreement in detection of clarithromycin and fluoroquinolone resistance was just 52.9% and 35.6%, respectively. In addition, there were a greater number of false positive mutations detected in the DNA isolated from the stool samples compared to DNA isolated from biopsy samples from the same patient. This would suggest that stool sample analysis using the GenoType HelicoDR assay is less sensitive than biopsy sample analysis, providing an explanation for the high rates of clarithromycin resistance observed using stool samples from UBT patients (96%, Section 3.3.4.2). The presence of large amounts of diverse commensal bacteria in the stool may hamper the specificity of the Genotype HelicoDR assay in detection of *H. pylori* antibiotic resistance-mediating mutations. Lastly, the sensitivity and specificity values of the assay for the detection of clarithromycin and fluoroquinolone resistance suggest it is currently unsuitable for the accurate detection of clarithromycin and fluoroquinolone resistance-mediating mutations in stool specimens.

Other studies have evaluated the use of GenoType HelicoDR assay on stool samples (148, 150, 377). A study conducted in Uganda reported positive results. However there is a flaw in the study design, as the authors did not compare the resistance profile obtained from stool to that of a profile obtained from a biopsy or from bacterial culture (377).

3.4.4 Virulence factors and resistance

The final aim of this study was to evaluate the impact of virulence factors on the prevalence of genotypic resistance to clarithromycin and fluoroquinolones. In addition, an update on the distribution of virulence factor genotypes in *H. pylori* strains in Ireland was provided.

In our patient population, less virulent strains of *H. pylori* are most prevalent. The overall frequency of *H. pylori* infections with strains containing the *cagA* gene was 22.4%. This has decreased since the distribution of the *cagA* genotype was last investigated in Ireland in 2009, with a frequency of 68% reported (50). This change in the pattern of *cagA* genotype could be explained by the changes in our population due to immigration, as the prevalence of virulence

factors is diverse among *H. pylori* strains isolated from different geographic areas and ethnic groups (378). Indeed it is also lower than distributions reported in Cuba and Iran (379, 380).

There is a well-known association between *cagA*-positive strains of *H. pylori* and peptic ulcer disease (38, 39). This relatively low frequency of *cagA*-positive genotype observed herein is not surprising given that the prevalence of peptic ulcer disease was also relatively low in our cohort at 13.3%, a decrease compared to the prevalence of peptic ulcer disease reported in the previous Irish study (17%) (50).

The distribution of *vacA* genotype in *H. pylori* strains in our patient population has not changed. The most prevalent *vacA* genotype in our cohort was S1/M2 at 44.8%, followed by S2/M2 (26.7%), S1/M1 (23.6%) and S2/M1 (4.8%). This pattern is similar to the pattern reported in Ireland in 2009 as well as the studies mentioned above (50, 379, 380). *CagA*-positive strains of *H. pylori* were more likely to possess the most virulent *vacA* genotype S1/M1 (37.8% vs 19.5%, p=0.03) and *cagA*-negative strains were more likely to possess the least virulent genotype S2/M2 (32% vs 8.1%, p=0.003). This is consistent with other studies which found the presence of *cagA* to be closely related to *vacA* S1 genotype (40, 43).

Interestingly, the frequency of the more virulent S1 genotype was significantly lower in those previously treated than the treatment-naïve group (58.3% vs 74.3%; p=0.04). Additionally, the frequency of the least virulent S2/M2 genotype was significantly higher in those previously treated previously (36.7% vs 21%; p=0.04). This is in accordance with a hypothesis described previously which suggests that more virulent strains elicit a stronger inflammatory response, enabling increased blood flow to the site of infection, therefore enhancing delivery of antibiotics and the potential for successful eradication (381). Another potential explanation is that a more virulent strain of *H. pylori* may replicate faster and is therefore more susceptible to antibiotics such as clarithromycin and fluoroquinolones, whose mechanism of actions are to inhibit bacterial replication (49).

We found an inverse relationship between the virulence of the infecting strain and the presence of genotypic clarithromycin resistance: the absence of *cagA,* and the less virulent *vacA* genotypes (S2/M1 and S2/M2), may be indicators of clarithromycin resistance, in particular in treatment-naïve patients. Given the differences in rates between phenotypic and genotypic clarithromycin resistance reported in Section 3.3.3.2, it would be interesting to determine whether the link between genotypic clarithromycin resistance and less virulent infections

extends to phenotypic clarithromycin resistance detected by culture-based methods in our patient cohort.

The prevalence of the more virulent *cagA*-positive genotype has decreased to 22.4% in our cohort and *cagA*-negative strains are significantly more likely to harbour clarithromycin resistance-mediating mutations. The increase in *cagA*-negative strains in our cohort over time is in line with the increase in clarithromycin resistance in our population.

The association between virulence factors and antibiotic resistance in *H. pylori* has been evaluated in other studies, with controversial results. Absence of *cagA* was found to be a risk factor for phenotypic metronidazole resistance (50) and other studies have found an association between clarithromycin resistance mutations and the less virulent *vacA* genotypes (382, 383). Another report revealed that *cagE* and *vacA* S1 correlated with clarithromycin and metronidazole resistance (384), while others found that neither *cagA* nor *vacA* was associated with resistance (39, 45, 385, 386). There may be no direct causation involving the presence of less virulent strains of *H. pylori* and antibiotic resistance. Rather, the presence or absence of virulence factors may cause physiological effects which create an environment in which antibiotic resistant strains of *H. pylori* can flourish, as outlined above (49). As less virulent strains are less immunogenic, an inadequate delivery of antibiotics may reach infected areas in the stomach and as a result, antimicrobial resistant strains may be selected for in the population of less virulent strains. It has been shown that *cagA*- strains may tend to acquire drug resistance *in vitro* (50).

H. pylori strains bearing the least virulent *vacA* s2 genotype were more likely to be resistant to fluoroquinolones when all patients were included in the analysis (30.8% vs 15%; p=0.02), however no difference was observed when patients were sub-divided based on treatment status. It's possible that the number of strains present after sub-division is not sufficient to achieve statistical significance. Further investigation into the influence of *vacA* genotype on fluoroquinolone resistance is required before a definitive conclusion can be made.

3.4.5 Summary

This study found a sharp increase in *H. pylori* resistance to commonly prescribed antibiotics in our centre. As discussed, rates are high compared to some studies, but similar to some very recent publications in Europe. The variation observed between local and European regions means local surveillance of existing and emerging antibiotic resistance is pertinent to the correct management of patients with *H. pylori* infection (64, 65, 88, 173, 174). This increase in local

resistance patterns has implications for treatment choice, as clarithromycin resistance has surpassed the 15% cut-off and dual clarithromycin and metronidazole resistance is high. This is discussed further in Chapter 4.

The GenoType HelicoDR assay was effective in detecting *H. pylori* DNA in gastric biopsy samples. Regarding its detection of genotypic resistance to clarithromycin and fluoroquinolones however, the assay displayed intermediate agreement with phenotypic methods. It displayed suboptimal sensitivity and specificity in detecting levofloxacin and clarithromycin resistance respectively. As such it cannot currently be recommended to directly replace culture-based antimicrobial susceptibility testing and should be reserved for cases when *H. pylori* culture is unsuccessful. Investigation into the accuracy of other genotypic methods of resistance detection is warranted.

Our study concluded that the GenoType HelicoDR assay is not suitable for the accurate detection of antibiotic resistance-mediating mutations using stool samples from *H. pylori* infected patients. Further studies are required to extend approaches for the non-invasive detection of *H. pylori* resistance to include multiple antibiotics. Recent advances in next generation DNA sequencing technologies may provide more robust opportunities for the accurate analysis of specific resistance-associated DNA regions. The successful optimisation of molecular-based antimicrobial susceptibility testing methods will enable resistance data obtained from patients managed by the 'Test and Treat' strategy to be utilised in choosing effective antibiotics for the treatment of *H. pylori*. In this way, eradication rates of *H. pylori* infection may be improved.

Finally, *cagA*-negative and *vacA* S1/M2 genotypes were the most prevalent in *H. pylori* strains in Ireland. There is a relationship between the less virulent strains of *H. pylori* (*cagA*-negative and *vacA* S2) and primary genotypic clarithromycin resistance and a possible relationship between *vacA* genotype and fluoroquinolone resistance. The relationship between less virulent strains of *H. pylori* and presence of antibiotic resistance found herein should be further investigated, as significant findings could allow the development of new strategies in the effort to improve eradication rates.

Chapter 4 Tailored treatment of *Helicobacter pylori* in a population with increasing rates of antibiotic resistance

4.1 Introduction

A key finding of Chapter 3 was a continuous increase in *H. pylori* resistance to commonly prescribed antibiotics in our centre. Primary *H. pylori* clarithromycin resistance was 36.1%, which exceeds the 15% prevalence cut-off for the use of clarithromycin-based first-line triple therapy, as recommended by the Maastricht V/Florence Consensus (64). *H. pylori* treatments have fallen below the recommended 80% ITT eradication rate in recent years (64, 282, 350). As such, necessary alternative treatment strategies should be explored to improve eradication rates.

Several alternative therapeutic regimens for first-line treatment of *H. pylori* have been suggested, which involve combinations of known antibiotics or extended treatment durations and are explained in detail in Section 1.7.2. However the increasing complexity of these regimens is associated with a reduction in compliance and increased risk of side effects (247).

In an effort to increase eradication rates, a strategy whereby tailoring treatment based on the antimicrobial susceptibility of an individual's infecting *H. pylori* strain has shown promise. Several prospective, retrospective and meta-analysis studies have demonstrated that tailored treatment achieves a better eradication rate than empirically chosen treatments (294-303), some of which are presented in Table 4.1. Zhou *et al*. compared eradication rates, safety, and compliance of tailored treatment to those of triple therapy plus bismuth and concomitant therapy for first-line treatment of *H. pylori.* They found that tailored therapy achieved significantly higher eradication rates and fewer side effects (297). However questions remain as to the feasibility of this treatment strategy- as detailed in Section 1.4.2.3, bacterial culture is currently not widely available, time-consuming, requires trained personnel and the success rate can vary. This may delay treatment for patients and the cost effectiveness of the treatment strategy is unclear.

Study	First-line therapies (ITT analysis)	
Cosme et al. (296)	Clarithromycin-based triple therapy 49%	Tailored treatment 88%
Cosme et al. (294)	Concomitant therapy 87%	Tailored treatment 94%
Cosme et al. (295)	NA	Tailored treatment 58.3%- 97.6%
Zhou et al. (297)	Bismuth quadruple 77.4% Concomitant therapy 78.3%	Tailored treatment 88.7%
Ferenc et al. (300)	Concomitant/ levofloxacin-based triple therapy 86.6%	Tailored treatment 95.5%
Park et al. (302)	Clarithromycin-based triple therapy 71.9%	Tailored Treatment 94.7%

Table 4.1: Recent studies investigating the effectiveness of first line tailored therapy compared to empirically chosen therapies.

The first aim of this study was to evaluate the efficacy of antimicrobial susceptibility-guided treatment (tailored treatment, TT) compared to standard clarithromycin-based triple therapy (STT) in first-line treatment of *H. pylori* infection. First-line treatment is the treatment given to patients who have never before received treatment for *H. pylori*. When this study commenced in March 2013, the standard treatment duration was 7-10 days (387) and patients in this study were treated with either 7-day STT or TT. In late 2016, new national and international guidelines on the treatment of *H. pylori* infection were released. Guidelines recommended 14 day duration of treatment as well as the use of higher dose, newer generation PPIs (64, 65). As such it was necessary to change the therapeutic regimens used in this study accordingly. As a result of this, the efficacy of STT and TT in those treated for 7 days and in those treated with 14 days will be presented separately in this study (with combined 7 and 14 day analysis included also).

Chapter 3 also investigated the performance of the GenoType HelicoDR assay in the detection of *H. pylori* antibiotic resistance and found significant variation in phenotypic and genotypic resistance to clarithromycin. In light of this, the second aim of this study was to investigate whether the presence of phenotypic and genotypic clarithromycin resistance impacts eradication rates.

Another finding of Chapter 3 was that less virulent *H. pylori* strains are now the most prevalent in our cohort. A relationship between the less virulent strains of *H. pylori* (*cagA*-negative and *vacA* S2) and primary genotypic clarithromycin resistance was also found. To further investigate whether this relationship has an impact on eradication rates, the impact of virulence factor genotype on treatment outcome was explored, as a potential association could be a strategy to explore in the effort to improve eradication rates for *H. pylori* infection.

The number of patients who require second-line or rescue treatment (after ≥2 failed eradication attempts) for *H. pylori* infection is increasing. Indeed, of the RUT positive patients in our cohort, 44.4% (n=118/266) had received 1 or more treatments for *H. pylori.* As first-line treatment for *H. pylori* is not standardised, it is difficult for consensus groups to recommend a specific subsequent treatment. Guidelines recommend that second-line treatment depends on what was prescribed first-line and should not be the same treatment (64, 87, 88, 173, 174). Use of either levofloxacin-based triple therapy, STT or BQT is recommended in Ireland and Europe (64, 65).

Although guidelines recommend that rescue treatment should be guided by susceptibility testing (64, 65, 87, 88), perhaps prescribing a tailored regimen based on antibiotic sensitivities upon first eradication failure is most effective. This would be a rational strategy, because if a patient fails first-line treatment, they are likely to now be resistant to the antibiotics they were prescribed initially. Studies have shown this strategy to be promising (388-390). A study on patients with refractory infection were retreated according to their susceptibility results found that susceptibility-guided treatment achieved a better eradication rate (86%) than empiric treatment (63%) (388). Other studies have shown no difference in eradication rates of tailored treatment in those with refractory infection and conclude that susceptibility testing is not necessary (391-393). The final aim of this study was to investigate the efficacy of susceptibility-guided treatment as a second-line and subsequent rescue treatment. As noted above, due to a change in guidelines for duration of treatment, the efficacy of second-line and rescue TT in those treated for 7 days and in those treated with 14 days will be presented separately.

4.1.1 Aims of the study

1. To compare the efficacy of TT versus STT for 7 or 14 days in first-line treatment of *H. pylori*
    a) Determine whether the test used to determine susceptibility affects eradication rate
    b) Determine the effect of antibiotic resistance on eradication rate.

2. To assess whether the presence of clarithromycin resistance impacts eradication rates of first-line STT (for 7 or 14 days)
    a) Assess whether the presence of phenotypic or genotypic clarithromycin resistance affects eradication rate of STT.

3. To assess whether virulence factor genotypes impact eradication rates of first-line STT
    a) The presence of the *cagA* and type of *vacA* genotypes and their impact on treatment outcome were investigated.

4. To examine the efficacy of 7 or 14 day tailored second-line and rescue treatments for *H. pylori* infection
    a) Determine the eradication rates of tailored second-line and rescue treatments across treatment attempts
    b) Determine whether the test used to determine susceptibility affects eradication rate.

4.2 Methods

4.2.1 Study design and ethics

The study was carried out in a tertiary referral teaching hospital (Tallaght University Hospital, formerly the Adelaide and Meath Hospital, incorporating the National Children's Hospital, Dublin) affiliated with Trinity College Dublin. The study received ethical approval from the Joint Research Ethics Committee of the Adelaide and Meath National Children's Hospital and St James Hospital (Ethics Committee Study Reference Number: 2013/23/04). Patients over the age of 18 years who were scheduled to undergo endoscopy as a routine part of their care were invited to participate in the study. Informed consent was obtained by either the PhD candidate or members of the endoscopy clinical team, as detailed in Section 2.2.1. It was explained to all participating patients that their biopsies would be used to perform bacterial culture-based antimicrobial susceptibility testing. Regarding treatment naïve patients, it was explained that they would be randomised to receive either STT or TT. Regarding patients previously treated for *H. pylori* infection, it was explained that they would receive TT. Following completion of antimicrobial susceptibility testing by the candidate, in those treated with TT (either first-line or subsequent treatment), the results were relayed to a clinician within the Department who prescribed treatment based on the individual patient's susceptibility profile. Those treated with first-line STT received the standard regimen detailed in Section 4.2.6. Patients were provided with the prescription for their treatment regimen approximately 7-10 days after their endoscopy. Tailored regimens were based on the patient's phenotypic susceptibility profile when bacterial culture was successful and their genotypic susceptibility profile when culture was unsuccessful. The candidate then arranged and performed a follow-up UBT 8 week's post-treatment on the patients, as described in Section 2.2.3.1. *H. pylori* eradication was defined as a negative UBT result. Compliance and adverse events were assessed at the follow-up UBT appointment. A compliance cut-off point was set as taking at least 90% of each drug.

4.2.2 Study population

Recruited patients described in Chapter 2 were included in this study. Inclusion and exclusion criteria were as described in Chapter 2, Section 2.2.2. For the study which compared the efficacy of TT versus STT in first-line treatment of *H. pylori,* treatment naïve patients only were included. For the study which examined the efficacy of tailored second-line and subsequent treatment for *H. pylori* infection, patients previously treated for *H. pylori* infection only were included.

4.2.3 Diagnosis of *H. pylori* infection

The RUT, histology and culture were performed as described in Sections 2.2.4.1, 2.2.4.2 and 2.2.4.3 respectively.

4.2.4 Culture and antimicrobial susceptibility testing

Gastric biopsy samples were collected for bacterial culture of *H. pylori* (as detailed in Section 2.2.4.3). Phenotypic susceptibility to clarithromycin, metronidazole, levofloxacin, amoxicillin, tetracycline and rifampicin was determined using Etest strips and MICs, as detailed in Section 3.2.5 (although rifabutin is used clinically, rifabutin Etests are not available routinely and rifampicin is used to screen for rifabutin resistance for rifabutin resistance (354)). Genotypic susceptibility to clarithromycin and levofloxacin was determined using the GenoType HelicoDR assay, as detailed in Section 3.2.7.

4.2.5 Virulence factor genotyping

To evaluate the impact of virulence factors on treatment outcome, PCR-based virulence factor genotyping was performed on DNA extracted from biopsies obtained from *H. pylori* positive patients as described in Section 3.2.8.

4.2.6 Therapeutic regimens

Using a random number table generated by the Stat Trek random number generator software (http://www.stattrek.com); patients were randomised to receive either STT or TT (based on antibiotic sensitivities). STT consisted of 500 mg clarithromycin, 1 g amoxicillin and PPI (either omeprazole (20 mg) or esomeprazole (40 mg), in accordance with guidelines that were relevant at time of treatment), all taken twice daily (Table 4.2). TT consisted of either STT, levofloxacin-based triple therapy, a regimen of 2 antibiotics of which the patient was found to be susceptible and omeprazole/esomeprazole, rifabutin-based triple therapy or BQT. In accordance with guidelines relevant at time of treatment, regimens were prescribed for either 7 or 14 days. Information on the dosage and administration of the drugs in these regimens are detailed in Table 4.2.

Treatment Regimen	Description	Duration[1]
Clarithromycin-based triple therapy	PPI[2] b.i.d. 500 mg clarithromycin b.i.d. 1 g amoxicillin b.i.d.	7/14 days
Levofloxacin-based triple therapy	PPI[a] b.i.d. 250 mg levofloxacin b.i.d. 1 g amoxicillin b.i.d.	7/14 days
Rifabutin-based triple therapy	PPI[a] b.i.d. 150 mg rifabutin b.i.d. 1 g amoxicillin b.i.d.	7/14 days
Bismuth quadruple therapy	PPI[a] b.i.d. 120 mg bismuth subcitrate potassium q.i.d. 400 mg metronidazole t.i.d. 500 mg tetracycline q.i.d.	7/14 days
Other	PPI[a] b.i.d. Two of: 500 mg clarithromycin b.i.d. 250 mg levofloxacin b.i.d. 1 g amoxicillin b.i.d. 250 mg levofloxacin b.i.d. 400 mg metronidazole t.i.d. 500 mg tetracycline q.i.d.	7/14 days

Table 4.2: *H. pylori* treatment regimens prescribed in this study. b.i.d., twice daily; q.i.d., four times a day; t.i.d., three times daily. [1] In accordance with guidelines relevant at time of treatment, regimens were prescribed for either 7 or 14 days; [2]In accordance with guidelines that were relevant at time of treatment, either omeprazole (20 mg) or the newer generation PPIs esomeprazole (40 mg b.i.d.) was given.

4.2.7 Sample size

The formula below was used to determine the required number of patients to be recruited to the study (394, 395). The baseline eradication rate for STT was estimated to be 70% (162). It was decided that a 10% increase in the eradication rate of TT compared to STT was considered a significant clinical difference. As such a sample size of 664 patients (332 in each arm) was required to detect a 10% difference between the treatment groups, using a two-tailed z-test of proportions with 85% power and a 5% level of significance.

The formula for calculating sample size is:

$$n = \frac{(Z_\alpha + Z_{1-\beta})^2 (p1\ (1-p1)\ +\ p2\ (1-p2))}{(p1-p2)^2}$$

where

- n: sample size required in each group
- p1: proportion of patients cured by STT therapy = 0.70
- p2: proportion of subject cured by TT= 0.80
- p1-p2: clinically significant difference = 0.10
- Z_α: level of significance = 1.96 for 5%
- $Z_{1-\beta}$: power= 1.0364 for 85% (394, 395)

4.2.8 Statistical analysis

Statistical analysis was carried out using GraphPad Prism (GraphPad Software Inc., CA, USA). Continuous variables are presented as arithmetic mean and SD. Categorical variables are presented as percentages and 95% confidence intervals (95% CIs). *P* values for categorical variables were calculated using the Fisher's exact test/ Pearson $\chi 2$-test. In all cases, P values less than 0.05 were considered to indicate statistically significant differences between groups.

Intention-to-treat (ITT) and per-protocol (PP) analysis were performed to compare *H. pylori* eradication rates in the two treatment groups. ITT analysis included all patients who were randomly assigned to one of the treatments, regardless of whether or not they completed the treatment and follow-up. In PP analysis, only those patients who completed the entire treatment, were compliant and returned for follow-up according to protocol were included in the analysis.

4.3 Results

4.3.1 The efficacy of TT compared to STT in first line eradication of *H. pylori*

4.3.1.1 Patient inclusion

A schematic flow chart of patient inclusion is presented in Figure 4.1. Of the 266 patients recruited and described in Section 2.3.4, 148 (55.6%) were treatment naïve. Of these, 34 (23%) patients opted to be treated elsewhere-by their referring consultants or General Practitioners and 10 (6.8%) patients could not be contacted to arrange treatment. As such, 104 patients were included in the study. The mean age of the study participants was 49.7 ± 15.8 years and 62.5% (n =65) were male. Further details of baseline characteristics of the patients included in this study are available in Supplementary Table 4.1 (Appendix 3). The number of patients recruited to this study did not meet the required sample size (as detailed in Section 4.2.7, n=332 was required in each treatment arm).

4.3.1.2 Eradication rates

The efficacy of tailoring therapy based on antimicrobial susceptibility was compared to STT as a first line treatment for *H. pylori* infection. During this study, national and international guidelines on the treatment of *H. pylori* infection were released with the recommendation of 14 day treatment duration (64, 65). As such, it was necessary to change the duration of treatment of STT and TT accordingly. A total of 78 (75%) study participants were treated for 7 days and 26 (25%) were treated for 14 days (Figure 4.1). Eradication rates of STT and TT in patients treated for 7 or 14 days are presented in Figure 4.1.

By ITT analysis, there was no statistically significant difference between eradication rates of STT and TT for 7 days (71.1% vs 75.0% respectively; p=0.8) or 14 days (83.3%, vs 85.7% respectively; p=1.0). Similarly by PP analysis, there was no statistically significant difference in the efficacy between treatments in those treated for 7 days (71.1% STT vs 81.1% TT, p=0.4) or 14 days (90.9% STT vs 92.3% TT; p=1.0). Confidence intervals of eradication rates are available in Supplementary Table 4.2 (Appendix 3).

Although not statistically significant at the numbers tested, treatment duration of 14 days improved the efficacy of STT when compared to duration of 7 days by ITT analysis (83.3% vs 71.1% respectively; p=0.5) and PP analysis (90.9% vs 71.1% respectively; p=0.3). Similarly (although not statistically significant), the efficacy of TT was improved with 14 days duration (85.7% vs 75.0% respectively; p=0.7, ITT and 92.3% vs 81.1% respectively; p=0.7, PP). Comparison tables for STT and TT are available in Supplementary Tables 4.3 and 4.4, respectively (Appendix 3).

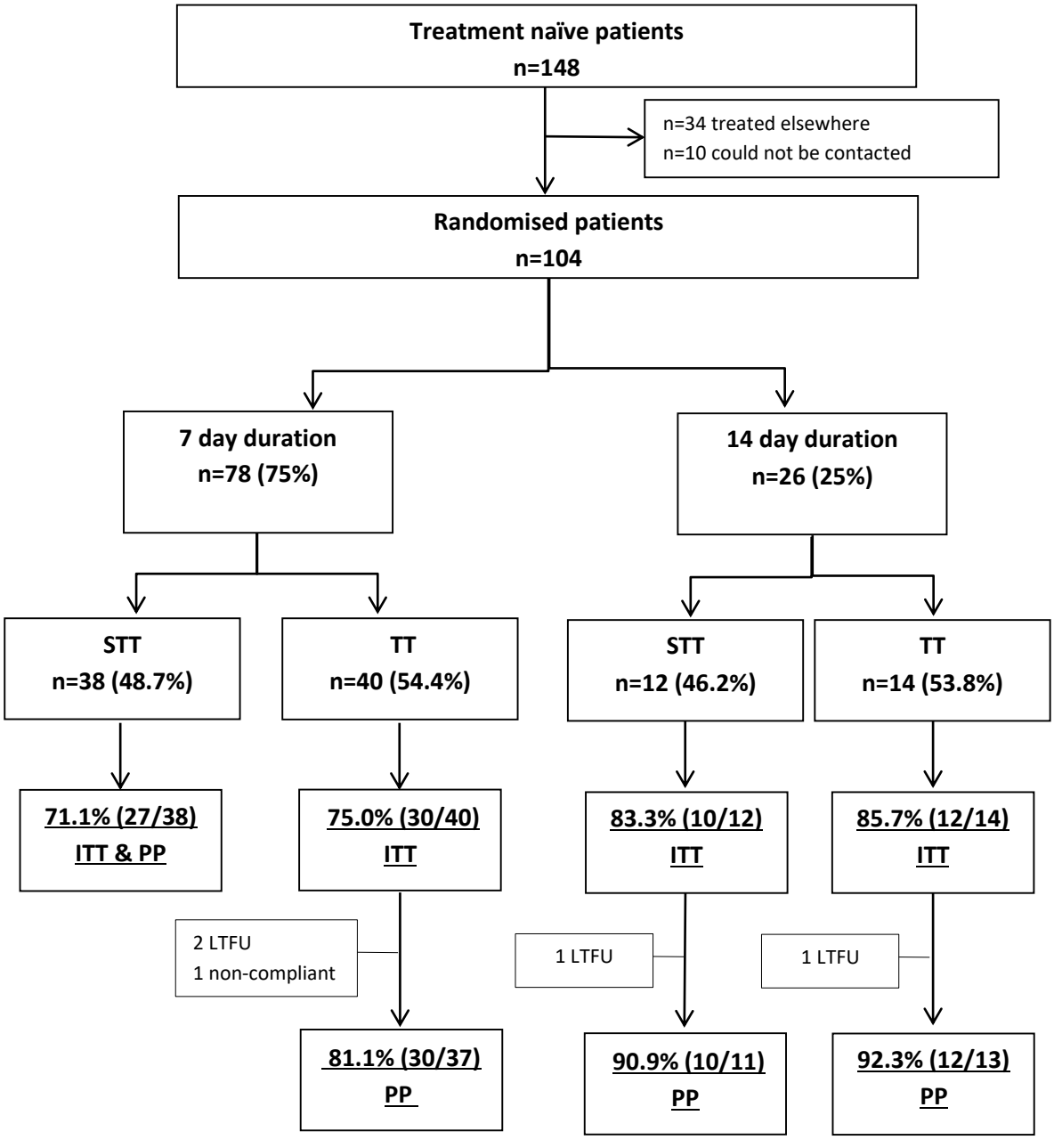


Figure 4.1: Flow chart of patient inclusion in those treated for 7 and 14 days and eradication rates in each group. STT: Standard triple therapy, TT: Tailored therapy, ITT: Intention to treat, LTFU: Lost to follow-up, PP: Per-protocol.

In patients whose treatment was tailored (n=54, 51.9%), the regimens prescribed, method of susceptibility testing and corresponding antibiotic resistance profile of patients is detailed in Table 4.3. Bacterial culture was successful in 35 (64.8%) and susceptibility to all 6 antibiotics was determined (described in Section 3.2.5). In those whose culture was unsuccessful (n=19), (35.2%) genotypic susceptibility to clarithromycin and levofloxacin was determined (described in Section 3.2.7). The respective eradication rates for each tailored regimen (by 7/14-day duration) are presented in Table 4.4.

Tailored regimen (n=54)	n	Method	n	Resistant to
CLAR- based triple therapy	**24**			
		Culture	9	None
			2	RIF
			5	MET
			1	MET, LEVO
		Genotype	6	None
			1	LEVO
LEVO-based triple therapy	**15**			
		Culture	5	CLAR, MET
			2	CLAR
			2	CLAR, MET, TET
		Genotype	6	CLAR
RIF-based triple therapy	**3**			
		Culture	2	CLAR, MET, LEVO, TET
		Genotype	1	CLAR,LEVO
Bismuth quadruple therapy	**3**			
		Culture	1	CLAR, MET, LEVO, AMOX, TET, RIF
		Genotype	2	CLAR,LEVO
CLAR, LEVO & PPI	**3**			
		Genotype	3	None
LEVO, RIF & PPI	**3**			
		Culture	**3**	CLAR, MET, AMOX, TET
LEVO, TET & PPI	**2**			
		Culture	**2**	CLAR, MET, AMOX, RIF
TET, RIF & PPI	**1**			
		Culture	**1**	CLAR, MET, LEVO, AMOX

Table 4.3: Tailored regimens, method of susceptibility testing and corresponding antibiotic resistance profile of patients who received TT. By culture, susceptibility to CLAR, MET, LEVO, AMOX, TET and RIF was determined. By genotype, susceptibility to CLAR and LEVO was determined.

Tailored Regimen n=54	7 day n=40, 74.1%		14 day n=14, 25.9%	
	n	ER	n	ER
CLAR- based triple therapy	20	**85%** (17/20) ITT **94.4%** (17/18) PP	4	**100%** (4/4) ITT & PP
LEVO-based triple therapy	9	**77.7%** (7/9) ITT & PP	6	**83.3%** (5/6) ITT **100%** (5/5) PP
RIF-based triple therapy	3	**100%** (3/3) ITT & PP	none	-
Bismuth quadruple therapy	2	**50%** (1/2) ITT **100%** (1/1) PP	1	**100%** (1/1) ITT & PP
CLAR, LEVO & PPI	3	**66.7%** (2/3) ITT & PP	none	-
LEVO, RIF & PPI	2	**50%** (1/2) ITT & PP	1	**0%** (0/1) ITT & PP
LEVO, TET & PPI	none	-	2	**100%** (2/2) ITT & PP
TET, RIF & PPI	1	**100%** (1/1) ITT & PP	none	-

Table 4.4: Eradication rates of each tailored regimen (by 7/14-day duration).

4.3.1.3 Compliance and adverse events

One patient treated with TT for 7 days was non-compliant. There was no significant difference in the rate of adherence to STT compared to TT (100% versus 98.1%, respectively p=1.0). 4 patients who received TT reported adverse events (3 patients were treated for 7 days and 1 patient treated for 14 days). The adverse events consisted of nausea and vomiting (n=3) and bloating (n=1). There was no significant difference in the incidence of adverse events in those whose treatment was tailored than for STT (7.4% vs 0%, respectively; p=0.1).

4.3.1.4 Penicillin allergy

6 (5.8%) patients were allergic to penicillin. 1 was randomised to receive STT (with metronidazole) and 5 received TT (3 received levofloxacin-based triple therapy with metronidazole and 2 received levofloxacin, tetracycline and PPI). Eradication was achieved in all 6 patients.

4.3.1.5 Does the type of test used to determine susceptibility impact eradication?

In patients whose treatment was tailored (n=54, 51.9%), 35 (64.8%) had their susceptibility determined phenotypically and 19 (35.2%) were determined genotypically as their bacterial cultures were negative. Eradication rates are presented in Table 4.5. There was no statistically significant difference in eradication in patients whose susceptibility was determined by phenotypic test compared to genotypic test for 7 or 14 days.

	Genotype HelicoDR n=19 (35.2%)	**Culture & Etest** n=35 (64.8%)	**p value**
	Overall		
ITT analysis	**73.7%** (14/19; 95% CI 51.2- 88.2)	**80.0%** (28/35; 95% CI 64.1- 90.0)	0.7[1]
PP analysis	**82.4%** (14/17; 95% CI 59.0- 93.8)	**84.8%** (28/33; 95% CI 69.1- 93.4)	1.0[1]
	7 day		
ITT analysis	**71.4%** (10/14; 95% CI 45.0- 88.7)	**76.9%** (20/26; 95% CI 57.6- 89.3)	0.7[1]
PP analysis	**76.9%** (10/13; 95% CI 49.1- 92.5)	**83.3%** (20/24; 95% CI 63.5- 93.9)	0.7[1]
	14 day		
ITT analysis	**80%** (4/5; 95% CI 36.0- 98.0)	**88.8%** (8/9; 95% CI 54.3- 99.9)	1.0[1]
PP analysis	**100%** (4/4; 95% CI 45.4 -100)		1.0[1]

Table 4.5: Eradication rates of TT (7/14 day) by resistance test used to determine susceptibility. [1]Fisher's exact test.

4.3.1.6 The impact of phenotypic resistance to any antibiotic on treatment outcome

The effect of phenotypic resistance to any antibiotic on first-line treatment outcome was evaluated. From 104 patients included in the study, culture and subsequent phenotypic resistance testing was successful in 59 (56.7%). Phenotypic resistance to at least one or more antibiotics was observed in 47 (79.7%) patients in the study and 12 (20.3%) patients were susceptible to all antibiotics (Figure 4.2). Interestingly, in those susceptible to all antibiotics that were treated with STT, high eradication rates were achieved in those treated for 7 days (100% ITT & PP) and in those treated for 14 days (66.6% ITT and 100% PP). In those resistant to one or

more antibiotic(s), there was no significant difference in eradication rate between STT and TT (7 or 14 days) by either ITT or PP analysis (Supplementary Table 4.5; Appendix 3).

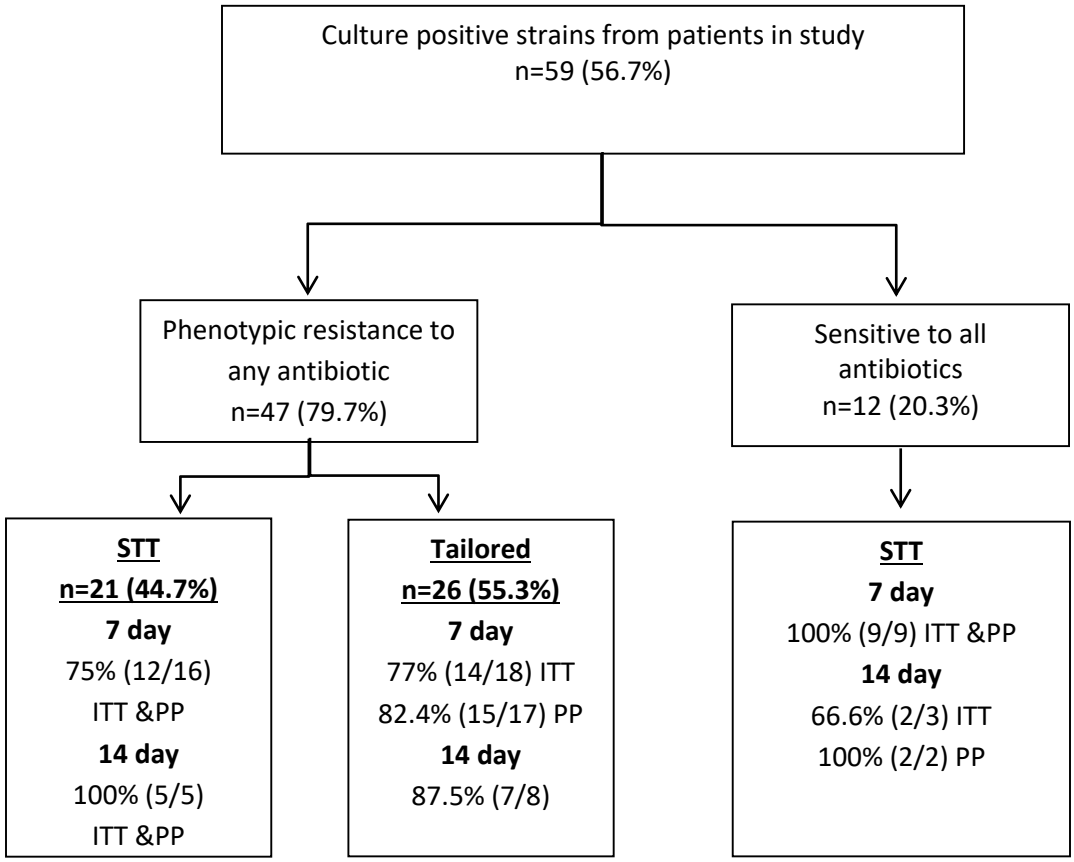


Figure 4.2: Eradication rates by phenotypic resistance to any antibiotic

In summary, there was no statistically significant difference in eradication rates of STT and TT (in those treated for 7 days and in those treated for 14 days). Although not significant at the numbers tested, treatment duration of 14 days improved the efficacy of both STT and TT when compared to duration of 7 days. Both treatments were well tolerated and adhered to. There was no statistically significant difference in eradication in those whose susceptibility was determined by culture and Etest versus those determined by genotypic test. Finally the presence of phenotypic resistance to any antibiotic did not have an impact on treatment outcome at the numbers tested.

4.3.2 The impact of clarithromycin resistance on treatment outcome for STT

Due to the rise in primary clarithromycin resistance (36.1%) and the significant variation in phenotypic and genotypic resistance to clarithromycin reported in Chapter 3, the impact of

phenotypic and genotypic clarithromycin resistance on first-line eradication rates was investigated.

All patients treated with first-line STT- regardless of treatment arm they were randomised to- were included in this analysis. Out of 104 patients, 74 (71.2%) received STT (either because they were deemed susceptible to clarithromycin or because they were randomised to receive standard therapy). When phenotypic resistance information was not available, genotypic resistance information was used to determine susceptibility. By PP analysis, overall eradication (7 and 14 day combined) was significantly lower in clarithromycin resistant patients than in sensitive (62.5% vs 87.2%, p=0.03, Table 4.6). When the analysis was stratified by treatment duration, a trend towards lower eradication was observed in clarithromycin resistant patients when compared to sensitive patients, although this difference was not statistically significant at the numbers tested (Table 4.6).

Eradication rate	**STT n=74**		**p value**
	Resistant to clarithromycin 24 (32.4%)	**Sensitive to clarithromycin 50 (67.6%)**	
	Overall		
ITT analysis		**82.0%** (41/50; 95% CI 69.2- 90.2)	0.09[1]
PP analysis	**62.5%** (15/24; 95% CI 42.7- 78.8)	**87.2%** (41/47; 95% CI 74.8- 94.0)	0.03[1]
	7 day		
ITT analysis		**79.5%** (31/39; 95% CI 64.2- 89.5)	0.1[1]
PP analysis	**57.9%** (11/19; 95% CI 36.2- 76.9)	**83.8%** (31/37; 95% CI 68.5- 92.7)	0.05[1]
	14 day		
ITT analysis		**90.9%** (10/11; 95% CI 60.1- 99.9)	1.0[1]
PP analysis	**80%** (4/5; 95% CI 26.6- 81.2)	**100%** (10/10; 95% CI 67.9- 100)	0.3[1]

Table 4.6: Eradication rate of STT in those susceptible and resistant to clarithromycin (tests combined). [1]Fisher's exact test.

When the data was stratified by the method of susceptibility testing, culture was successful in 41 (55.4%) and therefore susceptibility was determined phenotypically (Table 4.7). *H. pylori*

eradication by STT as assessed by genotypic testing is presented in Table 4.8. By both methods, a trend towards lower eradication was observed in clarithromycin resistant patients when compared to sensitive patients in those treated for 7 days, however this was not significant.

Eradication rate	**Culture and Etest**		**p value**
	n=41		
	Resistant to clarithromycin	**Sensitive to clarithromycin**	
	10 (24.4%)	**31 (75.6%)**	
	Overall		
ITT analysis	**70.0%** (7/10; 95% CI 39.7- 89.2)	**83.9%** (26/31; 95% CI 67.4- 92.9)	0.4[1]
PP analysis		**89.7%** (26/29; 95% CI 73.6- 96.4)	0.2[1]
	7 day		
ITT analysis	**62.5%** (5/8; 95% CI 30.4- 86.5)	**82.6%** (19/23; 95% CI 62.3-93.6)	0.3[1]
PP analysis		**86.4%** (19/22; 95% CI 65.8-96.2)	0.3[1]
	14 day		
ITT analysis	**100%** (2/2; 95% CI 29.0-100)	**87.5%** (7/8; 95% CI 50.8-99.9)	1.0[1]
PP analysis		**100%** (7/7; 95% CI 59.6-100)	1.0[1]

Table 4.7: Eradication rate of clarithromycin-based STT when clarithromycin susceptibility was determined phenotypically. [1]Fisher's exact test

Eradication rate	STT n=74		p value
	Resistant to clarithromycin 34 (45.9%)	Sensitive to clarithromycin 40 (54.1%)	
	Overall		
ITT analysis	**70.6%** (24/34; 95% CI 53.8-83.2)	**80.0%** (32/40; 95% CI 65.2-89.5)	0.4[1]
PP analysis	**70.6%** (24/34; 95% CI 53.8-83.2)	**86.5%** (32/37; 95% CI 72.0- 94.1)	0.1[1]
	7 day		
ITT analysis	**60.9%** (14/23; 95% CI 40.7- 77.9)	**80%** (28/35; 95% CI 63.8-90.3)	0.1[1]
PP analysis	**60.9%** (14/23; 95% CI 40.7- 77.9)	**84.8%** (28/33 95% CI 68.6-93.8)	0.06[1]
	14 day		
ITT analysis	**90.9%** (10/11; 95% CI 60.1-99.9)	**80%** (4/5; 95% CI 36.0-98.0)	1.0[1]
PP analysis	**90.9%** (10/11; 95% CI 60.1-99.9)	**100%** (4/4; 95% CI 45.4- 100)	1.0[1]

Table 4.8: Eradication rate of STT in those genotypically susceptible and resistant to clarithromycin. [1]Fisher's exact test

Treatment outcome of STT and both phenotypic and genotypic resistance status was available in 41 patients and data is presented in Table 4.9. Although not significant at the numbers tested, when resistance was detected both phenotypically and genotypically, the eradication rate of 7-day therapy was lower than in those who were both phenotypically and genotypically susceptible to clarithromycin (40.5% vs 87.5%; p=0.06, PP). All of these strains contained the A2147G mutation (the most commonly detected in our cohort, Table 3.12). According to point mutation, there was no difference in eradication between those harbouring the A2147G mutation and in those containing the remaining mutations (70.4%, n=19/27 vs 71.4%, n=5/7; p=1.0).

Clarithromycin resistance status	Eradication rate (%)	
	7 day	**14 day**
Phenotype:S **Genotype: S** (n=19)	**82.4%** (14/17) ITT **87.5%** (14/16) PP	**50%** (1/2) ITT **100%** (1/1) PP
Phenotype: R **Genotype: S** (n=2)	**100%** (2/2) ITT & PP	-
Phenotype: S **Genotype: R** (n=12)[1]	**83.3%** (5/6) ITT & PP	**100%** (6/6) ITT & PP
Phenotype: R **Genotype: R** (n=8)[2]	**40%** (2/5) ITT & PP	**100%** (2/2) ITT & PP

Table 4.9: Treatment outcome according to phenotypic and genotypic resistance status in culture positive strains. [1] 11 strains had the A2147G mutation and 1 strain had the A21426C mutation, [2]7 strains had the A2147G mutation and 1 strain had both A2147G+ A21426C mutations.

In summary, the presence of clarithromycin resistance had a significant effect on PP eradication rates overall (7 and 14 day combined). When the data was stratified by duration, although not statistically significant, the presence of clarithromycin resistance lowered the efficacy of TT for 7 and 14 days duration. Similarly, when stratified by the method of susceptibility testing (phenotypic or genotypic), although not statistically significant, the presence of clarithromycin resistance lowered the efficacy of STT in both groups. Finally there was a trend towards lower eradication rate of 7-day therapy in patients in whom resistance was detected both phenotypically and genotypically than in those who were fully susceptible to clarithromycin.

4.3.3 The impact of virulence factor genotype on treatment outcome of STT

As less virulent strains of *H. pylori* were associated with primary genotypic clarithromycin resistance (reported in Section 3.3.5), the impact of virulence factor genotype on treatment outcome was assessed. Of the 104 patients treated with first-line therapy, information on virulence factor genotype was available for 80 patients. 2 of these patients were excluded from the analysis as one was LTFU and the other was non-compliant. A total of 56 patients were treated with STT, with 48 treated for duration of 7 days and 8 treated for 14 days. Eradication was achieved in all 8 patients treated for 14 days and as there was no comparison data, this

analysis is not presented. The distribution of virulence factor genotype according to treatment outcome in those treated for 7 days is presented in Table 4.10. No significant association between virulence factor genotype and eradication was found.

	7 day (n=48)		
	Eradication Success n=34 (70.8%)	**Eradication Failure n=14 (29.2%)**	**p value**
cagA +	9 (26.5)	6 (42.9)	0.3[1]
cagA -	25 (73.5)	8 (57.1)	
***vacA* S1/M1**	12 (35.3)	3 (21.4)	0.5[1]
***vacA* S1/M2**	15 (44.1)	8 (57.1)	0.5[1]
***vacA* S2/M1**	0	1 (7.1)	0.3[1]
***vacA* S2/M2**	7 (20.6)	2 (14.3)	1.0[1]

Table 4.10: **Distribution of virulence factor genotype according to treatment outcome in patients treated with first-line STT for 7 or 14 days.** [1]Fisher's exact test

4.3.4 The efficacy of TT as a second-line and rescue therapy for *H. pylori* eradication

4.3.4.1 Patient inclusion

A schematic flow chart of patient inclusion is presented in Figure 4.3. Of the 266 patients recruited and described in Section 2.3.4, 118 (44.4%) had been previously treated for *H. pylori*. Of these, 39 (33.1%) patients opted to be treated elsewhere-by their referring consultants or General Practitioners and 9 (7.7%) patients could not be contacted to arrange treatment. On the day of their endoscopy, 15 recruited patients had reported that they had not been previously treated for *H. pylori* infection. However upon later review of the patient's referral letters, it was established that these patients had in fact been treated for *H. pylori* previously. These patients were omitted from both studies (and from Chapter 3). An additional 5 patients were removed from this study as it was established during analysis that the prescriptions for these patients were written based on the regimens that the patient had received previously instead of their resistance profile. As such, 50 patients were included in the study. The mean age of study participants was 45.4 ± 14.3 years and 38% (n =19) were male.

4.3.4.2 Eradication rates of tailored treatment in all patients previously treated for *H. pylori* infection

The efficacy of tailoring treatment based on antimicrobial susceptibility test results was evaluated in the total number of patients previously treated for *H. pylori* infection (second-line and rescue combined). As explained in Section 4.1, it was necessary to change the duration of treatment in this study from 7 to 14 days. A total of 37 (74%) study participants were treated for 7 days and 13 (26%) were treated for 14 days (Figure 4.3). Eradication rates of tailored therapy in those treated for 7 and 14 days are presented in Figure 4.3. Confidence intervals of eradication rates are available in Supplementary Table 4.6. Although not statistically significant at the numbers tested, treatment duration of 14 days improved the efficacy of tailored treatment when compared to duration of 7 days by ITT (76.9% vs 54.1%; p=0.2) and PP analysis (90.9% vs 57.1%; p=0.07).

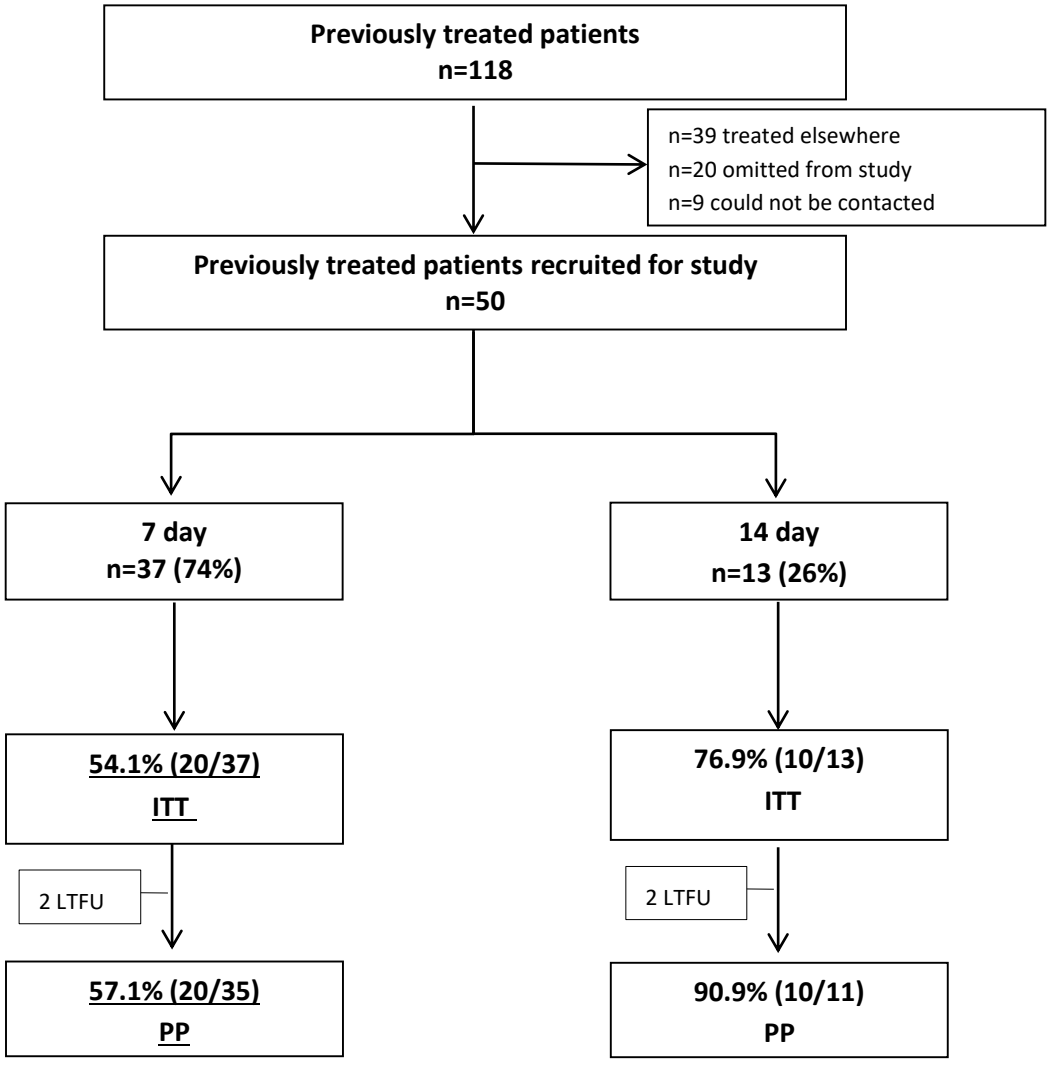


Figure 4.3: Flow chart of the tailored therapy in those treated for 7 and 14 days (all previously treated patients).

The tailored regimens, method of susceptibility testing and corresponding antibiotic resistance profile of patients are detailed in Table 4.11. Bacterial culture was successful in 28 (56%) and susceptibility to all 6 antibiotics was determined (described in Section 3.2.5). In those whose culture was unsuccessful (n=22, 44%), genotypic susceptibility to clarithromycin and levofloxacin was determined (described in Section 3.2.7). The respective eradication rates for each tailored regimen (by 7/14 day duration) are presented in Table 4.12.

Tailored regimen	n	Method	n	Resistant to
LEVO- based triple therapy	**26**			
		Culture	5	CLAR, MET
			4	CLAR
			2	CLAR, MET, RIF
			1	RIF
			1	MET
			1	CLAR, TET
			1	CLAR, MET, TET
		Genotype	10	CLAR
			1	NONE
Bismuth quadruple therapy	**7**			
		Culture	1	None
			2	CLAR, LEVO
		Genotype	4	CLAR, LEVO
CLAR-based triple therapy	**8**			
		Culture	5	NONE
			2	MET
		Genotype	1	NONE
TET, RIF & PPI	**2**			
		Culture	1	CLAR, MET, LEVO, AMOX
		Genotype	1	CLAR, LEVO
LEVO, TET & PPI	**3**			
		Genotype	3	CLAR
MET, TET & PPI	**2**			
		Genotype	2	CLAR, LEVO
LEVO, RIF & PPI	**1**			
		Culture	1	MET, AMOX
CLAR, TET & PPI	**1**			
		Culture	1	MET

Table 4.11: Regimens, method of susceptibility testing and corresponding antibiotic resistance profile of patients who received TT. By culture, susceptibility to CLAR, MET, LEVO, AMOX, TET and RIF was determined. By genotype, susceptibility to CLAR and LEVO was determined.

Tailored regimen	7 day n=37, 74%		14 day n=13, 26%	
	Rx (n)	ER	Rx (n)	ER
LEVO-based triple therapy	20	**65%** (13/20) ITT & PP	6	**83.3%** (5/6) ITT **100%** (5/5) PP
Bismuth quadruple therapy	5	**60%** (3/5) ITT & PP	2	**50%** (1/2) ITT &PP
CLAR-based triple therapy	5	**40%** (2/5) ITT **50%** (2/4) PP	3	**100%** (3/3) ITT & PP
TET, RIF & PPI	2	**50%** (1/2) ITT & PP		-
LEVO, TET & PPI	3	**66.7%** (2/3) ITT &PP		-
TET, MET & PPI	1	**0%** (0/1) ITT & PP	1	**0%** (0/1) ITT & PP
LEVO, RIF & PPI	1	**0%** (0/1) ITT & PP		-
CLAR, TET & PPI		-	1	**100%** (1/1) ITT & PP

Table 4.12: Eradication rates of each tailored regimen (by 7/14-day duration).

4.3.4.3 Compliance and adverse events

The rate of adherence to tailored treatment was 100%. Adverse events occurred in 1 patient (2%), which consisted of joint pain. This patient was treated for 14 days with clarithromycin, tetracycline and PPI.

4.3.4.4 Drug allergy

Allergy to penicillin was present in 2 (4%) patients; one was treated with levofloxacin, tetracycline and PPI and one with tetracycline, rifabutin and PPI. Allergy to levofloxacin was present in 1 (2%) and this patient was treated with tetracycline, metronidazole and PPI. *H. pylori* eradication was achieved in none of these patients.

4.3.4.5 Does the number of previous treatments impact eradication rates?

The efficacy of second-line TT (following one previous treatment attempt) and rescue TT (in those with ≥2 previous attempts) is detailed in Table 4.13. In patients treated for 7 days, per protocol analysis, eradication was significantly lower in patients who had received ≥2 previous

treatments compared to those who were receiving second-line treatment (33.3% vs 75%; p=0.02). The exact number of previous treatments that patients received and their respective eradication rates are detailed in Supplementary Table 4.7. Although not statistically significant, overall there was a reciprocal relationship between number of previous treatments received and eradication rate.

Eradication rate	Second-line treatment n= 27 (54%)	≥2 previous treatments n=23 (46%)	p value
	Overall		
ITT analysis	**70.4%** (19/27; 95% CI 51.4- 84.3)	**47.8%** (11/23; 95% CI 29.2- 67.0)	0.1[1]
PP analysis	**76%** (19/25; 95% CI 56.3- 88.8)	**52.4%** (11/21; 95% CI 32.4- 71.7)	0.1[1]
	7 day		
ITT analysis	**71.4%** (15/21; 95% CI 49.8- 86.4)	**31.3%** (5/16; 95% CI 13.9- 55.9)	0.08[1]
PP analysis	**75%** (15/20; 95% CI 52.8- 89.2)	**33.3%** (5/15; 95% CI 15- 58.5)	0.02[1]
	14 day		
ITT analysis	**66.6%** (4/6; 95% CI 29.6- 90.8)	**85.7%** (6/7; 95% CI 46.7- 99.5)	0.6[1]
PP analysis	**80%** (4/5; 95% CI 36- 98)	**100%** (6/6; 95% CI 55.7-100)	1.0[1]

Table 4.13: Eradication rate of tailored treatment according to number of previous treatments. [1]Fisher's exact test.

4.3.4.6 Does the type of test used to determine susceptibility impact eradication?

Of the 50 patients treated with tailored therapy, 28 (56%) had their susceptibility determined phenotypically and 22 (44%) were determined genotypically as their bacterial cultures were negative. Although not significant at the numbers tested, tailored therapy eradications trend higher for culture based tests for both 7 and 14 day duration (Table 4.14).

Eradication rate	Genotype HelicoDR n=22 (44%)	Culture & Etest n=28 (56%)	p value
	Overall		
ITT analysis	**50%** (11/22; 95% CI 30.7- 69.2)	**67.9%** (19/28; 95% CI 49.2- 82.2)	0.3[1]
PP analysis	**55%** (11/20; 95% CI34.2- 74.1)	**73.1 %** (19/26; 95% CI 53.7-86.5)	0.2[1]
	7 day		
ITT analysis	**47.3%** (9/19; 95% CI 27.3- 68.3)	**61.1%** (11/18; 95% CI 38.5- 79.8)	0.5[1]
PP analysis	**50%** (9/18; 95% CI 29- 71)	**64.7%** (11/17; 95% CI 41.2- 82.8)	0.5[1]
	14 day		
ITT analysis	**66.7%** (2/3; 95% CI 20.2- 94.4)	**80%** (8/10; 95% CI 47.9- 95.4)	1.0[1]
PP analysis	**100%** (2/2; 95% CI 29- 100)	**88.8%** (8/9; 95% CI 54.3- 99.9)	1.0[1]

Table 4.14: Eradication rates by resistance test used to determine susceptibility. [1]Fisher's exact test

In summary, although not statistically significant at the numbers tested, the efficacy of 14 day tailored treatment was improved compared to 7 day duration. For second-line treatment of *H. pylori* infection, the efficacy of 14 day TT was 66.6% and 80% by ITT and PP analysis respectively. For subsequent rescue treatment of *H. pylori* infection, the efficacy of 14 day TT was 85.7% and 100% by ITT and PP analysis respectively. Compliance to this treatment was good and there were minimal adverse events. In patients treated for 7 days, efficacy of TT in those who had received ≥2 previous treatments was significantly lower than in those who received second-line TT. Although not statistically significant, there was a trend towards higher eradication in those whose susceptibility was determined by culture and Etest versus those determined by genotypic test.

4.4 Discussion

4.4.1 Standard versus tailored first-line treatment of *H. pylori* infection

To ensure adequate power in this study, the optimal sample size was determined prior to commencement of this study (Section 4.2.7). It was established that recruitment of 664 patients (332 in each arm) was required to detect statistical significance. However, disappointingly, upon completion of this study, a total of 104 treatment naïve patients had been included. There were a number of contributing factors to this study not meeting the required sample size. Originally, this study was to be conducted in another Dublin-based Hospital which had the potential to recruit a significant proportion of the patients required to meet the sample size. Unfortunately early on in the project, our collaborator in this Hospital was not available to collaborate on this project due to illness. In our centre, there were a number of difficulties pertaining to patient recruitment. Firstly, the number of patients undergoing endoscopy who had a positive RUT indicative of *H. pylori* infection was low (22.6%, Section 2.3.4); of the eligible patients who had *H. pylori* infection, 44.4% of patients had been previously treated for *H. pylori* and therefore could not be included in the STT vs TT study. Finally, 23% of patients opted instead to be treated by their referring doctor.

As national and international guidelines on the first-line treatment of *H. pylori* infection were released in late 2016 which recommended a treatment duration of 14 days (64, 65), it was necessary to change this study protocol accordingly. This recommendation was based on many meta-analyses and observational studies which showed that increasing the duration of first-line STT increases eradication rates, with treatment durations of 14 days most effective (250-254, 396, 397). In this study, although not significant at the numbers tested, treatment duration of 14 days improved the efficacy of first-line STT when compared to 7 days (83.3% vs 71.1%; p=0.5, ITT and 90.9% vs 71.1%; p=0.3, PP). The efficacy of first-line TT also improved with 14 day duration (85.7% vs 75.0%; p=0.7, ITT and 92.3% vs 81.1% p=0.7, PP).

In this study, the eradication rate of 14 day TT and STT was 85.7% and 83.3% respectively by ITT analysis and 92.3% and 90.9% PP analysis (p=1.0 for both). The absence of a statistically significant difference in the efficacy of TT compared to STT may be due to the low numbers of patients included. As the study did not meet the required sample size, unfortunately a definitive conclusion on the effectiveness of TT vs STT in our cohort cannot be made. As detailed in Section 4.1, a number of other studies have shown antimicrobial susceptibility-guided treatment to be more effective than empirically chosen treatments (294-303). A meta-analysis of twelve studies on TT found that TT was more efficacious than empirical 7-10 day STT (299). While some

studies have shown TT to be more cost effective than STT (296, 304), others have suggested that the feasibility and cost-effectiveness of this strategy is unclear as most centres do not have the resources to carry out susceptibility testing (296, 304-306).

In those resistant to at least one antibiotic, there was no significant difference in eradication of STT and TT. Phenotypic resistance to at least one antibiotic was observed in 79.7% of patients included in this study. This level has also been seen in a study carried out by Binkowska et al. (185) which reported that 80.9% of adult patients were resistant to at least one antibiotic. They also reported primary clarithromycin and levofloxacin resistance in adults at 32% and 17% respectively- similar to the primary resistance rates reported in Chapter 3 (36.1% and 16.7% respectively). Additionally, a pre-print paper not yet accepted for publication (available on BioRxiv) reported 69.7% multi-drug resistance in Iran (398).

4.4.2 Clarithromycin resistance and first-line treatment outcome

Key findings of Chapter 3 were an alarming rise in primary clarithromycin resistance (36.1%) and a significant variation in phenotypic and genotypic resistance to clarithromycin. In light of this, the second aim of this chapter was to investigate the impact of clarithromycin resistance on eradication rates in our cohort. All patients treated with STT- regardless of the treatment arm they were randomised to- were included in this analysis.

Overall eradication (7 and 14 day combined) was significantly lower in clarithromycin resistant patients than in sensitive (62.5% vs 87.2%, p=0.03, PP). When the analysis was stratified by treatment duration, although not statistically significant, there was a trend towards lower eradication in clarithromycin resistant patients when compared to sensitive patients in those treated for 7 and 14 days. Further studies with increased patient numbers are required to investigate the impact of clarithromycin resistance on the extended 14 day treatment duration in our patient cohort.

When the patients were stratified according to their susceptibility test method, overall there was a trend towards lower eradication in clarithromycin resistant patients when compared to sensitive patients for both Etest and GenoType HelicoDR, but this difference was not statistically significant. Treatment outcome according to phenotypic and genotypic resistance status was then analysed (Table 4.9). Unfortunately the numbers of patients treated for 14 days in this analysis is too small to comment on. However in those treated for 7 days, the eradication rate of STT in fully sensitive patients (both phenotypically and genotypically) was 87.5% (PP) while in fully resistant patients, eradication was just 40% (PP; p=0.1). De Francesco et al. examined the

role of phenotypic and genotypic clarithromycin resistance on therapeutic outcome of 10 day STT and sequential therapy and found that in strains with phenotypic and genotypic resistance, eradication (for both therapies combined) was also low at 55% (374).

Clarithromycin resistance in our cohort is high, but overall, in those treated with 14 day clarithromycin-based therapy an acceptable eradication rate of >80% was achieved (Figure 4.1). In those treated for 7 days, an eradication rate of 71.1% was achieved. There a few factors which could potentially explain the eradication rates presented herein.

These patients were part of a research study where their diagnosis and treatment regimen was clearly explained to them. They were counselled as to the importance of compliance in taking the full course of treatment. These patients were properly followed-up to confirm eradication of infection. As discussed in Section 4.4.6, compliance is a very important factor in *H. pylori* eradication and structured patient counselling and follow-up can significantly improve *H. pylori* eradication rates (247, 399). In the studies on first-line and subsequent treatments, compliance rates of 99% and 100% respectively were achieved.

Second, it could in part be due to the action of amoxicillin and the PPI used as part of the STT regimen. In those patients who were resistant to clarithromycin and who received STT with amoxicillin, (n=24), eradication was achieved in 62.5% (PP).

As discussed in detail in Section 3.4.1, technical variations in the manner in which antimicrobial susceptibility testing is performed means reporting of resistance rates can vary. An earlier study from our centre in 2009 used a MIC cut-off of >1 mg/L to deem a strain resistant to clarithromycin (50), whereas a cut-off of >0.5 mg/L was used in this study, according to current EUCAST recommendations (354). Additionally, as discussed in Section 3.4.1, recently the EUCAST steering committee changed the definitions of the susceptibility testing categories S, I and R (S= susceptible, I= intermediate and R= resistant) (365). In 2019, the term “intermediate” would be replaced by “susceptible, increased exposure”. Clarithromycin was one of the antibiotics to be classified in this way, which has implications for determining clarithromycin resistance with regards to *H. pylori* infection.

Eradication in spite of resistance has been observed previously. Adachi et al. reported an eradication rate of 70.6% for 7 days clarithromycin triple therapy when the prevalence of clarithromycin resistance was 45% (400). De Francesco et al. have suggested that antibiotic MIC values impact treatment outcome. They tested whether different MIC values for clarithromycin and metronidazole play a role in the therapeutic efficacy of sequential therapy. They classified

the level of low and high levels of resistance by MIC values for clarithromycin (low: >0.5 to ≤8 mg/L and high: >8 to 256 mg/L) and for metronidazole (low: >8 to ≤32 mg/L and high: >32 to 256 mg/L). They found that even in the presence of dual clarithromycin and metronidazole resistance when MIC levels were low, the eradication rate was still high (92.7%). The eradication rate was reduced only when MIC levels of both antibiotics were high (83.9%) (401). In Chapter 3, it was found that 53.8% of primary clarithromycin resistant strains had low MIC values (Section 3.3.2.1, Figure 3.2(a)). Whether MIC values are associated with the outcome of clarithromycin-based triple therapy is worthy of further investigation.

Finally, in those treated according to the new guidelines, the high level of eradication could also be due to the use of newer generation high dose PPI's (257).

4.4.3 Considerations for first-line treatment of *H. pylori*

Similar to levels of clarithromycin resistance reported recently in other countries around the world (169, 183, 185, 188, 191, 192, 300, 360), primary clarithromycin resistance in our cohort is high, at 36.1%. Current guidelines recommend that in areas of high clarithromycin resistance (>15-20%), empirical STT should be abandoned and alternatives such as BQT or concomitant therapy be used (64, 173, 174). However primary dual resistance to clarithromycin and metronidazole in our cohort is 30.6% and concomitant therapy is not recommended when the primary rate of dual resistance is >15%.

BQT has shown promise in a number of studies, with reported eradication rates of up to 97% (186, 262, 263). BQT can overcome metronidazole resistance and it has been reported that clarithromycin resistance does not influence the effectiveness of BQT (246, 259). A very recent meta-analysis examining the effectiveness of 10-day treatment with Pylera®, (a single capsule containing bismuth, metronidazole and tetracycline) and a PPI reported an eradication rate of approximately 90% both in first- and second-line therapy. The study included patients with clarithromycin- or metronidazole-resistant strains and in those previously treated with clarithromycin (264). BQT however is not widely available in Ireland. De-Noltab (bismuth subcitrate potassium 120mg; Astellas Pharma, Leiden, The Netherlands), is licensed and marketed in the UK, but not in Ireland, where it is considered an Exempt Medicinal Product (65). As such, it needs to be sourced by pharmacists through a special wholesaler. The single capsule treatment Pylera (140 mg bismuth subcitrate potassium, 125mg metronidazole, 125 mg tetracycline hydrochloride; Aptalis Pharma, Houdan, France) is licenced but not marketed in Ireland, making it currently very difficult for pharmacies to obtain (65). Although BQT is

currently not an easily accessible option, efforts should be made to make this treatment available for patients in Ireland. If BQT becomes more widely available however, eradication rates should be monitored, given the rate of tetracycline resistance reported in Chapter 3 (18.1%).

The eradication rate of 7 day STT in this study was 71.1% ITT- below the 80% ITT deemed acceptable for a treatment by European guidelines (387). Therefore the use of 7 day STT is not recommended and this treatment strategy should be abandoned, in line with current guidelines (65). It is difficult however for clinicians to choose viable alternatives to STT. As discussed, the efficacy, feasibility and cost effectiveness of susceptibility guided treatment in our patient cohort is uncertain and BQT is not currently accessible for many patients in Ireland. In the small number of patients treated with 14 day STT in this study (n=12), an acceptable eradication rate of 83.3% ITT was achieved. As such, further investigation into the efficacy of 14 day STT is warranted. With larger study numbers, if the eradication rate of 14 day STT falls below 80% and clarithromycin resistance remains high, 14 day STT should be abandoned.

In light of the high rates of resistance to commonly used antibiotics reported in Chapter 3, continued surveillance of resistance as well as treatment success rates is vital, both locally and at a national level.

4.4.4 The efficacy of TT following previous eradication failure of *H. pylori* infection

Although not significant at the numbers tested, treatment duration of 14 days improved the efficacy of tailored treatment when compared to 7 day duration (76.9% vs 54.1%; p=0.2 ITT and 90.9% vs 51.7%; p=0.07 PP).

When stratified by number of treatments received, the efficacy of second-line 14 day TT was 66.6% and 80% by ITT and PP analysis respectively. For subsequent rescue treatment of *H. pylori* infection (>2 failed therapies), the efficacy of 14 day TT was 85.7% and 100% by ITT and PP analysis respectively- above the recommended 80% efficacy deemed acceptable for a treatment (387). Compliance to this treatment was good and there were minimal adverse events. This data supports the use of TT in patients who have had more than 2 failed therapies, in agreement with other consensus guidelines (64, 65, 87, 88). Further study is required in order to determine whether TT is more effective than the currently recommended second-line treatment regimens (detailed in Section 1.7.2).

Other studies which investigated the efficacy of both phenotypic and genotypic susceptibility guided treatment also found an acceptable treatment success rate of 80-90% (127, 402). On the other hand, two meta-analysis carried out recently which compared tailored and empirically chosen therapy in those previously treated found no difference in efficacy between the two (298, 299).

Overall, there was a reciprocal relationship between eradication and the number of previous treatments received. In patients treated for 7 days, the eradication rate in patients who received more than one previous treatment was significantly lower than those who had just one previous treatment (33.3% vs 75%; p=0.02, PP). This emphasizes the importance of eradicating *H. pylori* infection the first time round.

4.4.5 Compliance

Finally, compliance with therapy is a very important factor in *H. pylori* eradication and in the development of antibiotic resistance (247). Graham et al. reported that when patients took 60% or more of their medications, eradication levels of 96% were observed, compared to a significantly lower rate of 69% in those taking less than 60% of their medications (249). Treatment regimens for *H. pylori* can often involve multiple drugs and multiple dosing intervals. Emphasizing the importance of compliance to patients is vital as well as ensuring confirmation of eradication of infection. Indeed it has been found that structured patient counselling and follow-up can significantly improve *H. pylori* eradication rates and it has been recommended that this should be a routine part of therapy (399).

4.4.6 Summary

In line with numerous recently published consensus guidelines recommending 14 day duration for *H. pylori* therapies (64, 65, 87, 88, 173, 174, 403), a trend was observed towards higher eradication rates when 14 day therapies were used for first-line and subsequent therapies. Due to the study not meeting the required sample size, no conclusion can be made on the efficacy of 14 day TT versus STT for first-line treatment of *H. pylori*.

As well as recent reports of high primary clarithromycin resistance rates in other countries, there has been an alarming rise in primary clarithromycin resistance in our cohort. Going forward, it will be vital to continue monitoring antibiotic resistance rates as well eradication rates of treatment regimens, ideally to include different centres around the country. If the high

primary clarithromycin resistance rate prevails and 14 day STT eradication rates lie below the >80% cut-off, 14 day empirical STT should be abandoned and alternatives will need to be considered. If it becomes readily available in Ireland, BQT is an attractive alternative to STT.

Finally, the use of 14 day TT for more than 2 failed therapies is recommended.

Chapter 5 Discussion

5.1 Study Summaries

H. pylori infection is responsible for the development of peptic ulcer disease, gastric adenocarcinoma and tissue (MALT) lymphoma (4, 19, 404). To manage these disorders, guidelines recommend that *H. pylori* be sought and eradicated (64, 65, 87, 88, 173, 174, 403). As such diagnosis and treatment are critical factors in the management of *H. pylori* infection

5.1.1 Diagnosis of *H. pylori* infection

5.1.1.1 Non-invasive diagnostic tests

The prevalence of *H. pylori* infection has decreased in the developed world and as result, there is potential for the PPV of tests to diagnose the infection to be reduced (5, 32, 68). To ensure the most sensitive and specific tests are routinely used in a given centre, it is necessary to regularly evaluate the accuracy of diagnostic tests. We sought to locally evaluate invasive and non-invasive tests for *H. pylori* infection and to optimise their performance and diagnostic yield.

With regards to non-invasive diagnosis of *H. pylori* infection, guidelines recommend the use of the UBT or SAT (64, 87, 88). The UBT is the gold standard method for diagnosis and confirmation of eradication of the infection in Ireland (65) and numerous studies report high sensitivity (90-96%) and specificity (88-98%) of the test (89-95). As the accuracy of a stool antigen test was last evaluated in Ireland 13 years ago in 2006 (27), we evaluated the accuracy of the Premier Platinum HpSA Plus test but found that it was inferior to the UBT in our facility. The sensitivity, specificity, PPV and NPV of the HpSA versus the UBT for initial diagnosis of *H. pylori* infection were 58.2%, 89.8%, 75.4% and 80.0%, respectively. Based on the low sensitivity and specificity of the Premier Platinum HpSA Plus reported here, the UBT remains the gold-standard non-invasive diagnostic tool for both diagnosis and assessing eradication of *H. pylori* infection.

Some studies report that the DOB value used in the UBT is reflective of the degree of colonisation of bacteria in the stomach i.e. bacterial load (71-78) whereas other studies do not support this theory (309, 310). This study sought to determine whether the DOB value (as a surrogate marker for bacterial load) and the SAT value were associated with treatment outcome. It was found that neither value predicted treatment outcome in our cohort. Other studies on DOB value and association with treatment outcome report conflicting results (79-86).

5.1.1.2 Invasive diagnostic tests

Although bacterial culture of *H. pylori* is not widely available, it is vital in the effort to improve eradication of *H. pylori* infection, as it allows antimicrobial susceptibility testing. Antimicrobial

resistance surveillance allows regional antibiotic resistance patterns to be determined and in turn allows recommendation of suitable empirical treatment for that population. Indeed many consensus groups recommend that local *H. pylori* resistance rates be considered before an empirical first-line therapy is chosen (64, 65, 88, 173, 174). Susceptibility testing also allows an individual patient's treatment to be tailored, which is particularly important in refractory infection. Culture is highly specific but sensitivity can be low, as *H. pylori* is a fastidious organism with very specific growth requirements. This is reflected in the culture success rate of 59.4% reported in this chapter. We sought to improve culture success in our centre by enhancing our sampling protocol and optimising our method of transport of samples from the endoscopy suite to the research lab. Optimising *H. pylori* culture remains a high priority, given that the GenoType HelicoDR assay was found to be unsuitable as a direct replacement for culture-based AST, as discussed in Section 5.3.2 below.

The study found that the use of combined antral and corpus biopsies significantly improved culture rates when compared to the use of a single biopsy (64.4% vs 49.3%; p=0.04). As discussed in Chapter 2, PPI use and the presence of structural disease such as atrophy or intestinal metaplasia can cause a decrease in colonisation and patchy distribution in the stomach (104, 106, 344). Collecting biopsies for culture from both anatomical sites in the stomach may account for patchy distribution of the bacterium. Additionally, a single biopsy site cannot be considered representative of an individual's antibiotic susceptibility profile, as differences exist in the antimicrobial susceptibility between strains isolated from the corpus and those from the antrum (348, 349). We recommended the use of a combined biopsy protocol to improve diagnostic accuracy and potentially improve the detection of antibiotic resistance.

For culture to be successful, the conditions in which the biopsy is kept following endoscopy and prior to processing are critical. To optimise transport and storage of biopsy samples, we investigated the efficacies of different transport media. We found that Dent's transport media significantly reduced contamination and increased bacterial yield. We recommended the use of *H. pylori* specific transport media such as Dent's transport media and considering other reports on its efficacy (129-131), Portagerm pylori. To our knowledge this was the only study conducted in Ireland with the aim to improve culture success of *H. pylori*.

5.1.2 Detection of antibiotic resistance in *H. pylori* infection

5.1.2.1 Phenotypic antimicrobial resistance of *H. pylori*

An update on the local prevalence of *H. pylori* resistance to commonly prescribed antibiotics in Ireland was required, as this had last been carried out in 2008 and 2014 (175, 179). This study found a sharp increase in *H. pylori* resistance to commonly prescribed antibiotics in our patient population.

In terms of primary clarithromycin and metronidazole resistance, rates of 36.1% and 59.9% respectively were reported, which have significantly increased since the previous Irish study carried out in 2008 (175). Dual clarithromycin and metronidazole resistance was 30.6%. The clarithromycin and metronidazole resistance rates reported in this study have implications for choice of treatment. Consensus guidelines recommend that clarithromycin should be abandoned as a first-line triple therapy if resistance rates are above 15-20% (64, 87, 174). In areas of high dual clarithromycin and metronidazole resistance, BQT is the recommended first-line treatment (64).

Primary resistance to levofloxacin, amoxicillin, tetracycline and rifampicin was 16.7%, 22.2%, 18.1% and 11.1%, respectively, which have increased compared to the previous studies carried out in Ireland and Europe (163, 179).

Section 3.4.1 compared the *H. pylori* resistance rates in this study to the most recent rates reported in Europe and worldwide. There is considerable variation in resistance rates across different regions. While the rates reported in this study are high, they are similar to recent studies from countries in Italy, Poland, Spain, Iran, Peru and China (183, 185, 188, 191, 192, 300, 360). There is a strong link between primary *H. pylori* antibiotic resistance and previous antibiotic use (163, 358, 359). Differences in antibiotic prescribing patterns are likely to contribute to the emergence of *H. pylori* antibiotic resistance in different regions. Indeed Ireland has a proportionately higher use of macrolides and amoxicillin compared to many other countries in the EU (366). Technical differences in the manner in which antimicrobial susceptibility testing is performed could also contribute to the variations in resistance rates. For example, there are variations in the MIC cut-offs used to determine whether a strain resistant or sensitive. Indeed, an earlier study from our centre in 2009 used a MIC cut-off of >1 mg/L for clarithromycin resistance (44), compared to the >0.5 mg/L cut-off used in the current study, according to EUCAST recommendations. There are also variations in the MIC cut offs used to determine susceptibility to amoxicillin and rifampicin (50, 163, 354, 357, 362-364).

5.1.2.2 The GenoType HelicoDR assay in the detection of genotypic *H. pylori* antibiotic resistance

Unfortunately *H. pylori* is a fastidious bacteria and as presented in Section 2.3.5, the success rate of traditional culture and Etest in this study is low (59.4%). Molecular based methods have been suggested as an alternative (69); however insight into the clinical validity of molecular testing in Ireland was needed. We sought to evaluate the qualitative PCR-based GenoType HelicoDR assay in detection of genotypic resistance to clarithromycin and fluoroquinolones.

As detected by the GenoType HelicoDR assay, primary genotypic resistance to clarithromycin and fluoroquinolones was 58.0% and 15.2% respectively. There was a significant difference in clarithromycin resistance rates reported by culture and Etest and by the GenoType HelicoDR assay. The assay displayed suboptimal sensitivity and specificity in detecting levofloxacin and clarithromycin resistance, respectively. As such the GenoType HelicoDR assay cannot be recommended in our cohort to directly replace culture-based antimicrobial susceptibility testing and should be reserved for cases when *H. pylori* culture is unsuccessful. Until further investigation into the accuracy of other genotypic methods (examples listed below), culture based phenotypic testing should remain the method of choice for resistance surveillance and tailoring therapy.

These results show a significant difference between phenotypic and genotypic methods in resistance detection. It is unclear whether mutations observed genotypically will be translated into a phenotypic change resulting in resistance in the bacteria (201). Other molecular based methods such as the ClariRes real-time PCR assay (Ingentix, Vienna, Austria), the RIDA®GENE *Helicobacter pylori* assay (R-Biopharm AG, Darmstadt, Germany) and the AmpliDiag® *H. pylori* (MobiDiag, Espoo, Finland) are commercially available and may be more accurate in detecting resistance (145, 405). While there are an ever increasing number of publications reporting on the detection of genotypic resistance, oftentimes they do not assess the associated impact on treatment outcome (405, 406). In light of the findings of the current study, to accurately assess their potential, genotypic susceptibility testing methods should always be evaluated by assessing the impact of genotypic resistance on treatment outcome, as well as comparing to culture-based susceptibility testing.

5.1.2.3 The GenoType HelicoDR assay in non-invasive detection of *H. pylori* infection and antibiotic resistance

Optimisation of non-invasive detection of *H. pylori* resistance will enable resistance data to be gathered from patients managed by the 'Test and Treat' strategy, which will encompass all patients infected with *H. pylori*. More accurate recommendations on the correct treatment regimen for *H. pylori* could be provided and eradication rates of *H. pylori* infection may be improved. Data on the use of molecular methods, in particular the GenoType HelicoDR assay, for the analysis of stool samples was limited. Therefore we sought to evaluate the GenoType HelicoDR assay for the detection of clarithromycin and fluoroquinolone resistance using DNA isolated from stool samples compared to biopsy samples.

The study concluded that the GenoType HelicoDR assay was not suitable for the accurate detection of antibiotic resistance-mediating mutations using stool samples from *H. pylori* infected patients, as agreement between stool and biopsy samples was low (52.9% and 35.3% for clarithromycin and fluoroquinolone resistance respectively) and sensitivity and specificity values were inadequate. The high incidence of false positives seen in this study could be due to the presence of contaminating microbiota. In future it is likely that there will be further advances in resistance testing in stool samples. Based on the results from this study, there are a number of factors in resistance testing in stool samples that could be refined. For example, the procedure for collection to ensure minimal degradation of the sample and the reduction of false positives results from contaminating microbiota. As discussed above regarding genotypic testing using biopsies, it will be important to validate non-invasive susceptibility testing by comparison with results obtained from culture-based methods as well as determining the impact of genotypic resistance data obtained non-invasively on treatment outcome. To our knowledge, this study was one of the first to investigate the use of the GenoType HelicoDR assay on stool samples. Since our study was conducted other studies have been published which investigated this, with promising results (148, 150). The potential of accurately detecting *H. pylori* antibiotic resistance in stool for clinical use remains unclear but it is an exciting area that is worth pursuing.

5.1.2.4 Virulence factors and resistance

The virulence of *H. pylori* varies geographically and numerous studies have linked bacterial virulence factors to PUD and gastric cancer (40-42). Virulence may also be a factor in determining treatment outcome (38, 44, 46-49). This study sought to gain further insight into the virulence of *H. pylori* strains in our patient population.

Less virulent strains of *H. pylori* are now most prevalent in our cohort (*cagA*-negative and *vacA* S1/M2 genotypes at 77.6 % and 44.8% respectively). *H. pylori* strains containing the *cagA* gene have decreased since the distribution of the *cagA* genotype was last investigated in Ireland in 2009 (22.4% vs 68%) (50). Changes in our population due to immigration could explain this, as the prevalence of virulence factors is diverse among *H. pylori* strains isolated from different geographic areas and ethnic groups (378). Additionally, the low prevalence of PUD disease (13.3%, Table 3.27) reported in our patient cohort reflects the low numbers of strains containing the *cagA* genotype in this study.

The study reported a relationship between the less virulent strains of *H. pylori* (*cagA*-negative and *vacA* S2) and primary genotypic clarithromycin resistance and a possible relationship between *vacA* genotype and genotypic fluoroquinolone resistance. The increase in *cagA*-negative strains in our cohort over time is in line with the increase in clarithromycin resistance in our population.

To our knowledge, this study was one of the few which investigated the association between genotypic resistance to clarithromycin and fluoroquinolones by the GenoType HelicoDR assay and virulence genotype of *H. pylori*. Some other studies have reported on associations between metronidazole and clarithromycin resistance and the absence of *cagA* and less virulent *vacA* strains (50, 382-384), while others found that virulence was not associated with resistance (39, 45, 385, 386). In Section 5.1.2.2 above, it was recommended that currently, culture based phenotypic testing should be the method of choice for resistance surveillance and tailoring therapy. However, in future, if genotypic resistance testing becomes more clinically accurate and costs continue to decrease, there could be a clinical role for virulence testing of individual strains, which would allow us to fully characterise the strain and make informed decisions on the type of regimen to be prescribed.

5.1.3 Tailored treatment of *H. pylori* in a population with increasing rates of antibiotic resistance

5.1.3.1 First-line tailored treatment of *H. pylori* infection

In an effort to improve the current eradication rates of *H. pylori* infection, we sought to evaluate the efficacy of antimicrobial susceptibility-guided treatment (tailored treatment) in both first-line and subsequent treatments of *H. pylori* infection. Due to a difficulty in recruitment of patients, the study did not meet the required sample size. The absence of a statistically significant difference in the efficacy of 14 day tailored treatment versus standard clarithromycin-

based triple therapy (85.7% vs 83.3% ITT and 92.3% vs 90.9% PP; p=1.0 for both) could be explained by this. Therefore a definitive conclusion on the effectiveness of TT vs STT in our cohort cannot be made.

In accordance with several guidelines published during the course of the research presented herein, treatment duration was changed from 7 to 14 days (64, 65, 87, 88, 173, 174, 403). Although not statistically significant at the numbers tested, treatment duration of 14 days improved the efficacy of all treatments investigated in this study, when compared to 7 day duration (for e.g. 7 day STT: 71.1% vs 14 day STT: 83.3%, ITT).

5.1.3.2 Clarithromycin resistance and treatment outcome

Chapter 3 reported a sharp rise in primary clarithromycin resistance. In light of this, the impact of clarithromycin resistance on eradication rates of STT in our cohort was established. Overall eradication (7 and 14 day combined) was significantly lower in clarithromycin resistant patients than in sensitive patients (62.5% vs 87.2%, p=0.03, PP). When the analysis was stratified by treatment duration, the difference was not statistically significant. Further studies are needed to assess the impact of clarithromycin resistance on 14 day STT in our patient cohort. Continued surveillance of antibiotic resistance rates in combination with treatment success rates is vital, both in our centre and at a national level. With larger study numbers, if the eradication rate of 14 day STT falls below 80% and clarithromycin resistance remains high, empirical clarithromycin-based triple therapy should be abandoned and alternatives will need to be strongly considered.

BQT can overcome metronidazole resistance and clarithromycin resistance does not impact its efficacy (246, 259). It has shown promise in a number of studies, with reported eradication rates of up to 97% (186, 262, 263). In light of the increasing rates of both clarithromycin and dual clarithromycin and metronidazole resistance reported in this study, BQT is an attractive alternative to clarithromycin triple therapy and efforts should be made to make this treatment readily available for patients in Ireland.

5.1.3.3 The efficacy of tailored treatment as a second-line and rescue therapy for *H. pylori* eradication

Regarding second-line treatment, the efficacy of 14 day tailored treatment was 66.6% and 80% by ITT and PP analysis respectively. For subsequent rescue treatment of *H. pylori* infection, the efficacy of 14 day tailored treatment was 85.7% and 100% by ITT and PP analysis respectively- above the recommended 80% efficacy deemed acceptable for a treatment. Results of this study

support the continued use of tailored treatment after two failed treatment attempts. This study reported that in those treated for 7 days; there was reciprocal relationship between number of previous treatments received and eradication rate, emphasising the importance of eradicating *H. pylori* infection the first time round.

5.2 Future work

- At the time of commencement of this study, guidelines recommended treatment of *H. pylori* infection for a duration of 7 days (387). In late 2016, new guidelines were released which recommended 14 day duration of treatment as well as the use of higher dose, newer generation PPIs (64, 65). As such it was necessary to change treatment duration during the study. In light of the high clarithromycin and metronidazole resistance rates reported in this study, it is essential that eradication rates are monitored on a wider scale to determine whether the release of these guidelines significantly increases eradication rates and whether the increase in resistance reported in this study affects 14 day treatment outcome.
- Efforts should be made to ensure that both general practitioners and gastroenterologists have the most up-to-date knowledge in this area, as they ultimately decide and prescribe treatment for *H. pylori* infection. Although new guidelines on the treatment of *H. pylori* infection have been released, we are unsure as to whether clinicians are implementing these guidelines in practice. It would be useful to conduct a survey on current level of knowledge of the management of *H. pylori* infection, similar to those conducted elsewhere (407).
- As our study was conducted on samples obtained from patients attending two Dublin-based hospitals, it would be interesting to determine the rate of phenotypic and genotypic resistance in patients attending other hospitals in Ireland. However this may be difficult for smaller district general hospitals as they may not possess the resources to routinely perform susceptibility testing. Therefore a possible better use of resources would be the creation of a reference centre for susceptibility testing for *H. pylori* in Ireland. This has been done in the UK and France with success.
- In light of the emergence of resistance to amoxicillin, tetracycline and rifampicin reported in this study and elsewhere (169, 182, 184), genotypic methods should be further developed to include resistance detection for multiple antibiotics.

- It would be interesting to determine whether resistance-mediating mutations observed in Ireland are similar to those detected in other geographical locations. Additionally, as it becomes more readily available and affordable, perhaps next generation sequencing could be used to determine the mutations conferring resistance to metronidazole, amoxicillin, tetracycline and rifampicin found in this study.
- This study investigated the prevalence of *H. pylori* infection in symptomatic patients. It would be interesting to determine the prevalence of *H. pylori* infection in the general population. A study is currently being conducted in Europe which investigates population-based eradication of *H. pylori* in order to prevent gastric cancer (408). As eradication of *H. pylori* reduces the incidence of dyspepsia, peptic ulcer disease and gastric cancer, population screening for *H. pylori* is attractive (409). However as incidence of gastric cancer in Ireland is relatively low, screening of this kind may not be as important to healthcare policy makers.

5.3 Concluding remarks

The overall aim of this study was to evaluate strategies for the management of *H. pylori* infection, in particular in relation to diagnosis and treatment. Based on the findings presented herein, the main conclusions are as follows:

- Continued use of UBT is recommended as the gold-standard non-invasive diagnostic test for *H. pylori* infection in Ireland. The UBT DOB value did not predict treatment outcome in our cohort of patients.
- To maximise success of bacterial culture, we recommend that combined antral and corpus biopsies be used as well as a *H. pylori*-specific transport media, such as Dent's transport media.
- Rates of *H. pylori* primary and secondary phenotypic resistance to commonly prescribed antibiotics have increased sharply in our centre.
- Although the GenoType HelicoDR assay was effective in detecting *H. pylori* infection, it displayed suboptimal accuracy in the detection of resistance to clarithromycin and fluoroquinolones. Further investigation into other genotypic resistance testing methods is warranted.
- Currently, culture based phenotypic testing should remain the method of choice for resistance surveillance and tailoring therapy.
- The GenoType HelicoDR assay was inadequate in assessing antibiotic resistance non-invasively in stool samples from patients infected with *H. pylori*.

- Less virulent strains of *H. pylori* (those with a *cagA* -negative and *vacA* S1/M2 genotype) are the most prevalent in *H. pylori* strains in our cohort. Virulence factors did not influence clinical phenotype or treatment outcome.
- There is a relationship between the less virulent strains of *H. pylori* (*cagA*-negative and *vacA* S2) and primary clarithromycin resistance and a possible relationship between *vacA* genotype and fluoroquinolone resistance.
- For first-line treatment of *H. pylori*, a conclusion cannot be made on the efficacy of tailored treatment versus standard triple therapy in our patient cohort, as the study did not meet the required sample size.
- Continuous monitoring of antibiotic resistance rates and eradication rates is vital, ideally to include different centres around the country. If the high primary clarithromycin resistance rate is observed at a national level and 14 day eradication rates lie below the >80% cut-off deemed acceptable, empirical clarithromycin-based triple therapy should be abandoned. Bismuth quadruple therapy has been shown to be effective and this treatment needs to be made more readily available in Ireland.
- The continued use of susceptibility guided treatment after two 2 failed therapies is recommended.

Chapter 6 References

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Appendix 1: Information sheets and consent form

Patient Information sheet for UBT patients

SJH / AMNCH RESEARCH ETHICS COMMITTEE

Patient Information Sheet

1. **Title of study:** Helicobacter pylori infection & antibiotic resistance

2. **Introduction:** Helicobacter pylori is a bacterium that infects the stomach of up to 50 % of the world's population. In many people there are no symptoms of Helicobacter pylori infection. However, Helicobacter pylori may cause stomach inflammation (chronic gastritis) and ulcers. In recent years it has become more difficult to eliminate Helicobacter pylori infection due to an increase in antibiotic-resistant types of these bacteria.
   We hope to determine the rates of these antibiotic resistant bacteria in the local area, with a view to guide doctors in their future choice of treatment and improve patient outcomes.
   We hope to develop a non-invasive method of determining whether Helicobacter pylori is resistant to antibiotics, which could be an alternative to the invasive methods used routinely in the clinic. We will investigate whether this method can be carried out by testing the Helicobacter pylori present in stool.

3. **Procedures:** If you agree to take part, you will be asked to supply a stool sample. You will be supplied with a collection kit which contains the materials needed to collect the sample along with easy to follow instructions on how to use the kit. Collection of the stool sample can be done at home, 1 day prior to/on the morning of you dropping the sample to the hospital. You will also be given a suitable storage container and packaging in which the stool sample can be stored and which you will bring in with you when you are dropping the sample to the hospital. This process is detailed in the diagram below.

http://www.alphalabs.co.uk/cms/site/images/IFU_Alpha-Logo.jpg

The stool samples will be used to test for the presence of Helicobacter pylori in the laboratory. If Helicobacter pylori is present in your stool sample, the Helicobacter pylori will then be tested to determine whether it is resistant to antibiotics. The Helicobacter pylori will also be examined for the presence of certain characteristics which may be associated with antibiotic resistance.

4. **Benefits:** There may be no direct benefit to you participating. If Helicobacter pylori is detected in your sample, it will be tested to determine whether it is resistant to a number of antibiotics. This study will assess the level of resistance to antibiotics in the local community and will be useful in helping doctors choose the best treatments in future. Improving treatment of infection and the associated symptoms may aid in the prevention of serious long-term complications including peptic ulcers and gastric cancer.

5. **Risks:** The risks associated with the study are no greater than those associated with any other non-invasive test to diagnose Helicobacter pylori infection.

6. **Exclusion from participation:** You cannot participate in the study if any of the following are true. If you;

    a. are less than 18 years old or greater than 80 years old.
    b. are unable to give informed consent.
    c. are pregnant or breast-feeding.
    d. have used antibiotics within 4 weeks or strong antacids (PPI's) within 2 weeks of supplying the sample.

7. **Confidentiality:** Your identity will remain confidential. Your name will not be published or disclosed to anyone outside the research team. Any information relating to the study will be encrypted and kept on a secure computer for the duration of the study. All study records once finished will be destroyed after an appropriate period of time.

8. **Compensation:** Your doctors are covered by standard medical malpractice insurance. Nothing in this document restricts or curtails your rights.

9. **Voluntary Participation:** Participation in this study is voluntary. You may quit at any time. If you decide not to participate, or if you quit, you will not be penalised and will not give up any benefits which you had before entering the study.

10. **Stopping the study:** You understand that your doctor may stop your participation in the study at any time without your consent.

11. **Permission:** This trial has hospital Research Ethics Committee approval.

12. **Further information:** You can get more information or answers to your questions about the study, your participation in the study, and your rights, from Denise Brennan (01 8963828) or Dr Joseph Omorogbe (089 4134681). If your doctor learns of important new information that might affect your desire to remain in the study, he or she will tell you.

Patient Information sheet for endoscopy patients

SJH / AMNCH RESEARCH ETHICS COMMITTEE

Patient Information Sheet

1. **Title of study:** Helicobacter pylori infection & antibiotic resistance

2. **Introduction:** Helicobacter pylori is a bacterium that infects the stomach of up to 50 % of the world's population. In many people there are no symptoms of Helicobacter pylori infection. However, Helicobacter pylori may cause stomach inflammation (chronic gastritis) and ulcers. In recent years it has become more difficult to eliminate Helicobacter pylori infection due to an increase in antibiotic-resistant strains of the bacteria. We hope to determine the rates of these antibiotic resistant bacteria in the local area, with a view to guide doctors in their future choice of treatment and improve patient outcomes.

3. **Procedures:** If you agree to take part, you will be asked to allow tissue biopsies to be taken during your already planned camera test. The tissue sample will be stored and used to test for antibiotic resistant strains of Helicobacter pylori in the laboratory. The Helicobacter pylori will also be examined for the presence of certain characteristics which may be associated with antibiotic resistance. If *H. pylori* is detected on the camera test, you will be given a prescription for either standard treatment or a treatment based on resistance observed in your infection.

4. **Benefits:** There may be no direct benefit to you participating. However, if *H. pylori* is detected on the camera test and initial attempts to clear it fail, the information collected from your biopsies may help guide your doctors' future choice of antibiotics. This study will assess the level of resistance in the local community to antibiotics and will be useful in helping doctors choose the best treatments in future.

5. **Risks:** The risks associated with the study are no greater than those associated with any routine camera test, which may occur in a very low percentage of patients. These include bleeding, abdominal discomfort, infection and in very rare situations perforation.

6. **Exclusion from participation:** You cannot participate in the study if any of the following are true:
    a. are less than 18 years old or greater than 80 years old.
    b. unable to give informed consent.
    c. are pregnant or breast-feeding.
    d. have a significant co-existing illness (cardiovascular, gastrointestinal, immunological).
    e. have a bleeding problem or use blood thinning drugs
    f. antibiotic use within 4 weeks or strong antacids (PPI's) within 2 weeks of gastroscopy.
    g. have a condition or are taking a medication that the investigator believes would interfere with the study or poses a safety risk.

7. **Confidentiality:** Your identity will remain confidential. Your name will not be published or disclosed to anyone outside the research team. Any information relating to the study will be encrypted and kept on a secure computer for the duration of the study. All study records once finished will be destroyed after an appropriate period of time.

8. **Compensation:** Your doctors are covered by standard medical malpractice insurance. Nothing in this document restricts or curtails your rights.

9. **Voluntary Participation:** Participation in this study is voluntary. You may quit at any time. If you decide not to participate, or if you quit, you will not be penalised and will not give up any benefits which you had before entering the study.

10. **Stopping the study:** You understand that your doctor may stop your participation in the study at any time without your consent.

11. **Permission:** This trial has hospital Research Ethics Committee approval.

12. **Further information:** You can get more information or answers to your questions about the study, your participation in the study, and your rights, from Denise Brennan (01 8963828) or Dr Joseph Omorgbe (089 4134681). If your doctor learns of important new information that might affect your desire to remain in the study, he or she will tell you.

Consent Form

SJH / AMNCH RESEARCH ETHICS COMMITTEE

Patient Information Sheet

Title of research study: Helicobacter pylori infection & antibiotic resistance

This study and this consent form have been explained to me. My doctor has answered all my questions to my satisfaction. I believe I understand what will happen if I agree to be part of this study.
I have read, or have had read to me, this consent form. I have had the opportunity to ask questions and all my questions have been answered to my satisfaction. I freely and voluntarily agree to be part of this research study, though without prejudice to my legal and ethical rights. I have received a copy of this agreement and I understand that, if there is a sponsoring company, a signed copy will be sent to that sponsor.

PARTICIPANT'S NAME: _____

PARTICIPANT'S SIGNATURE: _____

Date: _____

Date on which the participant was first furnished with this form: _____

Where the participant is incapable of comprehending the nature, significance and scope of the consent required, the form must be signed by a person competent to give consent to his or her participation in the research study (other than a person who applied to undertake or conduct the study). If the subject is a minor (under 18 years old) the signature of parent or guardian must be obtained:-

NAME OF CONSENTOR, PARENT or GUARDIAN: _____
SIGNATURE: _____
RELATION TO PARTICIPANT: _____

Where the participant is capable of comprehending the nature, significance and scope of the consent required, but is physically unable to sign written consent, signatures of two witnesses present when consent was given by the participant to a registered medical practitioner treating him or her for the illness.
NAME OF FIRST WITNESS: _____
SIGNATURE: _____
NAME OF SECOND WITNESS: _____
SIGNATURE: _____

Statement of investigator's responsibility: I have explained the nature, purpose, procedures, benefits, risks of, or alternatives to, this research study. I have offered to answer any questions and fully answered such questions. I believe that the participant understands my explanation and has freely given informed consent.
Physician's signature: _____
Date: _____

Appendix 2: Dents Transport Medium

Dents transport medium is Brian Heart infusion (BHI) broth containing 2.5% (w/v) yeast extract, 5% sterile horse serum and Helicobacter Selective Supplement.

To prepare 100 ml of BHI broth, add 3.7 g BHI (Sigma Aldrich, Missouri, USA) and 2.5 g yeast extract (Fisher Scientific, Dublin, Ireland) to 100 ml of distilled di-ionised water and autoclave. Allow to cool, then add 5 ml sterile horse serum (Fisher Scientific, Dublin, Ireland) and 1 ml Helicobacter Selective Supplement (Fisher Scientific, Dublin, Ireland).

Appendix 3: Supplementary tables

Chapter 3

	n (%)			p value
	All patients	**Treatment Naïve**	**Previously**	
	130 (100)	**72 (55.4)**	**treated**	
			58 (44.6)	
Gender				
Female	57 (43.8)	20 (27.8)	37 (63.8)	0.0004[1]
Male	73 (56.2)	52 (72.2)	21 (36.2)	
Age				
mean ±SD	49.4 ± 15.1	51.3 ± 15.8	47 ±13.8	0.1[2]
Histology findings				
Chronic gastritis	106 (81.5)	58 (80.6)	48 (82.8)	0.8[1]
Intestinal metaplasia	15 (11.5)	8 (11.1)	7 (12.1)	1.0[1]
No data available	8 (6.2)	5 (6.9)	3 (5.2)	NA
Normal mucosa	1 (0.8)	1 (1.4)	0 (0.0)	1.0[1]
Endoscopic findings				
Gastritis	85 (65.4)	46 (63.9)	39 (67.2)	0.7[1]
Normal	29 (22.3)	16 (22.2)	13 (22.4)	1.0[1]
Gastric/ Duodenal Ulcer	14 (10.8)	10 (13.9)	4 (6.9)	0.3[1]
No data available	2 (1.5)	0 (0)	2 (3.4)	NA

Supplementary Table 3.1: Demographics and clinical characteristics of culture positive *H. pylori*-infected patients. [1]Fisher's exact test, [2]unpaired t-test,

Genotype	Number of gastric biopsy specimens n (%)			p value[1]
	All patients	**Treatment**	**Previously**	
	165 (100)	**Naïve**	**treated**	
		105 (63.6)	**60 (36.4)**	
Susceptible to clarithromycin (WT)				
	65 (39.4)	**52 (49.5)**	**13 (21.7)**	
Resistant to clarithromycin				**<0.01[2]**
	100 (60.6)	**53 (50.5)**	**47 (78.3)**	
Point mutations				
A2147G	78 (78)	44 (83)	34 (72.3)	
A2146G	8 (8)	3 (5.7)	5 (10.6)	
A2146C	6 (6)	3 (5.7)	3 (6.4)	
A2146C + A2147G	5 (5)	3 (5.7)	2 (4.3)	
A2146G + A2147G	2 (2)	0 (0)	2 (4.3)	
A2146G + A2146C	1 (1)	0 (0)	1 (2.1)	

Supplementary Table 3.2: Clarithromycin resistance rates and the distribution of resistance-mediating mutations of samples used in virulence factor study (Section 3.3.5). [1]comparison of resistance rates in treatment naïve versus previously treated patients by Fisher's exact test, [2]p=0.0005.

Genotype	Number of gastric biopsy specimens *n* (%)			*p* value[1]
	All patients	**Treatment**	**Previously**	
	165 (100)	**Naïve**	**treated**	
		105 (63.6)	**60 (36.4)**	
Susceptible to fluoroquinolones (WT)				
	132 (80)	**89 (84.8)**	**43 (71.7)**	
Resistant to fluoroquinolones				**0.07**
	33 (20)	**16 (15.2)**	**17 (28.3)**	
Point mutations				
*gyr*91 D91Y	18 (54.5)	10 (62.5)	8 (47.1)	
*gyr*91 D91N	6 (18.2)	2 (12.5)	4 (23.5)	
*gyr*91 D91G	2 (6.1)	0 (0)	2 (11.8)	
gyr91 D91N +*gyr91* D91G	2 (6.1)	1 (6.3)	1 (5.9)	
gyr91 D91N +*gyr91* D91Y	2 (6.1)	1 (6.3)	1 (5.9)	
gyr87 N87K	1 (3)	1 (6.3)	0 (0)	
gyr87 N87K +*gyr91* D91N +*gyr91* D91G	1 (3)	0 (0)	1 (5.9)	
gyr87 N87K +*gyr91* D91N +*gyr91* D91G +*gyr91* D91Y	1 (3)	1 (6.3)	0 (0)	

Supplementary Table 3.3: Fluoroquinolone resistance rates and the distribution of resistance-mediating mutations of samples used in virulence factor study (Section 3.3.5). [1]comparison of resistance rates in treatment naïve versus previously treated patients by Fisher's exact test

Genotype	Number of gastric biopsy specimens n (%)			*p* value[1]
	All patients 165 (100)	Treatment Naïve 105 (63.6)	Previously treated 60 (36.4)	
Susceptible (to both)	60 (36.4)	49 (46.6)	11 (18.3)	<0.01[2]
Resistant (to at least one)	105 (63.6)	56 (53.3)	49 (81.6)	
Susceptible/resistant to one	137 (83.0)	92 (87.6)	45 (75)	
Resistant to both	28 (17.0)	13 (12.4)	15 (25)	0.05

Supplementary Table 3.4: Antimicrobial susceptibility results for both clarithromycin and fluoroquinolone of samples used in virulence factor study (Section 3.3.5). [1]comparison of resistance rates in treatment naïve versus previously treated patients by Fisher's exact test, [2]p=0.0004.

Chapter 4

		n (%)		p value
	Total	**STT**	**TT**	
	104 (100)	**50 (48.1)**	**54 (51.9)**	
Gender				
Female	39 (37.5)	19 (38)	20 (37)	1.0[1]
Male	65 (62.5)	31 (62)	34 (63)	
Age				
mean ±SD	49.7±15.8	50.2±16.5	49.3±15.2	0.8[2]
Compliance	**103 (73.6)**	**50 (100)**	**53 (98.1)**	**1.0[1]**
Adverse events	**4 (3.8)**	**0 (0)**	**4 (7.4)**	**0.1[1]**
Nausea	3 (75)	0 (0)	3 (75)	1.0[1]
Bloating	1 (25)	0 (0)	1 (25)	1.0[1]

Supplementary Table 4.1: Demographic and clinical characteristics of patients in each therapeutic group. [1] Fisher's exact test, [2] unpaired t-test.

Eradication rate	Overall n=104		p value
	STT n= 50 (48.1%)	TT n=54 (51.9%)	
ITT analysis	**74%** (37/50; 95% CI 60.4- 84.1)	**77.8%** (42/54; 95% CI 65.1- 86.8)	0.8[1]
PP analysis	**75.5%** (37/49; 95% CI 61.9- 85.4)	**84.0%** (42/50; 95% CI 71.5- 91.7)	0.3[1]
	7 day n=78 (75%)		
	STT n= 38 (48.7%)	TT n=40 (54.4%)	
ITT analysis	**71.1%** (27/38; 95% CI 55.2 -83.0)	**75.0%** (30/40; 95% CI 59.8- 85.8)	0.8[1]
PP analysis	**71.1%** (27/38; 95% CI 55.2 -83.0)	**81.1%** (30/37; 95% CI 65.8- 90.5)	0.4[1]
	14 day n=26 (25%)		
	STT n=12 (46.2%)	TT n= 14 (53.8%)	p value
ITT analysis	**83.3%** (10/12; 95% CI 55.2- 97.0)	**85.7%** (12/14: 95% CI 60.1- 97.5)	1.0[1]
PP analysis	**90.9%** (10/11; 95% CI 62.3-99.5)	**92.3%** (12/13; 95% 66.7-99.6)	1.0[1]

Supplementary Table 4.2: Eradication rates of STT and TT. [1]Fisher's exact test.

Eradication rate	Standard triple therapy n=50 (48.1%)		p value
	7 day	14 day	
ITT analysis	**71.1%** (27/38; 95% CI 55.2- 83.0)	**83.3%** (10/12; 95% CI 55.2- 97.0)	0.5[1]
PP analysis	**71.1%** (27/38; 95% CI 55.2- 83.0)	**90.9%** (11/12; 95% CI 62.3- 99.5)	0.3[1]

Supplementary Table 4.3: Eradication rate of standard triple therapy by treatment duration. [1]Fisher's exact test**.**

Eradication rate	Tailored treatment n=54 (51.9%)		p value
	7 day	14 day	
ITT analysis	**75.0%** (30/40; 95% CI 59.8- 85.8)	**85.7%** (12/14; 95% CI 60.1- 97.5)	0.7[1]
PP analysis	**81.1%** (30/37; 95% CI 65.8- 90.5)	**92.3%** (12/13; 95% CI 66.7- 99.6)	0.7[1]

Supplementary Table 4.4: Eradication rate of tailored treatment by treatment duration.
[1]Fisher's exact test.

Eradication rate	Phenotypically resistant to any antibiotic n=47		p value
	Standard Triple Therapy 21 (44.7%)	Tailored Treatment 26 (55.3%)	
	Overall		
ITT analysis	**81.0%** (17/21; 95% CI 60.0- 93.3)	**76.9%** (20/26; 95% CI 57.9-89.0)	1.0[1]
PP analysis		**80.0%** (20/25; 95% CI 60.9-91.1)	1.0[1]
	7 day		
ITT analysis	**75%** (12/16; 95% CI 50- 90.2)	**77.7%** (14/18; 95% CI 54.3-91.5)	1.0[1]
PP analysis		**82.4%** (14/17; 95% CI 58.2- 94.6)	1.0[1]
	14 day		
ITT & PP analysis	**100%** (5/5; 95% CI 51.1- 100)	**87.5%** (7/8; 95% CI 50.1- 99.9)	1.0[1]

Supplementary Table 4.5: Eradication rates by phenotypic resistance to any antibiotic.
[1]Fisher's exact test.

Eradication rate	7 day n=37 (74%)	14 day n=13 (26%)	p value
ITT analysis	**54.1%** (20/37; 95% CI 38.4- 69)	**76.9%** (10/13; 95% CI 49.1- 92.5)	0.2[1]
PP analysis	**57.1%** (20/35; 95% CI 40.8- 72)	**90.9%** (10/11; 95% CI 60.1- 99.9)	0.07[1]

Supplementary Table 4.6: Eradication rates of tailored therapy in all patients previously treated for *H. pylori* infection, by 7 and 14 day duration. [1]Fisher's exact test.

Previous treatments	Patients n (%)	Eradication rate 7 day (n=37, 74%)	Eradication rate 14 day (n=13, 26%)
1	27 (54)	**71.4%** (15/21) ITT **75%** (15/20) PP	**66.6%** (4/6) ITT **80%** (4/5) PP
2	8 (16)	**25%** (1/4) ITT & PP	**75%** (3/4) ITT **100%** (3/3) PP
3	5 (10)	**0%** (0/4) ITT **0%** (0/3) PP	**100%** (2/2) ITT & PP
4	3 (6)	**50%** (1/2) ITT & PP	**100%** (1/1) ITT & PP
5	3 (6)	**33.3%** (1/3) ITT & PP	-
≥6[1]	4 (8)	**50%** (2/4) ITT & PP	-

Supplementary Table 4.7: Eradication rate of tailored therapy by number of previous treatments. [1]2 patients had 6 previous treatments, 1 patient had 7 previous treatments and 1 patient had 8 previous treatments.

Appendix 4: List of Abbreviations

^{13}C	Radio labelled Carbon-12
AMOX	Amoxicillin
AMOX-R	Amoxicillin resistant
APECED syndrome	Autoimmune polyendocrinopathy-candidiasis–ectodermal dystrophy
ATCC	American type culture collection
CAGA-F	CAGA forward primer
CAG-R	CAGA reverse primer
CI	Confidence interval
CLAR	Clarithromycin
CLAR-R	Clarithromycin resistant
CLO	*Campylobacter*-like organism
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
ELISA	Enzyme linked immunosorbent assay
EUCAST	European committee on antimicrobial susceptibility testing
FISH	Fluorescent in-situ hybridisation
HpSA	*H. pylori* stool antigen assay
IDA	Iron deficiency anaemia
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IHPWG	Irish *Helicobacter pylori* working group
ITT	Intention-to-treat
LEVO	Levofloxacin
LEVO-R	Levofloxacin resistant
M	Molar
MALT	Mucosa associated tissue lymphoma
MET	Metronidazole
MET-R	Metronidazole resistant
MgCl	Magnesium Chloride
MIC	Minimum inhibitory concentration
MIN	Minute(s)
NADPH	Nicotinamide adenine dinucleotide phosphate
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells

PBP	Penicillin binding proteins
PCR	Polymerase chain reaction
PP	Per-protocol
PPI	Proton pump inhibitor
PUD	Peptic ulcer disease
RIF	Rifampicin
RIF-R	Rifampicin resistant
RT	Room temperature
RUT	Rapid urease test
STT	Standard clarithromycin-based triple therapy
TET	Tetracycline
TET-R	Tetracycline resistant
TT	Tailored treatment
UNG	Uracil-N-glycosylase
VA1-F	VA1 forward primer
VA1-R	VA1 reverse primer
Vaca m1	*vacA* middle region
Vaca m2	*vacA* middle region
Vaca s1	*vacA* signal region
Vaca s2	*vacA* signal region
VAG-F	VAG forward primer
VAG-R	VAG reverse primer
WHO	World Health Organisation