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Helicobacter pylori Infection: In Vitro Diagnostic Methods, Antimicrobial Susceptibility Testing, Epidemiology of Recrudescence and Clarithromycin Resistance

A Thesis Submitted for the Degree of Doctor of Philosophy

at The University of Dublin, Trinity College

by

Denise K. Hyde

Sir Patrick Dunn Microbiology Research Laboratory,
Faculty of Health Sciences,
University of Dublin,
Trinity College.

September 1999
Declaration

I hereby declare that this thesis has not been previously submitted for a Degree at this or any other University and that it represents my own work except where duly acknowledged in the text.

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Denise K. Hyde
Summary

*H. pylori* is a Gram-negative, non-spore forming, curved bacterium that colonises the gastric epithelium of the human stomach. *H. pylori* is firmly established as an aetiological agent in peptic ulcer disease and successful eradication of this organism leads to duodenal ulcer cure.

Culture of selected biopsy specimens that are known to be presumptively positive for *H. pylori* by a urease test might generate both accurate and cost-effective protocols for the diagnosis of this infection in clinical practice. Hence, the feasibility of culturing *H. pylori* from antral biopsy specimens of positive CLO tests was assessed. *H. pylori* was cultured from routine antral biopsy specimens in 34 of 38 (89%) *H. pylori*-infected patients and from antral biopsy specimens from positive CLO tests in 30 (79%) of these patients. Therefore, optimal isolation rates were achieved with routine biopsies. From a practical viewpoint, it may be more reliable to store the routine microbiology biopsy at 4 °C until the CLO test yields a positive result before sending the routine biopsy for culture.

A 16S rRNA gene PCR technique was evaluated for the detection of *H. pylori* in paraffin-embedded gastric biopsy specimens from untreated and post-treatment patients and compared with the conventional diagnostic tests. A high number of discrepant PCR results were obtained in patients who did not harbour the infection. An evaluation of primer specificity demonstrated that the 16S rRNA gene primers were not specific for *H. pylori*. This may explain the high frequency of false-positive PCR results and the poor correlation observed with conventional tests.

The accuracy of the routine biopsy-based tests for *H. pylori* diagnosis from untreated and post-treatment patients was assessed. The CLO test and histology were the best tests for the accurate diagnosis of *H. pylori* infection in untreated patients and histology was the best single test for the assessment of *H. pylori* infection in post-treatment patients. Culture was the least sensitive routine test in the two patient populations studied.

The E-test was employed to determine the prevalence of metronidazole, clarithromycin and amoxicillin resistance among 51 consecutive clinical isolates of *H. pylori*. The
prevalences of metronidazole, clarithromycin and amoxicillin resistance were 29%, 3.9% and 0%, respectively. The incidence of metronidazole resistance in *H. pylori* did not differ significantly between men (8/31, 25.8%) and women (7/20, 35%). The reproducibility of the E-test was assessed by determining the MIC's of three *H. pylori* reference strains for the three antimicrobials on three separate occasions. Better reproducibility was observed with the MIC values obtained for metronidazole at the beginning and half-way through the study. However, higher MIC's in the region of ≥ 1.5 log₂ dilutions were obtained at the end of the study for some of the reference strains against clarithromycin.

The E-test and disc-diffusion methods were evaluated for the susceptibility testing of selected *H. pylori* isolates to clarithromycin by comparison with the reference agar dilution method. Susceptibility testing was performed on 28 *H. pylori* isolates from seven patients, consisting of six pre-treatment and 22 post-treatment isolates. The agreement between the E-test and the agar dilution methods was high (90% within ± 1 log₂ dilution step) and the correlation between 2 log₂ two-fold dilutions was 95%. Excellent correlation (100%) was observed between the agar dilution method and the disc-diffusion test. The disc-diffusion test is more cost-effective than the E-test and provides accurate, qualitative results that are sufficient for the clinical management of *H. pylori* infection.

Random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR), repeat sequence oligonucleotide analysis and *cagA* gene hybridisation techniques were used to genotype these 28 pre-treatment and recurrent *H. pylori* isolates. Molecular characterisation of these *H. pylori* isolates showed that *H. pylori* recurrence in four of five patients who had received eradication treatment was due to recrudescence of a temporarily suppressed infection rather than reinfection with a different strain. Patients may be infected with more than one strain/subtype at one site in the stomach and a mixed infection of *cagA*-positive and *cagA*-negative strains can co-exist in the same patient. Furthermore, with reference to the susceptibility testing data of these isolates, it was shown that acquired clarithromycin resistance was due to *in vivo* strain selection of a resistant population rather than reinfection with a different resistant strain.
The mechanism of clarithromycin resistance in *H. pylori* was investigated. Susceptibility testing of *H. pylori* isolates that were resistant to clarithromycin demonstrated cross-resistance to azithromycin. Twenty-four of the 28 *H. pylori* isolates were examined by 23S rRNA gene PCR. The nucleotide sequences of the 425 bp PCR products were determined. A-to-G point mutations at positions 2142 and 2143 in domain V, the peptidyltransferase region, of the 23S rRNA gene were stable *in vivo*. In the majority of isolates, clarithromycin resistance was associated with these mutations among Irish isolates. RFLP analysis of a small selection of the patient isolates, using a labelled 425 bp PCR product of the 23S rRNA gene of a clarithromycin-resistant isolate with an A-to-G 2143 mutation as a probe, confirmed the sequencing results.

There was an association between the type of point mutation observed and the level of clarithromycin resistance. The A-to-G 2142 mutation was detected in isolates with an MIC of ≥ 64 µg/ml and the A-to-G 2143 mutation was detected in isolates with an MIC of 16 – 32 µg/ml. There was some indication of a link between the type of treatment regimen employed for *H. pylori* eradication and the *in vitro* level of resistance. An MIC ≥ 64 µg/ml was observed in 2 of 3 patients who had received a dual therapy regimen consisting of a proton pump inhibitor and clarithromycin.

One patient harboured a mixed population of clarithromycin-susceptible and -resistant *H. pylori* strains. Point mutations were not observed in the 23S rRNA gene nucleotide sequence of one *H. pylori* isolate that demonstrated low-level clarithromycin and azithromycin resistance. Thus in this case, it is possible that another mechanism of clarithromycin resistance may be involved.
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## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>b.i.d.</td>
<td>Twice daily</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>CagA</td>
<td>Cytotoxin associated protein A</td>
</tr>
<tr>
<td>CCUG</td>
<td>Culture Collection at the University of Göteborg</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>CLO</td>
<td>Campylobacter-like organism</td>
</tr>
<tr>
<td>CTAB</td>
<td>Cetyltrimethyl ammonium bromide</td>
</tr>
<tr>
<td>dATP</td>
<td>Deoxyadenosine triphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>Deoxycytosine triphosphate</td>
</tr>
<tr>
<td>ddUTP</td>
<td>Dideoxyuridine triphosphate</td>
</tr>
<tr>
<td>dGTP</td>
<td>Deoxyguanosine triphosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Dinucleotide triphosphate</td>
</tr>
<tr>
<td>DU</td>
<td>Duodenal ulcer</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine-tetraacetic acid</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>g</td>
<td>Gravity</td>
</tr>
<tr>
<td>GC</td>
<td>Gastric cancer</td>
</tr>
<tr>
<td>GC*</td>
<td>GC agar</td>
</tr>
<tr>
<td>%G+C</td>
<td>Percentage guanine and cytosine</td>
</tr>
<tr>
<td>GU</td>
<td>Gastric ulcer</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>Abbr.</td>
<td>Description</td>
</tr>
<tr>
<td>-------</td>
<td>--------------------------------------------</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IM</td>
<td>Intestinal metaplasia</td>
</tr>
<tr>
<td>kb</td>
<td>Kilobase</td>
</tr>
<tr>
<td>l</td>
<td>Litre</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>MALT</td>
<td>Mucosa-associated lymphoid tissue</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum inhibitory concentration</td>
</tr>
<tr>
<td>µ</td>
<td>Micro</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µl</td>
<td>Microlitre</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>mJ</td>
<td>Millijoule</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitre</td>
</tr>
<tr>
<td>NCTC</td>
<td>National Collection of Type Cultures</td>
</tr>
<tr>
<td>NCCLS</td>
<td>National Committee for Clinical Laboratory Standards</td>
</tr>
<tr>
<td>NUD</td>
<td>Non-ulcer dyspepsia</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PAI</td>
<td>Pathogenicity island</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>pmol</td>
<td>Picomole</td>
</tr>
<tr>
<td>PPI</td>
<td>Proton pump inhibitor</td>
</tr>
<tr>
<td>PUD</td>
<td>Peptic ulcer disease</td>
</tr>
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VIII
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</tr>
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<tbody>
<tr>
<td>q.i.d.</td>
<td>Four times daily</td>
</tr>
<tr>
<td>RAPD</td>
<td>Random amplified polymorphic DNA</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>r.p.m.</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>s</td>
<td>Second</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris acetate EDTA buffer</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris borate EDTA buffer</td>
</tr>
<tr>
<td>TE</td>
<td>Tris EDTA buffer</td>
</tr>
<tr>
<td>t.i.d.</td>
<td>Three times daily</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>Isooctylphenoxypolyethoxyethanol</td>
</tr>
<tr>
<td>V</td>
<td>Volts</td>
</tr>
<tr>
<td>vol</td>
<td>Volume</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume per volume</td>
</tr>
<tr>
<td>w/v</td>
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1.1. Isolation and Discovery of *Helicobacter pylori*

The first reports describing the isolation of *Helicobacter pylori* by Warren and Marshall (Marshall, 1983; Warren, 1983) and the subsequent evidence that this bacterium is an aetiological agent in peptic ulcer disease is one of the most important medical breakthroughs of this century. However, throughout the nineteenth and twentieth centuries numerous reports had described the presence of spiral organisms and urease in animal and human stomach (Buckley and O’ Morain, 1998). As early as the 1890’s, gastric spiral bacteria were reported in dogs (Bizzozero, 1893) and cats and mice (Salomon, 1896), but the first description of spiral organisms in the human stomach was provided by Krienitz (1906) in a study of patients with gastric carcinoma. Spiral bacteria were later described in the stomachs of patients with gastric and duodenal ulcers (Rosenow and Sanford, 1915) followed by reports, some 23 years later, documenting the presence of spirochaetes in gastric autopsy specimens (Doenges, 1938) and in 37% of gastric resection specimens from patients with carcinoma or gastric ulcer (Freedberg and Barron, 1940). However, Palmer (1954) failed to identify spiral bacteria in gastric biopsy specimens from over 1000 humans and, consequently, interest in gastric bacteria declined leaving behind the general view that the bacteria seen were contaminants from the oral cavity.

The first evidence of urease activity in the human stomach was reported by Luck and Seth (1924). Fitzgerald and Murphy (1950) described urease activity in gastrectomy sections from patients with peptic ulcer disease but at that time the source of gastric urease was unknown. The role of a bacterial pathogen in peptic ulcer disease was proposed by Steer and Colin-Jones (1975) who reported bacteria in 88% of gastric biopsies from patients with gastric ulcer. Four years later, spiral bacteria were observed on the surface of epithelial cells in patients with chronic gastritis (Fung *et al.*, 1979).
However, it was not until 1983 when Warren and Marshall successfully isolated ‘campylobacter-like’ bacteria beneath the gastric mucosa and correctly associated the presence of the organism with antral gastritis, and with duodenal and gastric ulcers, that the role of this bacterium in upper gastrointestinal disease was recognised (Marshall, 1983; Warren, 1983; Marshall and Warren, 1984).

1.2. Nomenclature

The ‘campylobacter-like’ organism isolated by Warren and Marshall (1984) was initially named *Campylobacter pyloridis* due to its morphological resemblance to campylobacters and the anatomical site from which it was most commonly isolated, namely the pylorus of the stomach. In 1987, for Latin grammatical reasons, its name was changed to *Campylobacter pyloris* (Marshall and Goodwin, 1987a). However, ribosomal RNA sequencing studies later showed that this organism was related to but distinct from the genus *Campylobacter* and was therefore redesignated to a new genus *Helicobacter* (Goodwin et al., 1989).

1.3. Taxonomy

*Helicobacter* belongs to rRNA homology group VI which also includes *Campylobacter*, *Arcobacter*, *Wolinella* and *Flexispira*. To date, the *Helicobacter* genus consists of 20 species, the majority of which are associated with the gastric mucosa of a variety of mammals (see Table 1.1). *Helicobacter heilmannii*, formerly *Gastrospirillum hominis*, was first isolated in 1996 (Andersen et al., 1996) and is one of the few helicobacters, with the exception of *H. pylori*, that is able to colonise the human stomach. It accounts for a very small proportion of cases of human gastritis (see section 1.7.1). The culture, biochemical characterisation and ultrastructural histopathology of a *H. heilmannii* strain have been recently described (Andersen et al., 1999).
Table 1.1. Species within the genus *Helicobacter* and their hosts\(^a\).

<table>
<thead>
<tr>
<th>Species</th>
<th>Main host(s)</th>
<th>Primary site of isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. acinonyx</em></td>
<td>Cheetah</td>
<td>Gastric mucosa</td>
</tr>
<tr>
<td><em>H. bilis</em>(^b)</td>
<td>Mouse</td>
<td>Intestinal mucosa(^c)</td>
</tr>
<tr>
<td><em>H. bizzozeroni</em>(^b)</td>
<td>Dog</td>
<td>Gastric mucosa</td>
</tr>
<tr>
<td><em>H. canis</em></td>
<td>Dog, man</td>
<td>Intestinal mucosa</td>
</tr>
<tr>
<td><em>H. cholecystus</em>(^b)</td>
<td>Hamster</td>
<td>Intestinal mucosa</td>
</tr>
<tr>
<td><em>H. cinaedi</em></td>
<td>Man, hamster</td>
<td>Intestinal mucosa</td>
</tr>
<tr>
<td><em>H. felis</em></td>
<td>Cat, dog, man</td>
<td>Gastric mucosa</td>
</tr>
<tr>
<td><em>H. fennelliae</em></td>
<td>Man</td>
<td>Faeces</td>
</tr>
<tr>
<td><em>H. heilmanni</em>(^b)</td>
<td>Cat, dog, man</td>
<td>Gastric mucosa</td>
</tr>
<tr>
<td><em>H. hepaticus</em></td>
<td>Mouse</td>
<td>Intestinal mucosa(^c)</td>
</tr>
<tr>
<td><em>H. muridarum</em></td>
<td>Rat, mouse</td>
<td>Intestinal mucosa</td>
</tr>
<tr>
<td><em>H. mustelae</em></td>
<td>Ferret, mink</td>
<td>Gastric mucosa</td>
</tr>
<tr>
<td><em>H. nemestrinae</em></td>
<td>Macaque</td>
<td>Gastric mucosa</td>
</tr>
<tr>
<td><em>H. pametensis</em></td>
<td>Bird, pig</td>
<td>Faeces</td>
</tr>
<tr>
<td><em>H. pullorum</em></td>
<td>Bird, man</td>
<td>Caecum</td>
</tr>
<tr>
<td><em>H. pylori</em></td>
<td>Man</td>
<td>Gastric mucosa</td>
</tr>
<tr>
<td><em>H. rappini</em>(^b)</td>
<td>Dog, sheep</td>
<td>Intestinal mucosa</td>
</tr>
<tr>
<td><em>H. salomonis</em></td>
<td>Dog</td>
<td>Gastric mucosa</td>
</tr>
<tr>
<td><em>H. suis</em>(^b)</td>
<td>Pig</td>
<td>Gastric mucosa</td>
</tr>
<tr>
<td><em>H. trogontum</em></td>
<td>Rat</td>
<td>Colonic mucosa</td>
</tr>
</tbody>
</table>

\(^a\) Adapted from Owen, 1998 and Jalava *et al.*, 1997

\(^b\) Species has not been formally validated

\(^c\) Also isolated from the liver
1.4. Bacteriology

*H. pylori* is a Gram-negative, non-spore forming, slow growing, spiral bacterium that colonises the gastric epithelium of the stomach and is often found beneath the mucus layer that overlies the gastric epithelium where the oxygen tension is low. Successful isolation of *H. pylori* is achieved under microaerophilic conditions in an atmosphere of 5 – 10% CO₂. However, some strains may become sufficiently aerotolerant such that they can grow aerobically (Xia *et al.*, 1994c). *H. pylori* growth occurs within a temperature range of 33 – 40 °C but optimal growth is achieved at 37 °C (Goodwin and Armstrong, 1990). The bacterium is acid-sensitive and can grow within a pH range of 5.5 – 8.5 but it grows best between pH 6.9 and 8.0 (Owen, 1998). When cultured on solid medium, three to five day-old colonies of *H. pylori* are grey, convex, circular (1 – 2 mm in diameter) and translucent in appearance.

*H. pylori* typically exhibits a spiral morphology (2.5 – 5 μm long and 0.5 – 1 μm wide) *in vivo* but assumes a curved rod-like morphology *in vitro*. It possesses 4 – 6 sheathed unipolar flagella that are essential for motility of the bacterium through the gastric mucus. In the presence of unfavourable conditions or after prolonged culture, *H. pylori* undergoes morphological transformation from a bacillary to a coccoid form (compare Fig. 1.1a, b and c), a feature akin to the morphological transformation exhibited by *Campylobacter jejuni* (Moran and Upton, 1986; Moran and Upton, 1987; Catrenchich and Makin, 1991). While the stimuli prompting coccoid cell formation in *C. jejuni* remain incompletely defined, recent studies suggest that the formation of degenerate forms may be as a consequence of oxidative stress (Thomas *et al.*, 1999). In the case of *H. pylori*, there is evidence to suggest that the coccoid forms may be metabolically active (Nilius *et al.*, 1993) but studies have found that these forms are difficult to culture. It is unclear whether the coccoid cell represents a degenerate form or whether these forms can revert...
Figure 1.1. Scanning electron micrographs of *H. pylori* cells.

*H. pylori* reference strain NCTC 11638 was grown on blood agar for 3 days. (a) Cells with typical curved rod-like morphology; (b) and (c) Coccoid forms. Two coccoid cells appear as U-shaped bacilli in panel (b). Bars below panels represent 2 μm.
to the usual vegetative form. Therefore, until the stimuli prompting and the mechanisms effecting coccoid cell formation in *H. pylori* are elucidated, these coccoid forms are likely to remain non-culturable.

*H. pylori* produces many enzymes, including catalase, cytochrome oxidase, acid phosphatase, alkaline phosphatase and urease, the latter is an important phenotypic marker used for laboratory identification of the bacterium (Méraud *et al.*, 1985).

### 1.5. Epidemiology

Up to 50% of the world's population is infected with *H. pylori* but the incidence of the infection varies considerably from country to country and between populations. Many individuals acquire the infection in childhood or early adulthood and following *H. pylori* colonisation, the infection is generally life-long unless the individual is treated or until the individual develops severe atrophic gastritis that leads to the gradual loss of the infection.

In developing countries, analysis of the prevalence rates among different age groups shows that there is rapid acquisition of the infection in childhood with 50% infected under the age of 10 years, whereas the prevalence of *H. pylori* infection among the adult population is 60 - 95%. In developed countries, the prevalence of the infection in children is low and gradually increases at a rate of approximately 0.3 - 1% per year. However, *H. pylori* infection is present in 20% of individuals below the age of 40 years and increases to 50% in individuals over 50 years old. The substantial increase in prevalence rates among those aged over 50 cannot be explained by the low rate of *H. pylori* acquisition or by the low spontaneous eradication rate of the infection. Therefore, it is generally considered that this is in fact an age 'cohort effect' that can be explained by
the higher rate of exposure to the infection in the past when poorer living conditions and overcrowding may have facilitated the spread of the infection. Epidemiological studies have provided evidence to support this hypothesis and have shown a strong association between *H. pylori* seropositivity and lower socioeconomic status, overcrowding and sharing a bed during childhood (Mendall *et al*., 1992; Webb *et al*., 1994). A better understanding of the role of the host may be important for resolving the discordance between the expected rate of transmission and the actual rate of infection.

1.6. Transmission

The only known reservoir of *H. pylori* is the human stomach and, although *H. pylori* infection in cats and non-human primates has been described, it is unlikely that these are reservoirs of the infection (Sahay and Axon, 1996). Furthermore, an environmental reservoir of the microorganism has not been conclusively identified (Feldman *et al*., 1997). The evidence to support person to person transmission of the infection has been provided by numerous seroepidemiological studies. These studies have found an increased seroprevalence of *H. pylori* infection among institutionalised patients, gastroenterologists (Mitchell *et al*., 1989) and families (Drumm *et al*., 1990; Dominici *et al*., 1999). With respect to the latter, molecular genotyping studies have found that some family members may be infected with a common *H. pylori* strain (Bamford *et al*., 1993; Wang *et al*., 1993b; Georgopoulos *et al*., 1996; van der Ende *et al*., 1996) and may even serve as a source of reinfection (Schütze *et al*., 1995). Therefore, it is likely that transmission of *H. pylori* infection occurs from person to person but to date the actual route of transmission is unknown. The published data suggest that transmission of *H. pylori* occurs by either the oral-oral, faecal-oral or gastro-oral route. Iatrogenic
transmission of *H. pylori* infection via endoscopes has been reported (Langenberg *et al.*, 1990).

The evidence for transmission via an oral-oral route is confined to the occasional isolation of *H. pylori* from saliva (Ferguson *et al.*, 1993) and the reports of isolation of the bacterium from dental plaque (Krajden *et al.*, 1989; Shames *et al.*, 1989; Majmudar *et al.*, 1990; Khandaker *et al.*, 1993). The inconsistent *H. pylori* isolation rates in individual studies may reflect the presence of low numbers or non-viable bacteria in the mouth. Alternatively, *H. pylori* infection in the oral cavity may be transient. It is possible that *H. pylori* may be transmitted by episodes of gastro-oesophageal reflux into the mouth but whether the mouth is an actual reservoir for the bacterium is speculative. Moreover, dentists do not appear to have an increased infection risk. The faecal-oral route has been proposed as a possible transmission route and two studies have reported the isolation of *H. pylori* from faeces (Thomas *et al.*, 1992; Kelly *et al.*, 1994). However, the organisms isolated by Kelly *et al.* (1994) had an unusual colonial morphology and some of these strains did not possess the urease gene. Moreover, Hazel *et al.* (1994) have shown that the prevalence of *H. pylori* infection among different populations does not correlate with the prevalence of hepatitis A, a virus which is known to be transmitted by the faecal-oral route.

The polymerase chain reaction (PCR) has been used for the detection of *H. pylori* in the oral cavity and faeces to elucidate the transmission route of *H. pylori* and in epidemiological studies to identify possible reservoirs of the infection (Nguyen *et al.*, 1993; Mapstone *et al.*, 1993a; Mapstone *et al.*, 1993b; van Zwet *et al.*, 1994; Wahlfors *et al.*, 1995; Namavar *et al.*, 1995; Hultén *et al.*, 1996; Doré-Davin *et al.*, 1999). However,
the use of PCR in such studies is controversial because this technique is unable to distinguish between viable and non-viable bacteria.

1.7. Consequences of *H. pylori* Infection

*H. pylori* colonisation of the normal gastric mucosa results in acute gastritis that is histologically characterised by the mucosal infiltration of neutrophils and mononuclear cells (Calam, 1996; Crabtree, 1996). During the initial infection the degenerate changes in the epithelium, e.g. mucin depletion, are probably due to a combination of bacterial factors and the production by the host of chemokines (IL-8) and other cytokines that are involved in the inflammatory response. The acute gastritis is usually short-lived and is associated with a transient period of hypochlorhydria.

In the vast majority of individuals the host immune response fails to eliminate the infection and consequently, the individual develops chronic gastritis of varying severity. Chronic gastritis is characterised histologically by the infiltration of lymphocytes and plasma cells into the gastric epithelium. The topographical pattern of chronic gastritis in an individual may influence the outcome of peptic ulcer disease. For example, patients in whom an antral gastritis predominates are most likely to develop duodenal ulcer, whereas patients with chronic pangastritis involving the entire stomach are likely to develop gastric ulcer and gastric cancer (see below). However, it is unclear why some individuals develop more severe disease, which is often associated with upper abdominal symptoms, while others remain asymptomatic. It is likely that the reasons for the differences in disease outcome are possibly due to the differences in the virulence of bacterial strains, host characteristics (e.g. blood group antigens), environmental factors and possibly the age of individuals at acquisition of the infection (see Fig. 1.2).
Figure 1.2. Consequences of *H. pylori* infection.
Four gastro-duodenal diseases are known to be associated with *H. pylori* infection, namely duodenal ulcer, gastric ulcer, adenocarcinoma of the distal stomach and gastric mucosa-associated lymphoid tissue (MALT) lymphoma.

**1.7.1. H. pylori infection and gastro-duodenal disease**

It is thought that approximately 10% of individuals who are infected with *H. pylori* subsequently develop duodenal or gastric ulcer disease. The organism is present in approximately 70% of patients with gastric ulcer. Of the remaining 30% in whom *H. pylori* infection is absent, ulcer disease can be accounted for by the use of aspirin or non-steroidal anti-inflammatory drugs (NSAID). *H. pylori* infection is present in 90% of patients with uncomplicated duodenal ulcer disease. Moreover, eradication of the infection prevents ulcer relapse (Logan *et al.*, 1995b) and leads to duodenal ulcer cure (Coghlan *et al.*, 1987). However, an important study by Jhala *et al.* (1999) found that *H. heilmannii* infection was present in 4 (0.42%) of 946 patients with duodenal ulcer and in 2 (0.71%) of 281 patients with NSAID-associated gastric ulcers. The findings of this study demonstrate that *H. heilmannii* infection is associated with peptic ulcer disease in a small minority of patients.

It is clear that duodenal ulcer disease is a multifactorial process and that the levels of acid in the stomach may be an additional factor that contributes to the progression of duodenitis to duodenal ulcer. Studies have found that the gastric acid output in patients with duodenal ulcers is elevated and that these patients possess approximately twice the number of acid-secreting parietal cells, whereas the level of gastric acid output in patients with gastric ulcers is lower than that secreted by normal controls (Calam, 1996). It is thought that acid-induced gastric metaplasia of the proximal duodenum, *i.e.*
transformation of the epithelium from the intestinal to the gastric type, enables *H. pylori* to colonise the duodenum and consequently induce active inflammation.

Non-ulcer dyspepsia (NUD) refers to dyspeptic symptoms related to the upper gastrointestinal tract for greater than three months where investigations do not reveal any cause for these symptoms. In developed countries, approximately 50% of patients with NUD are infected with *H. pylori*. However, to date, the role of *H. pylori* in NUD remains controversial. Similarly, the role of *H. pylori* infection in gastro-oesophageal reflux disease in patients on long-term antisecretory treatment is unclear (McNamara and O'Morain, 1999), although some studies have found that patients receiving this treatment may be at increased risk of developing atrophic gastritis of the corpus of the stomach (Kuipers et al., 1995a; Kuipers et al., 1996).

**1.7.2. *H. pylori*, gastric cancer and MALT lymphoma**

An investigation of the prevalence of *H. pylori* seropositivity in 17 populations in Europe, Japan and the United states found that the differences in gastric cancer rates were directly related to the prevalence of *H. pylori* infection. Furthermore, studies have shown that *H. pylori* infection is associated with a 3 – 6 fold increased risk of developing gastric cancer of the distal stomach (Parsonnet et al., 1991; Forman et al., 1991; Nomura et al., 1991). However, this may be an over-estimate; when the data from over 44 seroepidemiological studies including more than 4000 cases were accumulated, the presence of *H. pylori* infection increased the risk of gastric cancer 2 to 3 fold (Danesh, 1999).

The association between chronic atrophic gastritis and gastric cancer, the second commonest cause of death from cancer worldwide, has long been recognised. Long-term
H. pylori-associated chronic gastritis eventually leads to atrophic gastritis which may occur in approximately 50% of the H. pylori-infected population (Kuipers et al., 1995b). Atrophic gastritis describes the loss of gastric glands and fibrosis from the mucosa due to repeated mucosal injury and their replacement with an intestinal type metaplastic epithelium (intestinal metaplasia). During this process there is a gradual disappearance of H. pylori infection from the gastric mucosa. Therefore, in 1994 H. pylori was classified as a grade I (definite) carcinogen because of its causal role in the development of chronic atrophic gastritis which is an early preneoplastic stage required for the development of gastric cancer (IARC, 1994).

Studies in Mongolian gerbils have demonstrated that H. pylori infection induces gastritis and eventually leads the development of gastric adenocarcinoma. Furthermore, individuals with H. pylori infection have an increased epithelial cell proliferation rate compared with non-infected individuals. This evidence suggests that the chronic inflammation induced by H. pylori may contribute to the pathogenesis of gastric cancer (for reviews see Ernst, 1999; Scheiman and Cutler, 1999).

However, it is clear that other factors apart from H. pylori infection are involved in the progression of atrophic gastritis to gastric cancer. A number of genetic alterations, most of which are unknown, are thought to play a part in neoplastic change. It is known that gastric atrophy leads to hypochlorhydria and that, as a consequence, low concentrations of gastric ascorbic acid, a scavenger for nitrate, are present in the gastric lumen. This environment may facilitate gastric colonisation with other bacteria capable of metabolising nitrate to potentially carcinogenic nitroso compounds. Hence, this may be a contributing factor in the progression of atrophic gastritis to gastric cancer.
The most convincing evidence linking \textit{H. pylori} infection with gastric malignancy is the association of this infection with MALT lymphoma. Long-term \textit{H. pylori} infection, in conjunction with other factors, may cause MALT lymphoma of the B-cell type in a small minority of patients. Wotherspoon \textit{et al.} (1991) showed that nearly all patients with B-cell gastric MALT lymphoma were infected with \textit{H. pylori}. The important studies by Carlson \textit{et al.} (1996) have documented the progression of \textit{H. pylori} chronic gastritis to MALT lymphoma. Moreover, eradication of \textit{H. pylori} infection leads to the histological regression of low-grade MALT lymphoma (Wotherspoon \textit{et al.}, 1993; Neubauer \textit{et al.}, 1997) and hence, eradication treatment is now considered the first-line therapy for patients diagnosed with this malignancy. However, careful staging procedures are necessary before treatment is prescribed.

\textbf{1.8. Pathogenicity Determinants of \textit{H. pylori}}

Virulence factors may be categorised as (1) those that facilitate colonisation (urease, flagella, adhesins), (2) those that enable the bacterium to evade host defence mechanisms thereby allowing prolonged colonisation and survival [catalase, lipopolysaccharide (LPS)] and (3) those that cause damage to the host (VacA cytotoxin). It is clear that the aetiology of \textit{Helicobacter}-associated gastritis is a complex multifactorial process. Bacterial components and an inappropriate host immune response, which fails to eliminate the infection, contribute to \textit{Helicobacter}-associated disease (for reviews see Moran and O'Morain, 1997; Dunn \textit{et al.}, 1997; Shimoyama and Crabtree, 1998; Covacci \textit{et al.}, 1999).

\textbf{1.8.1. Urease}

A remarkable feature of this bacterium is its ability to produce copious amounts of the enzyme urease (urea amidohydrolase E.C. 3.5.1.5), which catalyses the hydrolysis of urea.
to ammonia and carbonic acid. The *H. pylori* urease is a multimeric, nickel metalloenzyme of 550 kDa and is composed of two subunits of 26.5 kDa (UreA) and 60.3 – 61.6 kDa (UreB) (Mobley *et al.*, 1997). This enzyme has a high affinity for urea (K<sub>m</sub> 0.2 – 0.3 mM) which is present in low concentrations in the gastric mucosa. Urease is constitutively expressed by all *H. pylori* isolates and it is thought to aid buffering of the acid in the gastric mucosa by the production of ammonia, thereby facilitating colonisation and aiding survival of the bacterium in this environment. Indeed, Clyne *et al.* (1995) found that an acidic environment was an essential requirement in vitro for the survival of *H. pylori* in the presence of urea. Moreover, urease-negative isogenic mutants of *H. mustelae* were unable to colonise ferrets (Andrutis *et al.*, 1995; Solnick *et al.*, 1995). However, it is thought that urease does not play a role in the adherence of *H. pylori* to the gastric epithelium (Clyne and Drumm, 1996). The bacterial production of ammonia, a by-product of urea hydrolysis, may cause direct epithelial cell damage. In addition, indirect epithelial cell damage may be caused by the recruitment of inflammatory cells.

### 1.8.2. Flagella

The importance of the flagella for colonisation and persistence of *H. pylori* infection has been demonstrated in animal studies that have found that non-motile *Helicobacter* mutants lacking the flagellin genes *flaA* and *flaB* that encode the two major proteins of the flagellar filaments are unable to colonise the piglet or ferret stomach (Josenhans and Suerbaum, 1997). The helical shape of the bacterium and the flagella aid *H. pylori* motility through and beneath the viscous mucus where the pH of the environment is closer to neutrality. Furthermore, *H. pylori* requires motility to maintain sufficient
populations in the gastric mucus and to avoid the continuous peristalsis and the high turnover rate of mucus and epithelial cells.

1.8.3. Adhesins

While the majority of the *H. pylori* population are free-living within the gastric mucus, a small proportion demonstrate adherence to the epithelial cell surface. A number of putative receptors including sialylated receptors, fucosylated Lewis b blood group antigen, sulphated receptors and extracellular matrix receptors, *e.g.* laminin, have been described but the specific adhesins involved in the binding to epithelial cells are poorly understood (Ilver *et al.*, 1997). To date, the BabA adhesin of *H. pylori* is the best characterised. The gene encoding this adhesin was recently cloned by Ilver *et al.* (1998) and three alleles have been identified. The presence of a 10 bp repeat sequence motif within the signal peptide sequence of the babA2 allele suggests that this gene may be subject to phenotypic phase variation. Alternatively, the sequence similarity between the N-terminal and C-terminal domains of the BabA protein and those of other outer membrane proteins suggests that recombination events leading to mosaicism within the gene may be possible (Tomb *et al.*, 1997; Ilver *et al.*, 1998).

1.8.4. LPS

The LPS of *H. pylori* is known to induce changes in gastric mucin and consequently leads to the disruption of the gastric mucosa and barrier integrity. However, apart from the direct deleterious effect of the LPS on the host, the lipid A moiety of the LPS of *H. pylori* is associated with low proinflammatory activity and this may contribute to persistence of the infection. Moreover, the surface-exposed O-specific polysaccharide chains of the LPS of 80% of *H. pylori* isolates mimic fucosylated glycoconjugates that are identical to the Lewis x and Lewis y antigens expressed on erythrocytes and human
epithelial cells (Appelmelk et al., 1996). The molecular mimicry of the Lewis blood group antigens may be an important determinant that allows *H. pylori* to effectively evade the host immune response. Furthermore, autoantibodies directed against the human gastric mucosa and antibodies to the Lewis antigens may play an important role in autoimmunity.

1.8.5. **Vacuolating cytotoxin**

All *H. pylori* strains possess the vacuolating cytotoxin gene (*vacA*), although only approximately 40% of strains actually secrete detectable levels of the active toxin (Massari et al., 1997). This toxin causes cell damage by vacuole formation in HeLa cells and gastric epithelial cell lines *in vitro*. It is thought that vacuole formation is due to interference with intracellular membrane fusion. The *vacA* gene encodes for a 140 kDa protoxin. Processing of the protoxin during export from the bacterium produces an 87 kDa polypeptide that is released into the surrounding milieu. The C-terminal 50 kDa precursor remains associated with the bacterium as two fragments of approximately 35 kDa and 14 kDa. The 87 kDa polypeptide is further processed by cleavage of an exposed loop to produce a 35 kDa N-terminal and 55 kDa C-terminal fragment. The toxin of *H. pylori* is activated at low pH and is therefore particularly suited to the gastric environment.

Molecular analysis of the *vacA* gene has shown that considerable DNA sequence variation exists between different *H. pylori* strains and this may be an important means of identifying more virulent or ulcerogenic strains of *H. pylori* (Atherton, 1998; Atherton et al., 1999). Sequence diversity occurs in two regions of the *vacA* gene namely, the ‘signal sequence’ (three different types: s1a, s1b or s2) which is required for secretion of the toxin and a ‘mid-region’ (two types: m1 or m2) that encodes the C-terminus of the
toxin and is closely associated with cytotoxin activity in vitro (Atherton et al., 1995a). Recombination (mosaicism) within the vacA gene leading to type s1/m1 is closely associated with the increased production and activity of the toxin compared to types s1/m2 and s2/m2. Among the s1/m1 type, increased vacuolating activity is observed in H. pylori strains with the s1a/m1 type than the s1b/m1 type. The s1 type is also associated with the cagA gene (see below). Gastric epithelial cell damage is more profound in patients harbouring H. pylori strains possessing the vacA mid region type m1 compared to m2 but increased gastric mucosal inflammation, including neutrophil and lymphocytic infiltration, and peptic ulcer disease are associated with the vacA signal sequence s1a > s1b > s2 (Atherton et al., 1997).

1.8.6. Cytotoxin-associated gene A

Production of the cytotoxin is closely associated with the presence of a 120 – 140 kDa immunodominant protein (Covacci et al., 1993; Tummuru et al., 1993) encoded by the cytotoxin-associated gene A (cagA). The size of this protein varies between H. pylori strains and is dependent on the number of direct repeats present in the gene. The function of this protein is unknown and the role of CagA in gastroduodenal pathology remains controversial. The cagA gene has been cloned and sequenced (Covacci et al., 1993). The gene is present in approximately 60 – 85% of H. pylori strains and the protein is expressed in nearly all of those that possess it. Strains that carry the gene are considered to be more virulent due to a higher prevalence of these strains in duodenal ulcer patients (Cover et al., 1990; Crabtree et al., 1991; Covacci et al., 1993). Furthermore, the CagA protein upregulates epithelial IL-8 expression in vitro (Crabtree et al., 1995) and in vivo (Peek et al., 1995) and CagA expression is associated with more severe grades of gastritis.
Nucleotide sequencing of the DNA regions around the \textit{cagA} gene has found that the gene forms part of a \textit{cag}-pathogenicity island (\textit{cag-PAI}; Censini \textit{et al.}, 1996). The \textit{cag-PAI} is acquired by horizontal gene transfer and consists of a 40 kb locus containing 31 genes that are inserted into the glutamate racemase gene of \textit{H. pylori}. In some \textit{H. pylori} strains the entire \textit{cag-PAI} is present, whereas in others it may be partially deleted or reorganised. Six of these genes exhibit sequence similarity to genes of other bacterial species and encode a type IV export system that is involved in the transfer of complexes across the bacterial membrane (Covacci \textit{et al.}, 1999). There is a growing consensus of opinion that the \textit{cagA} gene is linked to other genes that are more directly involved in the induction of gastritis than the \textit{cagA} gene itself and that the \textit{cagA} gene may serve as a marker for these genes.

\textbf{1.8.7. Mucinase, protease and phospholipase}

The presence of a \textit{H. pylori} gene with high DNA sequence similarity to the mucinase gene of \textit{Vibrio cholerae} suggests that the expression of this gene \textit{in vivo} may contribute to the disruption of the gastric mucus layer (Dunn \textit{et al.}, 1997). In addition, the bacterium possesses phospholipases which may degrade the phospholipid layer of the mucus. The protease of \textit{H. pylori} may be involved in the degradation of the gastric mucus and may be an important factor that contributes to duodenal ulcer disease (Moran, 1996).

\textbf{1.8.8. Superoxide dismutase and catalase}

\textit{H. pylori} is thought to resist phagocytic killing by a mechanism of oxidative burst by the production of the antioxidant superoxide dismutase (Dunn \textit{et al.}, 1997). Furthermore, the catalase enzyme produced by \textit{H. pylori} may protect the bacterium from hydrogen peroxide produced by polymorphonuclear leucocytes.
1.9. Clinical Diagnosis of H. pylori Infection

Numerous diagnostic tests are available for determining the presence of H. pylori infection. These tests are based on either an invasive endoscopic procedure, thus allowing the collection of gastric mucosal biopsy specimens, or non-endoscopic methods, e.g. serology or the urea breath test (UBT). The sensitivity of a particular test, and therefore its applicability, may vary depending on whether it is performed before treatment or 4 – 6 weeks after H. pylori eradication treatment. Following eradication treatment, even if the infection is still present, the bacterial density will be much lower and this will alter the sensitivity and specificity of a test. However, no single routine test is ideal for the detection of H. pylori infection (see Table 1.2) and hence, a combination of the results derived from two tests with acceptable sensitivity and specificity is recommended for accurate diagnosis (WPEHPSG, 1997).

1.9.1. Tests that require invasive endoscopy

A number of invasive tests are employed for H. pylori diagnosis which include culture, Gram stain of a tissue smear, the rapid urease test (RUT) and histological examination. All of these biopsy-based tests are subject to the limitations of sampling error due to the uneven distribution of H. pylori in the gastric mucosa and multiple biopsy samples are often taken to improve detection of the infection.

1.9.1.1. Rapid urease test

The H. pylori urease is an important phenotypic marker that positively identifies H. pylori in gastric biopsy specimens. The rapid urease test is based on the principle that urease catalyses the hydrolysis of urea to ammonia and carbonic acid with a concomitant increase in pH. Although a number of commercially available rapid urease tests are available (Pyloritek, HP-fast, HUT-test, Jatrox-HPtest), the CLOtest is the most widely
Table 1.2. Comparison of test sensitivities (SN) and specificities (SP) before treatment in clinical practice.

<table>
<thead>
<tr>
<th></th>
<th>Cutler et al. 1995 (n=268)</th>
<th>Lerang et al. 1998 (n=351)</th>
<th>Thijs et al. 1996 (n=105)</th>
<th>Anderson et al. 1998 (n=97)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SN</td>
<td>SP</td>
<td>SN</td>
<td>SP</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Culture</td>
<td>nd</td>
<td>nd</td>
<td>93%</td>
<td>100%</td>
</tr>
<tr>
<td>Rapid urease test</td>
<td>89.6%</td>
<td>100%</td>
<td>80%</td>
<td>100%</td>
</tr>
<tr>
<td>C-UBT</td>
<td>90.2%</td>
<td>95.8%</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>C-UBT</td>
<td>nd</td>
<td>nd</td>
<td>95%</td>
<td>95%</td>
</tr>
<tr>
<td>Serology</td>
<td>91.3%</td>
<td>91.6%</td>
<td>99%</td>
<td>91%</td>
</tr>
<tr>
<td>Gold standard</td>
<td>Combination of 4 tests</td>
<td>Combination of 3 tests</td>
<td>Combination of 2 tests</td>
<td>Combination of 2 tests</td>
</tr>
</tbody>
</table>

*a* Warthin Starry stain of gastric antral biopsy specimens

*b* Haematoxylin & eosin and Giemsa staining of antral and corpus biopsy specimens

*c* Haematoxylin & eosin and immunohistochemistry of antral and corpus biopsy specimens

*d* CLOtest result recorded at 24 h

*e* ‘In house’ urease test

*f* IgG serology

nd, not determined
used, endoscopy-based test for *H. pylori* diagnosis. A gastric biopsy specimen is placed into an agar gel containing urea and a pH indicator. The change in pH due to the presence of *H. pylori* is indicated by a colour change of the agar from yellow to magenta. The time taken for this colour change to occur is dependent on the number of bacteria present in the biopsy specimen. Maximal urease activity is achieved at a temperature of 43 °C (Mobley *et al.*, 1988) and hence, the speed of the reaction may be increased by performing the test above room temperature (Laine *et al.*, 1996a). In most cases the test will yield a positive result within a couple of hours of inoculation, although in practice these tests are read up to 24 h. This increases the sensitivity of the test but is associated with a decrease in specificity due to the presence of other urease-producing bacteria and may give rise to a false-positive result. False-negative results may arise in older patients (> 60 years old) where the presence of gastric atrophy or intestinal metaplasia reduces the ability of *H. pylori* to colonise the gastric mucosa (Abdalla *et al.*, 1998).

The sensitivity of the CLOtest is approximately 90% in untreated patients and may be improved by using two antral biopsy specimens in one CLOtest slide (De Boer, 1997) or by incubating the test at or above 37 °C. However, the sensitivity of the rapid urease test depends on the number of bacteria present in the biopsy sample, a high number (at least \(10^5\)) of organisms being required to yield a positive result (Xia *et al.*, 1994a). Therefore, this test may be inappropriate for the assessment of *H. pylori* status in post-treatment patients because of the presence of low numbers of residual bacteria when eradication has been unsuccessful (Méraud, 1996b).

1.9.1.2. Culture

The success rate of isolation of *H. pylori* by culture is dependent on the transport conditions from the endoscopy room to the microbiological laboratory and the handling
of the gastric biopsy specimen during processing. Gastric biopsies are transported in a number of transport media, such as nutrient or Brucella broth, Stuart’s medium, saline or in a commercially available transport medium (e.g. Portagerm, Bio Merieux). It is important to maintain the specimen at 4 °C to avoid desiccation and to prevent the overgrowth of accompanying bacteria. Gastric biopsies may be inoculated directly onto solid microbiological media by smearing the biopsy (mucosal side downwards) over the surface of the agar but higher isolation rates and a more homogenous growth are achieved when the biopsy specimen is homogenised with a tissue grinder (Méraud, 1996b; WPEHPSG, 1997). Various media are available for isolation including Mueller-Hinton agar, Columbia agar, Wilkins Chalgren agar, Brain Heart Infusion agar and GC* agar (Oxoid) which are supplemented with either horse, sheep or human blood at concentrations ranging from 5 to 10% (v/v). Contamination compromises the sensitivity of the test and, therefore, selective supplements (vancomycin, nalidixic acid, trimethoprim, amphotericin B) may also be added to the agar to inhibit the growth of other bacteria and fungi. The choice of a particular medium will depend on local facilities and preferences but a combination of a selective and a non-selective medium should be used to increase sensitivity (Tee et al., 1991; Piccolomini et al., 1997a). Plates are incubated at 37 °C under microaerophilic conditions in 98% humidity for five to seven days. Incubation may be prolonged for up to 10 days in the case of a post-treatment evaluation.

The specificity of culture is the best (100%) of all of the diagnostic tests because isolation of the bacterium allows accurate identification. In order to minimise the risk of sampling error, it is advisable that two antral biopsy samples are taken for culture in untreated patients and that biopsy samples from both the antrum and corpus are collected for the post-treatment assessment of *H. pylori* status (WPEHPSG, 1997; Glupczynski,
High sensitivity is achieved in certain centres with extensive expertise and experience of the method (van Zwet et al., 1993; Lerang et al., 1998) but the published data suggest that the majority of investigators fail to reach high sensitivity rates in clinical practice.

Microbiological culture is essential for antimicrobial susceptibility testing (see section 1.13) and provides important information for the management of *H. pylori* infection. Isolation of the organism is also required to investigate microbe-host interactions, for molecular biology techniques, *e.g.*, the detection of the presence of certain *H. pylori* genes by the polymerase chain reaction (PCR, see section 1.9.1.5), and for the genetic comparison of strains (see section 1.15.2).

1.9.1.3. *Microscopy*

A Gram stain of a gastric biopsy smear on a sterile glass slide (touch preparation) is also used for the rapid detection of *H. pylori* and provides a cost-effective method of laboratory diagnosis (Montgomery et al., 1988).

1.9.1.4. *Histology*

As a diagnostic test, histology can estimate the extent of the *H. pylori* infection and is the only test that simultaneously determines the histopathological state of the gastric mucosa. It has been recommended that two biopsy samples should be taken from each of the antral and corpus sites of the stomach for the histological assessment of *H. pylori* infection (Price, 1991). Gastric biopsy specimens are transported to the laboratory in a fixative where they are processed and subsequently sectioned and stained for examination. Routine haematoxylin and eosin stain offers the best histological assessment of gastritis but the identification of *H. pylori* may be inaccurate when there is
debris on the gastric surface or when *H. pylori* infection is scanty (Price, 1996). The Giemsa stain is widely used and is an easy, rapid and effective staining method. Although these stains are not specific for *H. pylori*, the shape and the association of the bacteria with the luminal surface of the gastric epithelium allow distinction between *H. pylori* and other upper digestive tract bacteria that are occasionally present. Immunocytochemistry is available for the specific identification of *H. pylori* infection (Dunn et al., 1997).

The quality of the biopsy specimen will significantly affect the sensitivity and specificity of the histological test. Therefore, it is essential that full thickness mucosal biopsy specimens are taken for histological assessment and that they are properly orientated and processed. Furthermore, the efficacy of the test will, to a large extent, also depend on the experience of the observer. Inter-observer variability is high, particularly when the infection is scanty. When biopsy specimens have been examined by several pathologists the concordance of results has been poor (Christensen et al., 1992; Kolts et al., 1993; Maconi et al., 1999).

### 1.9.1.5. PCR

The PCR technique is widely available for the detection of various bacteria including *H. pylori*. The reaction cycle is composed of three different temperature-dependent steps. Double-stranded target DNA is heated to 94 °C to allow the complete separation of the two complementary DNA strands. The temperature is then lowered to allow annealing of two short single-stranded DNA primers (usually 10 to 30 bases long and of opposite polarity) which form a stable DNA duplex. The specificity of the PCR is governed by the degree of homology between the primer sequence and the DNA template. Following primer annealing, the temperature is then raised to 72 °C and the DNA that spans
between the primers is extended by the incorporation of deoxynucleotides by a heat-
stable DNA polymerase. Thereafter, the cycle of DNA denaturation, primer annealing
and DNA extension is repeated resulting in the exponential amplification of the target
DNA. The DNA amplification products of the reaction are visualised by agarose gel
electrophoresis.

A considerable number of PCR primers have been developed (Valentine et al., 1991; Ho
et al., 1991; Clayton et al., 1992; Hammar et al., 1992; Wahlfors et al., 1995; Thoreson
et al., 1995; Kawamata et al., 1996) and evaluated for the detection of \textit{H. pylori} DNA in
a wide range of specimens including gastric biopsy material (van Zwet et al., 1993;
Bickley et al., 1993; Wang et al., 1993a; Morera-Brenes et al., 1994; Weiss et al., 1994;
Fabre et al., 1994; Lage et al., 1995; Chong et al., 1996; Lu et al., 1999), gastric juice
aspirates (Kawamata et al., 1996), dental plaque (Bamford et al., 1998; Song et al.,
1999), and faeces (van Zwet et al., 1994; Gramley et al., 1999). In theory, PCR is a rapid
and accurate technique that can detect one copy of the target DNA. The nature of the
technique is such that it detects \textit{H. pylori} DNA and as a consequence, the method does
not distinguish between viable and non-viable bacteria. In clinical practice, the technique
has high sensitivity but false-positive results may occur in biopsy specimens that contain
\textit{H. pylori} DNA derived from endoscopes that have not been adequately washed
(Roosendaal et al., 1994). At present, PCR is regarded largely as a research tool and has
no routine clinical application.

1.9.2. Non-endoscopic methods

The UBT and serology, by their nature, overcome the limitation of sampling error. These
tests have the added advantage in that an invasive endoscopic procedure is not required
(for reviews see Vaira et al., 1999; Savarino et al., 1999).
1.9.2.1. Urea breath test (UBT)

The urea breath test, like the rapid urease test, depends on the ability of the \textit{H. pylori} urease to hydrolyse urea. Two carbon isotopes are currently used for the UBT, either $^{13}$C, which is a non-radioactive isotope, or radioactive $^{14}$C. Carbon-labelled urea is given to the patient. In the presence of \textit{H. pylori} urease, the urea is hydrolysed and labelled carbon dioxide is absorbed into the bloodstream and exhaled in the breath. Breath samples are collected by blowing into a tube and the amount of $^{13}$CO$_2$ or $^{14}$CO$_2$ is measured by a mass spectrometer or a scintillation counter, respectively. A distinct advantage of this test is that a positive UBT result indicates current infection.

A European standard protocol has been developed for the $^{13}$C-UBT (Logan \textit{et al.}, 1991). The sensitivity and specificity of the UBT is generally very high and in clinical practice this test is particularly useful for the assessment of \textit{H. pylori} eradication. However, false negative results may occur if the patient has recently received treatment with antibiotics, bismuth or proton pump inhibitors (PPI). PPI’s suppress \textit{H. pylori} infection and may give rise to false-negative UBT results (range 17 – 61%) if the test is performed within one week of treatment (Logan \textit{et al.}, 1995a; Savarino \textit{et al.}, 1999).

1.9.2.2. Serology

\textit{H. pylori} infection induces both local and systemic immune responses in the host, the latter yielding serum antibodies mainly of the IgG1 subclass that are directed against the outer bacterial surface proteins, the flagella and other bacterial proteins such as urease. Due to the chronic nature of the infection and the constant systemic IgG response induced, an enzyme-linked immunosorbent assay (ELISA) can be used to detect the antibodies produced. It is essential that each serological test is validated for the local population in which it is to be used to determine an appropriate cut-off value that
accurately distinguishes between *H. pylori*-infected and non-infected individuals. Different antigen preparations may be used to coat the ELISA wells and include crude preparations of antigens prepared by sonication or by glycine-extraction. A number of commercial kits are available but their performances vary considerably. The differences in accuracy observed with these tests may be due to the different antigen preparations used. Poor specificity has been observed with the first commercially available kits due to cross reactivity of *Helicobacter* flagellar antigens with those of *Campylobacter* species (De Boer, 1997). New second-generation kits employ a mixture of purified antigens and these increase specificity (Breslin and O'Morain, 1997).

Serology is not recommended for routine post-treatment assessment of *H. pylori* status as IgG levels may take six months or longer to fall to levels within the uninfected range following treatment success. Serology may be useful for monitoring the long-term effect of treatment in the clinical trial setting, although 2% of *H. pylori*-infected individuals do not mount a systemic IgG response (Méraud, 1996b). It is important to test simultaneously the pre- and post-treatment sera and to demonstrate a fall in antibody titre rather than seronegativity. A titre reduction of >50% is usually a reliable indicator of eradication as titres differing by ±20%, irrespective of treatment success, may merely represent *H. pylori* suppression (WPEHPSG, 1997).

Enzyme immunoassay and agglutination kits have been commercially developed for the detection of *H. pylori* infection in the physician's office. Unlike the laboratory-based ELISA's, which are both quantitative and qualitative, office-based tests are entirely qualitative. Lower sensitivity is observed with these tests and their accuracy still needs to be determined (Glupczynski, 1998).
1.10. Treatment of *H. pylori* Infection

Accurate diagnosis of *H. pylori* infection is an essential requirement before eradication treatment is prescribed.

### 1.10.1. Criteria for eradication therapy

It is universally accepted that patients who are infected with *H. pylori* who have past or present peptic ulcer disease, and patients with complicated bleeding ulcers should receive *H. pylori* eradication treatment (NIH, 1994; EHPSG, 1997). However, *H. pylori* research has proceeded at a rapid pace and the indications for *H. pylori* eradication have expanded. Eradication treatment is unequivocally recommended for *H. pylori*-positive patients with low-grade MALT lymphoma of the B-cell type and while treatment is not recommended for patients with terminal gastric cancer, it is strongly recommended in those patients with advanced or progressively worsening forms, *i.e.* intestinal metaplasia or glandular atrophy and erosive or hypertrophic forms of gastritis, and in *H. pylori*-positive patients after resection of early gastric cancer. Treatment remains equivocal for *H. pylori*-positive patients with NUD in whom no other causes of symptoms can be identified or in patients who are treated with long-term antisecretory treatment for gastro-oesophageal reflux disease (EHPSG, 1997; Malfertheiner *et al.*, 1997).

### 1.10.2. Combination eradication therapy

The first triple treatment regimen used for *H. pylori* infection consisted of a combination of three agents, namely bismuth, metronidazole and tetracycline or amoxicillin, and was found to give high success rates of 80% in motivated patients (Borody *et al.*, 1989). However, the high dose regimen and long duration (2 weeks) of this 'classical' triple treatment were associated with significant side effects, and consequently poor patient compliance, and the development of metronidazole-resistant strains (see Table 1.3).
which significantly compromised the clinical efficacy of this treatment (Axon, 1996). Bazzoli et al. (1994) were the first to introduce PPI-based triple therapy consisting of omeprazole, clarithromycin and tinidazole. This one-week treatment regimen was better tolerated and clinically efficacious, giving consistent eradication rates in the region of 90% in large multi-centre studies (Lind et al., 1996; Lind et al., 1999). Dual therapy regimens are available, e.g., a PPI and clarithromycin or a PPI and amoxicillin, with good eradication rates of 84% and 73%, respectively, have been achieved in some centres (Axon, 1996). However, comparison of the eradication rates achieved in individual studies shows a high degree of inconsistency and success rates are generally lower than those obtained with PPI-based triple therapy.

Currently, \textit{H. pylori} treatment regimens are simple, well tolerated and cost-effective and achieve eradication rates of >80%. The recommended treatment is a PPI-based triple treatment for seven days in combination with two of the following antimicrobials – clarithromycin, a nitroimidazole (metronidazole or tinidazole) and amoxicillin (EHPSG, 1997). Although about one-third of patients experience diarrhoea and a proportion experience taste disturbance, which appears to be clarithromycin dose dependent, all of these treatment regimens achieve consistent eradication rates of >90% in large multi-centre studies (Lind et al., 1996; Lind et al., 1999). While it is known that PPI monotherapy suppresses \textit{H. pylori} infection but does not eradicate the organism (Cariani et al., 1992), these multi-centre studies have found that omeprazole increases the eradication rate when this agent is used in the triple treatment regimen. It is possible that omeprazole, by reducing the secretion of acid from the gastric parietal cells of the corpus, may enhance the antimicrobial effect of clarithromycin and amoxicillin.
<table>
<thead>
<tr>
<th>Treatment regimen&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Duration (days)</th>
<th>Eradication rate (%)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Prevalence of Mtz resistance (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mtz&lt;sup&gt;s&lt;/sup&gt;</td>
<td>Mtz&lt;sup&gt;r&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td><strong>‘Classical’ Triple Therapy</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cbs+Mtz+Amox</td>
<td>14</td>
<td>91</td>
<td>63</td>
<td>26</td>
</tr>
<tr>
<td>Cbs+Mtz+Tet</td>
<td>14</td>
<td>90</td>
<td>32</td>
<td>20</td>
</tr>
<tr>
<td><strong>PPI-Based Triple Therapy</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPI+Amox+Mtz</td>
<td>10</td>
<td>96</td>
<td>77</td>
<td>30</td>
</tr>
<tr>
<td>PPI+Amox+Tin</td>
<td>7</td>
<td>93</td>
<td>64</td>
<td>14</td>
</tr>
<tr>
<td>PPI+Amox+Mtz</td>
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<td>96</td>
<td>75</td>
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<tr>
<td>PPI+Mtz+Clr</td>
<td>10</td>
<td>94</td>
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<td>30</td>
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<tr>
<td>PPI+Mtz+Clr</td>
<td>7</td>
<td>98</td>
<td>57</td>
<td>36</td>
</tr>
<tr>
<td>PPI+Mtz+Clr</td>
<td>7</td>
<td>95</td>
<td>76</td>
<td>27</td>
</tr>
</tbody>
</table>

<sup>a</sup> Cbs, colloidal bismuth subcitrate; Mtz, metronidazole; Amox, amoxicillin; Tet, tetracycline; PPI, proton-pump inhibitor; Tin, tinidazole

<sup>b</sup> Mtz<sup>s</sup>, metronidazole susceptible; Mtz<sup>r</sup>, metronidazole resistant
Metronidazole resistance significantly compromises the eradication rates in patients who have received a PPI-based treatment regimen containing this agent (see Table 1.3). A recent meta-analysis by Houben et al. (1999) found that metronidazole resistance reduces the clinical efficacy of PPI- and bismuth-based triple therapy by as much as 50%. However, the variation in clinical outcome observed in individual studies may be due, to some extent, to the different levels of primary resistance in the *H. pylori* population (see section 1.14.3), patient compliance and the different methods employed for microbiological susceptibility testing in each study (see section 1.13.1).

Clarithromycin resistance has a significant effect on the success rate of a given treatment (Houben et al., 1999; Realdi et al., 1999). Moreover, of concern is the emergence of secondary acquired clarithromycin resistance in *H. pylori* (see section 1.14.3). Wurzer et al. (1997) found that clarithromycin resistance had developed in 2 (33%) of 6 *H. pylori* strains recovered from patients treated with a PPI, amoxicillin and clarithromycin and in 26 (81%) of 32 strains from patients treated with a dual therapy regimen consisting of a PPI and clarithromycin. Moreover, studies have found that the emergence of clarithromycin resistance in *H. pylori* is high, especially when there is a high prevalence of metronidazole resistance. A Dublin study document secondary resistance in 58% of unsuccessful treatment cases (Buckley et al., 1997). This is consistent with other groups who have reported acquisition of resistance in up to two-thirds of patients with persistent infection after clarithromycin-based therapy (Bazzoli et al., 1994; Lee et al., 1999).

1.10.3. Management of patients in whom eradication therapy for *H. pylori* infection fails

The Maastrict Consensus Report strongly recommends a tailored treatment approach (see Table 1.4) consisting of a PPI and two antimicrobials, where the choice of antimicrobial
Table 1.4. Currently recommended one week PPI-based treatment regimens used for the eradication of *H. pylori* infection and the most appropriate regimen to use in the presence of antimicrobial resistance.

<table>
<thead>
<tr>
<th>Treatment regimen&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Indication in the presence of antimicrobial resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPI plus Metronidazole or Tinidazole (400 mg b.i.d) plus Clarithromycin (250 mg b.i.d)</td>
<td>None</td>
</tr>
<tr>
<td>PPI plus Amoxicillin (1 g b.i.d) plus Clarithromycin (500 mg b.i.d)</td>
<td>Advisable when nitroimidazole resistance likely</td>
</tr>
<tr>
<td>PPI plus Amoxicillin (1 g b.i.d) plus Metronidazole (400 mg b.i.d)</td>
<td>Advisable when clarithromycin resistance likely</td>
</tr>
</tbody>
</table>

<sup>a</sup> PPI, proton-pump inhibitor twice daily which can be either omeprazole (20 mg), lansoprazole (30 mg) or pantoprazole (40 mg)
is based on the predicted or observed rate of antimicrobial resistance (EHPSG, 1997). Ideally, patients in whom *H. pylori* eradication treatment has failed should undergo endoscopy to obtain gastric biopsies for culture so that susceptibility testing of the *H. pylori* strain isolated may guide further therapy. The choice of second-line treatment will depend on the previous therapy used or the outcome of *in vitro* susceptibility tests when available or both. Some studies have found that quadruple treatment (‘classical’ triple therapy plus omeprazole) may be suitable for patients in whom first-line eradication treatment for *H. pylori* infection has failed.

1.10.4. Definitions of clinical efficacy of *H. pylori* eradication treatment

1.10.4.1. Eradication

Eradication is defined as the failure to detect *H. pylori* infection either by conventional biopsy-based diagnostic tests (either the rapid urease test, histology, culture and microscopy) or a non-invasive $^{13}$C or $^{14}$C-urea breath test, at least four weeks after the end of treatment.

1.10.4.2. Recurrence

Recurrence of the infection is the situation where *H. pylori* infection is not detected by biopsy-based methods or the breath test one month after completing eradication treatment but these tests are subsequently positive again at a later stage. Recurrence of the infection may be due to either recrudescence or reinfection.

1.10.4.3. Recrudescence

The situation where the original pre-treatment *H. pylori* strain, that had been merely suppressed by treatment and was undetected four weeks post-treatment, thereafter
replicates to detectable levels in the stomach i.e. relapse of the infection by the same strain.

1.10.4.4. Reinfection

Reinfection by \textit{H. pylori} is defined as the situation where the patient in whom the original \textit{H. pylori} strain was successfully eradicated, later becomes infected with a new \textit{H. pylori} strain. However, reinfection by a strain from a common original source that is genetically identical to that present prior to treatment cannot be excluded.

1.11. Vaccines

Vaccination may provide another possible means for the treatment and prevention of \textit{H. pylori} infection. Oral immunisation with a number of \textit{Helicobacter sp} immunogens in conjunction with mucosal adjuvants produces a significant antibody response in a variety of animal models (Dunn \textit{et al}., 1997; Covacci \textit{et al}., 1999). While it is recognised that animal models have their limitations, important studies are in progress to assess whether the results in animals apply to humans. Recombinant \textit{H. pylori} urease is a potential immunogen for human vaccination studies. Phase I clinical trials aiming to assess the safety of orally administered recombinant urease in human \textit{H. pylori}-infected volunteers found that this preparation was well tolerated. However, in the absence of an adjuvant, the volunteers did not mount a sufficiently high immune response to eliminate the infection (Kreiss \textit{et al}., 1996). Phase II clinical trials using recombinant urease in combination with an adjuvant are currently in progress. The publication of the complete genome sequence of \textit{H. pylori} may allow identification of a range of potential vaccine candidates that may be employed in future vaccination studies (Tomb \textit{et al}., 1997; Alm \textit{et al}., 1999).
1.12. H. pylori Recurrence

A higher number of H. pylori recurrences occur within the first year of treatment and the recurrence rate decreases with increasing follow-up period. An important study by Bell and Powell (1996) has shown that of 57 ‘reinfections’ observed during the follow-up of 1182 patients totalling 1533.5 patient years of observation, the reinfection or late recrudescence rate one year after eradication therapy was 3.7% per year. However, the ‘reinfection’ rate was 9.5% per year, 1.3% per year and 0.58% per year, within the first six months, after six months and after one year following treatment, respectively. This suggests that these ‘reinfections’ actually represent late recrudescences of the infection. A number of different molecular genotyping methods have been applied to more accurately distinguish between reinfection and recrudescence (see section 1.15.2).

1.12.1. Factors associated with the recurrence of H. pylori infection

In order to accurately distinguish between recrudescence and reinfection, it is important to reliably establish that cure of the H. pylori infection has occurred. There are a number of factors, e.g., limitations of the diagnostic tests, sampling error and ineffective treatment regimens, that contribute to a high recurrence rate. Most recurrences likely represent recrudescence of H. pylori infection rather than true reinfection.

1.12.1.1. Limitations of the diagnostic tests for H. pylori infection

Tests for the detection of H. pylori should not be performed too early after eradication treatment as this may result in an over-estimate of cure rate and a high recurrence rate. Therefore, it is generally accepted that H. pylori eradication should be assessed four weeks after the completion of treatment. During this time, any residual bacteria will be able to replicate to numbers that may be detected by the conventional diagnostic tests. Although this is an arbitrary time period, a recent study has shown that the rate of
duodenal ulcer recurrence does not differ when \textit{H. pylori} eradication assessment is performed at either four weeks or \( \geq 12 \) weeks after treatment (Hopkins \textit{et al.}, 1996).

The sensitivity of the biopsy-based tests for detecting the presence of \textit{H. pylori} infection in post-treatment patients is compromised by sampling error in the presence of low bacterial numbers. This may result in failure to detect the infection at the follow-up endoscopy. Low bacterial numbers are also present in patients with gastric atrophy of the mucosa and intestinal metaplasia or after eradication treatment with a regimen containing bismuth or omeprazole. It is, therefore, recommended that at least two biopsy-based tests should be used to assess \textit{H. pylori} eradication (Hopkins \textit{et al.}, 1996). Furthermore, treatment with omeprazole changes the intragastric distribution of \textit{H. pylori} colonisation from the predominant antral site to either the fundal or the corporal sites (Logan \textit{et al.}, 1995a; Kuipers \textit{et al.}, 1995a). Consequently, the sensitivity of antral biopsy specimens for the detection of \textit{H. pylori} infection can be significantly reduced in patients who have received a PPI-based treatment regimen. Accordingly, biopsies from the corpus and fundus should be included for the assessment of \textit{H. pylori} eradication (WPEHPSG, 1997).

Some studies have found that the sensitivity and specificity of the urea breath test when solely used to assess \textit{H. pylori} eradication is lower than the combination of culture and histological examination (van der Ende \textit{et al.}, 1997) and may be due to the inhibition of \textit{H. pylori} urease activity following PPI-based treatment. Conversely, false-positive results may arise with the UBT (Cutler \textit{et al.}, 1995) which may be due to urease production by oropharyngeal bacteria. These findings suggest that the detection of \textit{H. pylori} infection in post-treatment patients should not be solely based on the urease-based
tests (either the UBT or the CLOtest) but rather that combination of the UBT with the endoscopy-based tests is recommended (Xia et al., 1997).

1.12.1.2. Clinical efficacy of treatment regimens

Currently, PPI-based triple therapy regimens are the most effective treatment used for *H. pylori* eradication (see section 1.10.2). The eradication rates obtained with dual therapy regimens vary between 50 to 75% and hence these regimens are inappropriate for *H. pylori* eradication, especially when more effective treatments are available. There is an inverse association between the recurrence rate and the clinical efficacy of a treatment regimen, *i.e.*, the more effective the treatment, the lower the recurrence rate (Bell and Powell, 1996; Xia et al., 1997; Gisbert et al., 1998). Indeed, Bell and Powell (1996) found that if six months was used as the time to define successful eradication and treatment was 80% effective, 1.7% 'reinfections' was observed, whereas with a treatment regimen that was 60 – 70% effective, 4.6% 'reinfections' was observed.

1.13. In Vitro Antimicrobial Susceptibility Testing of *H. pylori*

Several techniques may be used to determine the susceptibility of *H. pylori* to antimicrobial agents (for review see Méraud, 1997a). Antimicrobial susceptibility tests may be either quantitative, which facilitates the determination of the minimum inhibitory concentration (MIC) value, *i.e.*, the lowest antimicrobial concentration that will inhibit bacterial growth, or qualitative, where organisms are simply categorised as susceptible, moderately susceptible or resistant.

Quantitative methods include the agar and broth dilution methods. These methods are considered to be the 'gold standard' of *in vitro* susceptibility test methods in that they are reproducible to within ±1 log₂ dilution. However, factors such as the culture medium,
inoculum density (Berger *et al.*, 1993; Henriksen *et al.*, 1996), incubation atmosphere and duration of incubation (Henriksen *et al.*, 1996) will directly influence the growth of the organism and consequently the MIC value obtained. The broth dilution method offers an added advantage in that it may be used for the determination of the minimal bactericidal concentration (MBC) and also facilitates examination of both synergistic and antagonistic interactions between antimicrobial agents.

Qualitative susceptibility results are obtained using the disc-diffusion method. This method is cost-effective, easy to perform and more suitable for routine practice. However, this procedure requires strict standardisation. The correlation between the diameters of inhibitory zones obtained with the disc-diffusion test and the MIC’s obtained with the agar dilution method must be established. More recently, the epsilometer (E) test has been developed but unlike other diffusion methods, the E-test is based on the provision of an antimicrobial gradient and therefore, allows the determination of an actual MIC value.

**1.13.1. Correlation between in vitro susceptibility testing and clinical effectiveness**

The purpose of susceptibility testing is to assist the clinician with the choice of a suitable therapeutic antimicrobial or to explain treatment failure with selected agents. Interpretive categories are used to predict the response of the patient who has been treated with a particular antimicrobial agent. The susceptible category implies that the organism is likely to respond to treatment and, conversely, isolates classified as resistant are, in general, unlikely to respond to treatment. The intermediate interpretative category suggests that an indeterminate or intermediate response to treatment may be expected. However, in certain circumstances high doses or the use of antimicrobials that concentrate at the site of infection may result in a favourable response.
In vitro susceptibility of *H. pylori* does not necessarily correlate with treatment success. To date, there is no single method generally accepted for susceptibility testing of this organism. Factors such as culture media, inoculum density suitable control organisms, duration of incubation and the incubation atmosphere must be standardised to allow for accurate and reproducible susceptibility test results. The disc-diffusion test and the E-test are particularly influenced by medium composition and inoculum size. This is a particular problem with susceptibility testing of *H. pylori* to metronidazole and it is now suggested that metronidazole susceptibility results determined by these methods should be confirmed using the agar dilution method (WPEHPSG, 1997). The problems associated with in vitro susceptibility testing of *H. pylori* to metronidazole also extend to other antimicrobials.

Clarithromycin exhibits a bimodal distribution of MIC values and strains are easily defined as susceptible or resistant and, thus, susceptibility results correlate with clinical outcome. Since the MIC values between clarithromycin-susceptible and resistant populations are so distinct, any of the in vitro susceptibility testing methods are applicable. However, within the clarithromycin-susceptible strains, a small proportion possess relatively higher MIC values, the clinical significance of which is unclear (Méraud, 1996a). Moreover, a breakpoint MIC which differentiates susceptibility and resistance has not been adequately defined for clarithromycin and, coupled with the lack of a standardised susceptibility testing method, the comparison of resistance rates among different centres is difficult.

Apart from the limitations of *H. pylori* susceptibility testing methods, additional factors may account for the poor correlation between antimicrobial susceptibility and clinical success. Single colony analysis of *H. pylori* isolates recovered from single and multiple
biopsies has found that patients may harbour strains of \textit{H. pylori} which are heterogeneous in their susceptibility profile. Hence, susceptibility testing of sweep cultures may underestimate the true rate of antimicrobial resistance especially when less reliable methods of susceptibility testing are used (Jorgensen \textit{et al.}, 1996; Weel \textit{et al.}, 1996; Dore \textit{et al.}, 1998a; van der Wouden \textit{et al.}, 1999).

Furthermore, \textit{H. pylori} occupies a unique niche interposed between the epithelium and gastric mucus layer and the bacteria may, therefore, survive eradication attempts in so-called sanctuary sites (Atherton \textit{et al.}, 1995b). In addition, pharmacological resistance may play an important role. In this case, a strain is defined as resistant when the “antimicrobial concentration that it can tolerate is higher than the concentration achieved \textit{in vivo} at the site of infection” (Méraud, 1997a). Moreover, gastric acidity has also been shown to reduce antimicrobial activity and diffusion of the antimicrobial in the gastric mucosa is limited. Consequently, bacteria in sequestered sites may be exposed to sub-inhibitory levels of antimicrobials, thus generating an environment where antimicrobial resistance might be induced.

Equally, \textit{in vitro} resistance does not necessarily correlate with therapeutic failure, especially when combination therapy is used. The effect of antimicrobial resistance to one of the components of the treatment regimen on the eradication rate is unpredictable.


Resistance to an antimicrobial is an important factor that reduces the therapeutic value of many antibacterial agents (for reviews see Goddard and Logan, 1996; Méraud, 1997a; Méraud, 1998). Resistance of a strain may be either intrinsic or acquired.
1.14.1. **Intrinsic resistance**

When considering the bacterial population as a whole, intrinsic or natural resistance to an agent denotes that all of the bacterial species are resistant and have always been resistant. Therefore, the use of certain antimicrobial(s) for treatment is inappropriate. *H. pylori* is naturally resistant to vancomycin (glycopeptide), sulphonamides and trimethoprim (2,4-diaminopyridine). *H. pylori* is also resistant to first generation quinolones, cefsulodin (a β-lactam), polymixins and antifungal compounds, although some strains may be susceptible (Méraud, 1994).

1.14.2. **Acquired resistance**

Acquired resistance to an agent denotes that the bacterial species is usually susceptible but may become resistant. Resistance may be acquired in some bacterial cells either by alteration of the existing chromosome (mutational resistance) or by the transfer of DNA between cells (transmissible resistance).

1.14.2.1. **Transmissible resistance**

This type of acquired resistance describes the acquisition of resistance genes by a susceptible recipient cell from a resistant donor bacterial cell. The genes conferring antibiotic resistance may be acquired either by the uptake of chromosomal DNA or extrachromosomal elements such as a plasmid or by infection with a bacteriophage. Bacterial cells harbouring resistance determinants may have a survival advantage in the presence of the antimicrobial agent and may subsequently become widespread in the absence of selective antimicrobial pressure.

The mechanisms by which genetic information is transferred from one bacterial cell to another include natural transformation, conjugation and transduction (known collectively
as horizontal gene transfer). Transformation involves the lysis of bacterial cells and the uptake of the released naked DNA from the surrounding environment by a competent recipient cell. Studies have found that *H. pylori* is naturally competent for DNA uptake (Nedenskov-Sorensen et al., 1990) and this may be an important mechanism that contributes to genetic diversity. The transfer of DNA may also involve conjugation whereby physical contact between two bacterial cells permits the unidirectional transfer of DNA from a donor to a recipient cell. In this instance, the ability to conjugate is dependent on the carriage of an appropriate plasmid or transposon by the host cell. Studies by Kuipers et al. (1998) have identified a conjugation-like mechanism of DNA transfer in *H. pylori*. The third mechanism, namely transduction, involves a bacteriophage vector but to date, this mechanism has not been reported in *H. pylori*.

1.14.2.2. Mutational resistance

Within a bacterial population spontaneous events in the DNA replication process, *e.g.*, deletions, additions or substitutions of one or a few nucleotide bases, may occur at a frequency of between $10^{-2}$ and $10^{-10}$ per cell division. These mutational events may lead to spontaneous resistance in a small proportion of the bacterial population. Such mutants may have a survival advantage in the presence of the antimicrobial agent, whereas the susceptible population would be killed. In the subsequent absence of selective antimicrobial pressure, the surviving resistant cells may proliferate such that they are representative of the population as a whole.

1.14.3. Clinical definitions of resistance

Primary resistance refers to the selection of resistant mutants (spontaneous mutants) already present within a mixed population of an individual strain at the onset of antimicrobial therapy. Secondary resistance refers to the emergence of resistance in a
previously sensitive bacterial population through spontaneous mutation after the introduction of an antimicrobial agent (Goddard and Logan, 1996). However, in the clinical setting, acquired resistance may be considered as ‘primary’ to indicate that resistance has arisen due to selection by previous exposure to an antimicrobial that is to be considered for treatment, even if the patient has no recollection of taking the drug. Hence, it is essential in initiating a therapeutic regimen that adequate concentrations of the antimicrobial agent are achieved at the site of infection to prevent the emergence of resistance.

In order to prevent the rapid development of resistance, the current PPI-based triple therapy regimens employed for *H. pylori* eradication consist of a combination of two antimicrobial agents with different modes of action and consequently, independent mechanisms of resistance. However, poor patient compliance is an important cause of treatment failure and contributes to the emergence of resistant strains (Graham *et al.*, 1992).

**1.14.4. *H. pylori* resistance to macrolides**

Macrolide resistance (erythromycin and clarithromycin) has been reported among *H. pylori* isolates.

**1.14.4.1. Epidemiology of resistance**

The prevalence of primary clarithromycin resistance is generally low and may reflect the use of this agent and the newer macrolides, such as spiramycin, roxithromycin and azithromycin, for the treatment of respiratory infections and sexually transmitted diseases. The prevalence rates of resistance in European studies vary from 0 to 10%, *e.g.*
10% in France (Méraud, 1997a), whereas in the USA, the prevalence rate is estimated to be 7%.

1.14.4.2. Mechanism of resistance

Erythromycin, clarithromycin and azithromycin are among some of the members of the macrolide group. These antibiotics bind to the 50S ribosomal subunit and consequently inhibit polypeptide chain elongation. The mechanisms by which resistance to macrolides can arise are (a) by inactivation or destruction of the antibiotic, (b) by alteration of the target site thereby reducing or eliminating the binding of the antibiotic to the target site, or (c) by a reduction of the cell surface permeability or inhibition of the mechanism by which the antibiotic enters the cell (for reviews see Eady et al., 1990; Weisblum, 1998).

In *Escherichia coli*, target site modification is mediated by the action of *N*-methyltransferase, an enzyme that specifically mono- or di-methylates the *N*'-amino group of adenine at residue 2058 in the 23S rRNA, leading to reduced binding affinity of erythromycin to its binding site on the ribosome (see Fig. 1.3a). The *erm* (erythromycin ribosomal methylase) gene that encodes for this methylase was first described in *Staphylococcus aureus* and has since been identified in a number of Gram-negative and Gram-positive microorganisms.

Target site modification can also arise due to point mutations in domain II and domain V of the 23S rRNA of *E. coli*. A specific A-to-G point mutation at position 2058 in domain V of the 23S rRNA reduces the binding affinity of erythromycin to the 50S ribosomal subunit. Point mutations in domain V of the 23S rRNA at two positions cognate to positions 2058 and 2059 in *E. coli* (see Fig. 1.3b), which were named 2143 and 2144 and are now revised as 2142 and 2143, respectively (Taylor et al., 1997), confer
Figure 1.3. Comparison of the domain V loops of the 23S rRNA molecules of E. coli and H. pylori strain UA802.

The adenine residues within the 23S rRNA molecules of (a) E. coli and (b) H. pylori strain UA802 associated with erythromycin resistance and clarithromycin resistance, respectively, are indicated by the numbers and arrows. Adapted from Taylor et al., 1997.
clarithromycin resistance in *H. pylori* (Versalovic *et al.*, 1996; Stone *et al.*, 1996; Debets-Ossenkopp *et al.*, 1996; Hultén *et al.*, 1997; Occhialini *et al.*, 1997; García-Arata *et al.*, 1999). The point mutation can be a transition (A-to-G) or a transversion (A-to-C), but the former is much more frequent.

Goldman *et al.* (1994) confirmed that the 50S ribosome of *H. pylori* is the target site for erythromycin, clarithromycin and its 14-hydroxy metabolite. Moreover, studies by Occhialini *et al.* (1997) found that virtually no binding of erythromycin was observed with 70S ribosomes derived from clarithromycin-resistant *H. pylori* strains. Clarithromycin resistance is associated with cross-resistance to other chemically related members of the macrolide group and can be explained by the fact that these agents have a similar structure and mode of action and thus mechanism of resistance. Furthermore, cross resistance to macrolides, lincosamides and streptogramin (MLS) antibiotics also occurs. These agents are chemically distinct but they have a similar resistance mechanism and have overlapping but not identical binding sites on the ribosome. Two types of cross resistance have been identified in *H. pylori*. Studies by Wang and Taylor (1998) have demonstrated that *H. pylori* isolates possessing the A-to-G 2142 point mutation were associated with high-level cross resistance to all of the MLS antibiotics (type I), and those strains with the A-to-G 2143 mutation were associated with an intermediate level of resistance to clarithromycin and clindamycin but no resistance to streptogramin B (type II).

The number of rRNA genes is an important determinant for the expression of the 23S rRNA point mutation and consequently the selection of resistant mutants. In the presence of a high number of 23S rRNA genes where only one 23S rRNA gene possesses the mutation, the expression of the mutation may be masked. A low 23S rRNA gene copy
number (e.g. n=1) enables the selection of resistant mutants in the presence of selective antibiotic pressure, whereas the resistance mutation is recessive in most organisms that possess >1 23S rRNA gene. *H. pylori* possesses two copies of the 23S rRNA gene which are located in separate regions in the genome (Bukanov and Berg, 1994; Tomb *et al.*, 1997; Taylor *et al.*, 1997; Alm *et al.*, 1999). Furthermore, studies have found that a small minority of *H. pylori* strains may be heterozygous for the mutation, i.e. only one allele is mutated (Versalovic *et al.*, 1996; Stone *et al.*, 1997). Therefore, in the majority of the *H. pylori* population, mutations in both copies of the 23S rRNA gene are required to confer clarithromycin resistance. In contrast, studies of *Streptomyces ambofaciens*, which has four copies of the rRNA gene, have found that a point mutation in only one of these genes appears to be sufficient to confer phenotypic resistance (Weisblum, 1998).

### 1.14.5. *H. pylori* resistance to nitroimidazoles

*H. pylori* resistance to nitroimidazoles, including metronidazole and tinidazole, has been reported and, akin to macrolides, cross resistance occurs between the members of the nitroimidazole group.

#### 1.14.5.1. Epidemiology of resistance

The prevalence of primary nitroimidazole resistance is related to its use in the local community and as a consequence prevalence rates vary enormously both within and between from countries (Glupczynski *et al.*, 1990; Glupczynski, 1992; Banatvala *et al.*, 1994). In general, metronidazole resistance is more prevalent in developing countries, with resistance rates reported to be as high as 90% because it is widely prescribed to patients with parasitic diseases such as giardiasis, amoebiasis and other protozoan infections. In European countries metronidazole resistance rates range from 10 to 50% (Glupczynski, 1992). Furthermore, metronidazole resistance is more prevalent in women
than in men which may be related to the use of metronidazole for gynaecological conditions (Weil et al., 1990; Banatvala et al., 1994).

Metronidazole resistance in \textit{H. pylori} develops rapidly \textit{in vitro} (Haas et al., 1990) and \textit{in vivo} (Goodwin et al., 1988; Rautelin et al., 1994). Although there have been few surveillance studies documenting metronidazole resistance over time, it appears that the prevalence of metronidazole-resistant \textit{H. pylori} strains is increasing (Banatvala et al., 1994). A five-year study in Dublin found a marked increase in the prevalence of metronidazole-resistant \textit{H. pylori} from 31.8% in 1991 to 46.3% in 1995 (Xia et al., 1996b). However, this increase was not observed in a six-year prospective study conducted in Belgium where metronidazole resistance was 29% in 1990 – 1992 and 26% in 1993 – 1995 (De Koster et al., 1996).

\subsection{1.14.5.2. Mechanism of resistance}

Entry of the nitroimidazole into the bacterial cell allows the reduction of the nitro group of the imidazole ring and the formation of an active short-lived hydroxylamine derivative. This radical induces strand breakage in the bacterial DNA that subsequently leads to cell death (for reviews see Edwards, 1993; Samuelson, 1999). Metronidazole resistance in \textit{H. pylori} has been explained by a null mutation in a gene (rdxA) encoding oxygen insensitive NADPH nitroreductase (Goodwin et al., 1998).

\subsection{1.14.6. \textit{H. pylori} resistance to fluoroquinolones}

Ciprofloxacin was once employed for the treatment of \textit{H. pylori} infection but this agent is no longer used due to the rapid emergence of resistant strains following treatment.
1.14.6.1. Mechanism of resistance

Ciprofloxacin resistance in *E. coli*, *C. jejuni* and *H. pylori* arises due to mutations in the *gyrA* gene which encodes GyrA, the A-subunit of DNA gyrase. The enzyme consists of a tetramer composed of two subunits A and B and functions by introducing negative-superhelical turns into DNA to allow DNA replication and RNA transcription to take place. The amino-terminus of the GyrA protein contains a determining region for quinolone resistance and mutations in this region at amino acids 67 – 106 lead to high-level quinolone resistance.

1.14.7. *H. pylori* resistance to other antibiotics

Reports documenting tetracycline resistance in *H. pylori* have appeared in the literature but, to date, nothing is known about the mechanism of resistance in *H. pylori* (Midolo *et al.*, 1996a). However, of concern is the recent study by van Zwet *et al.* (1998) describing the emergence of *H. pylori* strains with stable resistance to amoxicillin. The actual mechanism of β-lactam resistance in *H. pylori* is unknown, although it is likely that the mechanism may involve either a decrease in outer membrane permeability or an alteration in the penicillin-binding proteins (Krishnamurthy *et al.*, 1999; Dore *et al.*, 1999a). In theory, enzymic inactivation of the antibiotic is another possible mechanism of resistance but this would require the acquisition of a β-lactamase gene by means of horizontal gene transfer from other bacterial species. Indeed, the published studies have found that this is an unlikely mechanism of penicillin resistance since β-lactamase was not detected in amoxicillin-resistant strains of *H. pylori* by the nitrocephin assay (van Zwet *et al.*, 1998; Dore *et al.*, 1999b).

The studies by Dore *et al.* have identified *H. pylori* strains with unstable tolerance to amoxicillin which is defined as “the decreased bacterial killing by growth-inhibiting
concentrations of the drug” (Dunn et al., 1997; Dore et al., 1997). Moreover, clinical studies have found that amoxicillin resistance is an important factor affecting clinical efficacy (Dore et al., 1998b).

There is concern that the indiscriminate use of antibiotics for *H. pylori* eradication may lead to widespread resistance in the environment. The available scientific data emphasises the importance of *H. pylori* susceptibility testing in clinical practice. Thus, local surveillance programmes are needed to monitor the evolution of *H. pylori* resistance to antimicrobial agents utilised in eradication regimens.

1.15. *H. pylori* Genome

The genomes of two *H. pylori* strains, namely, strains 26695 and J99, have been sequenced (Tomb et al., 1997; Alm et al., 1999). The *H. pylori* genome consists of a relatively small single circular chromosome of 1.7 Mb size compared with those of *E. coli* (4.6 Mb) and *Pseudomonas aeruginosa* (5.8 Mb). In strains 26695 and J99, the *H. pylori* genome is approximately 1.67 Mb and 1.64 Mb with 1590 and 1495 predicted open reading frames respectively, two-thirds of which are of known function.

*H. pylori* possesses few regulatory genes of the type that co-ordinate the expression of genes by an 'ON/OFF’ switch and this suggests that the pathways that are required for its survival are continually ‘switched on’ (Covacci et al., 1999). The mechanisms that allow an organism to adapt to changes in its environment, *e.g.*, pH, temperature and osmolarity, include the stringent response and two-component regulatory systems, which are absent and rare, respectively, in the *H. pylori* genome sequence. Only four two-component regulatory systems were identified in the *H. pylori* genome compared with, for example,
90 in *Ps. aeruginosa*, an organism that is able to survive in a number of different environments (Covacci *et al.*, 1999). This suggests that *H. pylori* has evolved to inhabit one environment, the human stomach. *H. pylori* has well developed systems for motility, chemotaxis and iron acquisition. Indeed, with respect to the latter, a number of genes coding for iron-scavenging pathways were identified in the genome sequence. This highlights the critical role for iron in the survival of *H. pylori* in the stomach. A well developed restriction and modification system also exists that is involved in the breakdown of foreign DNA.

### 1.15.1. Genetic diversity

*H. pylori* exhibits variation in genome size and the presence or absence of plasmids. Studies have found that plasmids are present in about 45% of *H. pylori* strains and their sizes range from 1.8 to 63 kbp (Kleanthous *et al.*, 1991).

Comparison of the genomic sequence of strain 26695 (Tomb *et al.*, 1997), recovered from a patient with gastritis, with the genome sequence of strain J99 (Alm *et al.*, 1999), recovered from a patient with duodenal ulcer, has provided important information concerning the structure and order of genes in *H. pylori*. Of interest is the finding that there is a highly conserved gene order between these two virulent strains. This contrasts with pulsed-field gel electrophoresis (PFGE) studies which found that the variability of gene order observed between five *H. pylori* strains was a factor that contributed to genetic diversity (Taylor *et al.*, 1992; Jiang *et al.*, 1996). Sequence analysis showed that the variation observed with the PFGE and PCR-restriction fragment length polymorphism (PCR-RFLP) techniques is probably due to silent mutations (*i.e.*, mutations that are not associated with phenotypic change) and that these methods probably overestimate the extent of genetic diversity in *H. pylori* (Alm *et al.*, 1999).
variation detected in the genome sequence between strains 26695 and J99 is due to DNA
inversion and translocation and to changes at the third base of codons. These events
modify the DNA but do not modify the protein. Indeed, comparison of the two genome
sequences has found that only 7% of the proteins are unique to one strain.

The mechanisms involved in genomic diversity include nucleotide mutations, the
presence or absence of non-conserved DNA, e.g. the cag-PAI, the transposition of
insertion elements, recombination with DNA from other H. pylori strains and horizontal
gene transfer of new genes (Marshall et al., 1998; Kersulyte et al., 1999). Genomic
diversity in H. pylori has been observed within individual conserved genes (for reviews
see Logan and Berg, 1996; Marshall et al., 1998), e.g., vacA gene mosaicism (see section
1.8.2.2), internal duplication and truncation in the vacA gene (Ito et al., 1998) and
reassortment of sequences in the flaA and flaB genes that encode the flagellin (Forbes et
al., 1995) and in the ureA, ureB and ureC urease structural genes (Kansau et al., 1996).

The generation of genetic diversity may also be the result of the exchange of DNA
between different strains by horizontal gene transfer (see section 1.14.2.1). Moreover,
regions within the H. pylori genome that are organised differently and possess
significantly lower %G+C content compared to the rest of the genome suggest that these
regions have been acquired by horizontal DNA transfer. The cagA-PAI (see section
1.8.6) is an example and this region has been identified in the two published genome
sequences of H. pylori.

The presence of repetitive DNA sequences and insertion sequences may allow
recombination events that result in chromosomal rearrangements, thereby contributing to
genetic diversity in H. pylori. Recombination within the genes encoding the outer
membrane proteins may be a putative mechanism that leads to antigenic variation.
Variation in the expression of the surface antigens may be a means by which *H. pylori* evades the host immune response (for review see Henderson *et al.*, 1999). Furthermore, the presence of homopolymeric tracts and dinucleotide CT or AG repeats, particularly in the genes encoding outer-membrane proteins, suggests that slipped-strand mispairing may be another mechanism that mediates phase variation in *H. pylori* (Marshall *et al.*, 1998). Slippage within DNA repeats results in the frequent shifting into and out of frame relative to the upstream translation initiation codon and this leads to ON-OFF switching of the gene product. This mechanism may form the basis of LPS phase variation in *H. pylori* (Appelmelk *et al.*, 1998). The O-antigen of the LPS of *H. pylori* strains contains polymorphic fucosylated glycoconjugates that are identical to the Lewis x and Lewis y antigens expressed on human gastric epithelial cells and erythrocytes. The presence of a 14-C homopolymeric tract in the α-1,2-fucosyltransferase gene, which is required for the formation of the Lewis y epitope, suggests that slipped-strand mispairing may result in the altered expression of Lewis y antigen (Marshall *et al.*, 1998; Saunders *et al.*, 1998; Alm *et al.*, 1999). The molecular mimicry exhibited between the LPS of *H. pylori* and host Lewis antigens may allow the bacterium to evade the host immune response.

1.15.2. Molecular genotyping analysis of *H. pylori* strains

Several molecular typing systems have been employed to characterise strains of *H. pylori* and have provided important information regarding population genetics. Typing techniques must be discriminatory, reproducible, standardised, rapid, easy to perform and cost-effective to allow accurate interpretation of the typing data (Struelens, 1996). Among the DNA fingerprinting techniques that have been employed in epidemiological studies, oligonucleotide fingerprinting (Marshall *et al.*, 1996a) and the PCR-based methods such as random amplified polymorphic DNA-PCR (RAPD-PCR) analysis (Akopyanz *et al.*, 1992) and repetitive element-PCR (Rep-PCR) appear to be the most
discriminatory for *H. pylori* isolates from unrelated individuals. *Hind*III-restriction fragment length polymorphism (RFLP) (Langenberg *et al.*, 1986), PCR-RFLP analysis of the urease gene of *H. pylori* (Foxall *et al.*, 1992; Clayton *et al.*, 1993; Fujimoto *et al.*, 1994), ribotyping (Rautelin *et al.*, 1994; Owen *et al.*, 1994) and amplified-fragment length polymorphism (AFLP) (Gibson *et al.*, 1998) are among the techniques that have been applied in clinical studies to discriminate between relapsing infection and re-infection. However, epidemiological analysis of unrelated *H. pylori* isolates using ribotyping and PCR-RFLP has lower discriminatory power. However, it is apparent that *H. pylori* is highly heterogeneous, to such an extent that the fingerprint profiles generated from *H. pylori* isolates from different individuals are unique by most of the genotyping methods.

1.15.2.1. Restriction endonuclease analysis (REA)

REA involves the complete cutting (restriction) of intact chromosomal or plasmid DNA with one or more endonucleases. The fragments of cleaved DNA are separated by agarose gel electrophoresis and the resulting band profile is visualised by staining the gel with ethidium bromide. However, this method has several limitations in that the fingerprints generated are complicated and interpretation within a single gel is subjective (intra-gel analysis). Furthermore, comparison of fingerprints between different gels (inter-gel analysis) is complicated and limits the applicability of this method for the analysis of a large number of isolates.

1.15.2.2. Oligonucleotide fingerprinting

The eukaryotic genome contains multiple stretches of repetitive DNA sequence motifs. Oligonucleotide probes of complementary sequences, including (GACA)$_n$, (GGAT)$_n$, (GATA)$_n$, (GTG)$_n$, and (GT)$_n$, have been employed in fingerprinting among *Candida*
species (Sullivan et al., 1993), and in prokaryotic microorganisms such as *Mycobacterium tuberculosis* (Wild et al., 1994) and *H. pylori* (Marshall et al., 1996a).

Oligonucleotide fingerprinting initially involves REA followed by transfer of the cleaved DNA to a nylon membrane by Southern transfer. The DNA fragments are fixed to the membrane and the DNA fingerprint is visualised by hybridisation with either a non-radioactive or radioactive labelled oligonucleotide probe of repetitive DNA sequence. The technique is reproducible and discriminatory and generates hybridisation profiles containing 16 – 35 bands. Unique DNA fingerprints differing by at least four hybridisation bands are generated from *H. pylori* isolates recovered from unrelated individuals (Marshall et al., 1996a). However, this method is labour-intensive and technically more demanding compared to other simpler and rapid fingerprinting methods.

1.15.2.3. RAPD-PCR

The RAPD-PCR technique or arbitrarily primed PCR (AP-PCR) is a polymerase chain reaction-based fingerprinting method simultaneously developed by Williams et al. (1990) and Welsh and McClelland (1990) and later applied by Akopyanz et al. (1992) to *H. pylori* analysis. The RAPD technique is performed under low stringency conditions to allow random annealing of the primer (or combination of primers of arbitrary sequence) to the DNA on both strands which by chance match or almost match the sequence of the primer. Primers that have annealed within a few kilobases of each other initiate DNA polymerisation and are amplified by the PCR. The nature of the technique is such that it may be applied to a number of different species for which there is limited genome sequence information available.
1.15.3. Multiple strain colonisation

The evidence for multiple strain colonisation has been provided by molecular typing studies of *H. pylori* isolates from consecutive biopsies in individual patients and multiple single colony isolates from biopsy specimens from one or multiple gastric sites. Some studies have found that a single infecting strain is present at one site in the stomach (Marshall *et al.*, 1995; Salama *et al.*, 1995) while other studies have found that the majority of patients are infected with a predominant strain. However, a review of the literature suggests that approximately 5 to 20% of patients harbour subpopulations of a predominant strain at one site (Oudbier *et al.*, 1990; Taylor *et al.*, 1995; Weel *et al.*, 1996; van der Ende *et al.*, 1996) or at different sites in the stomach (Cellini *et al.*, 1996; Jorgensen *et al.*, 1996; Dore *et al.*, 1998a; Miehlke *et al.*, 1999). PFGE analysis of single colonies of *H. pylori* isolated from multiple biopsy specimens from the antral and corporal gastric sites found that 6% of patients were infected with different *H. pylori* strains (Hirschl *et al.*, 1994). However, most of these studies did not define the number of band differences required to define an isolate as a subtype or a different strain. Nonetheless, it would appear that the majority of patients are infected with a predominant *H. pylori* strain and that some patients harbour subtypes, *i.e.*, the DNA fingerprints generated are very similar to those generated by the predominant strain, while the frequency of multiple genotypically different strains is low.
1.16. Aims and Objectives

The purpose of the present study was as follows;

(1) To assess the feasibility of culturing *H. pylori* from biopsies used for the CLOtest in untreated patients.

(2) To evaluate a 16S rRNA gene PCR method for the detection of *H. pylori* in paraffin-embedded gastric biopsy specimens in both untreated and post-treatment patients and to assess the accuracy of conventional tests for the diagnosis of *H. pylori* in these two patient groups.

(3) To determine the prevalence of metronidazole, clarithromycin and amoxicillin resistance among clinical isolates of *H. pylori* by the E-test method for susceptibility and to evaluate the E-test and disc-diffusion methods for susceptibility testing of *H. pylori* for clarithromycin by comparison with the reference agar dilution method.

(4) To determine whether recurrence of *H. pylori* infection was due to reinfection or recrudescence by molecular characterisation of the pretreatment and recurrent isolates from patients who had been treated with *H. pylori* eradication therapy and, with reference to the susceptibility testing data of these isolates, determine whether acquired antimicrobial resistance was due to *in vivo* strain selection.

(5) To investigate the mechanism, frequency and stability of point mutations in the 23S rRNA gene that confer clarithromycin resistance among paired Irish *H. pylori* isolates, to determine the association, if any, of these mutations with the level of clarithromycin resistance, to investigate cross-resistance among macrolides, and to determine the
possible relationship between the types of point mutations observed and the treatment regimen prescribed.
Chapter 2

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2.1. Primary Isolation of \textit{H. pylori} from Gastric Biopsies

Clinical \textit{H. pylori} isolates were obtained from gastric biopsy specimens by Dr Huaxiang Xia (St. James’s Hospital, Dublin) prior to July 1995 from patients undergoing routine upper gastrointestinal endoscopy at the Meath and Adelaide Hospitals, Dublin, Ireland. Endoscopic procedures were performed by the attending registrars, Department of Gastroenterology, Meath and Adelaide Hospitals. Biopsies were inoculated, mucosal side downwards, on to the surface of two fresh blood agar plates consisting of Columbia agar base (LAB M, UK) supplemented with 7\% horse blood (Jones, 1984). Plates were subsequently incubated at 37 °C for 4 – 7 days under microaerophilic conditions by flushing the anaerobic jars with CO$_2$. \textit{H. pylori} was identified by a Gram stain of typical small translucent colonies and by urease activity (Christensen’s agar slopes containing 40\% urea; Oxoid Ltd., Basingstoke, UK).

2.1.1. Storage and recovery of \textit{H. pylori} isolates

\textit{H. pylori} isolates were subcultured in duplicate on to blood agar and incubated under microaerophilic conditions for 3 days. Using a sterile loop, the bacterial colonies were harvested and placed into a sterile 1.5 ml freezer vial (Sarstedt, Germany) containing 1 ml of storage medium consisting of Brain Heart Infusion broth (Oxoid Ltd., UK), 20\% (v/v) glycerol and 10\% (v/v) filtered horse serum (Oxoid Ltd., UK). Storage vials were briefly vortexed and then stored at -70 °C.

When required, storage vials were removed from the freezer and allowed to defrost at room temperature. To aid bacterial recovery, 10 μl of the suspension was removed with a sterile loop and four 2-cm$^2$ areas of two blood agar plates were inoculated. Plates were incubated for 4 – 7 days and subcultured at least twice before use.
2.2. DNA Extraction

2.2.1. Genomic DNA

H. pylori DNA was purified according to the method described by Ausubel et al. (1987) with some modifications. Bacterial growth was harvested from 3 day-old blood agar plates and resuspended in 1 ml phosphate buffered saline pH 7.3. The bacterial cells were pelleted by centrifugation at 14,000 r.p.m. for 5 min using a bench-top microcentrifuge. This procedure was repeated and the pellet was resuspended in 567 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) to which 30 µl of 10% SDS (w/v) and 3 µl of proteinase K (20 µg/ml) were added. The suspension was incubated at 37 °C in a waterbath. After 1 h, 100 µl of 5 M NaCl was added and the suspension was mixed thoroughly. Then 80 µl of cetyltrimethyl ammonium bromide (CTAB)-NaCl solution [10% (w/v) CTAB in 0.7 M NaCl] was added and the suspension was incubated for 10 min at 65 °C in a waterbath. An equal volume of chloroform:isoamyl alcohol (24:1) was then added to the solution, mixed thoroughly and centrifuged at 14,000 r.p.m. for 5 min. The upper aqueous layer containing the DNA was collected and an equal volume of phenol:chloroform:isoamyl alcohol (24:24:1) was added. The solution was thoroughly mixed, centrifuged and the aqueous layer was collected as before. Isopropanol (0.6 vol) was added and the microcentrifuge tube was gently inverted to precipitate the DNA. The DNA was pelleted by centrifugation in a bench-top centrifuge for 15 min at 4 °C. The pellet was washed twice with 1 ml of 70% (v/v) ethanol and the pellet air-dried for 15 min. The DNA was resuspended in 50 µl of sterile distilled water overnight and the amount of DNA recovered was quantitated by agarose gel electrophoresis using lambda DNA (Promega, USA), between the range of 0.06 – 1µg. The DNA was stored at -20 °C until required.
2.2.2. Plasmid DNA

Transformed *E. coli* (see section 6.2.4) were grown overnight at 37 °C in 3 ml of L-broth medium (tryptone, 10 g/l; yeast extract, 5 g/l; NaCl, 5 g/l) supplemented with 50 µg of ampicillin (Sigma, USA) per ml in an orbital shaker at 250 r.p.m. Cells were pelleted at 2, 500 r.p.m. for 5 min in an IEC Centra GP8 centrifuge. Recombinant plasmid DNA was purified using a Qiagen plasmid mini-kit (Qiagen Ltd., UK) according to the manufacturer's instructions. The purified plasmid DNA was washed twice with 70% ethanol and the amount of DNA isolated was quantitated by agarose gel electrophoresis using a DNA molecular mass ladder (Boehringer Mannheim GmbH, Germany).

2.3. Agarose Gel Electrophoresis

DNA was routinely separated in 0.7 – 2% (w/v) agarose gels in 0.5 X Tris borate-EDTA (TBE) buffer (0.045 M Tris, 0.045 M boric acid, 0.01 M EDTA, pH 8.0) containing 0.5 µg of ethidium bromide per ml. To separate PCR products, 2% agarose gels were used whilst 0.7 to 1% agarose gels were used to separate restriction-digested DNA fragments and total genomic DNA. The DNA was visualised using an ultraviolet (UV) light transilluminator (Chromato-vue, Model TL-33, UVP Inc., CA, USA).

2.4. Southern Transfer from Agarose Gels

2.4.1. Southern transfer of restriction endonuclease-digested *H. pylori* DNA

Southern blot transfer was performed using standard techniques (Sambrook *et al.*, 1989). Genomic DNA (2 – 5 µg) was digested with the appropriate restriction enzyme according to the method of Sambrook *et al.* (1989) and incubated at the temperature recommended by the manufacturer. Digested DNA samples and molecular weight markers (1 kb plus DNA ladder, Gibco BRL, USA.; Digoxigenin-labelled DNA marker III, Boehringer Mannheim GmbH, Germany) were separated by electrophoresis on a 0.7 – 1% agarose
gel at 30 V overnight. The DNA was visualised under UV light and the position of the markers was indicated by perforating the gel with a sterile pin. The DNA in the agarose gel was depurinated for 10 min in several volumes of 0.2 M HCl with gentle agitation. Then the DNA was denatured by submerging the gel in denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 30 min with gentle shaking. This step was repeated for 15 min with fresh denaturation solution. The gel was then rinsed in distilled water and the DNA was neutralised by submerging the gel in neutralisation solution (1 M Tris-HCl, pH 7.4, 1.5 M NaCl) for 30 min with gentle agitation. This step was repeated for 15 min and the DNA was transferred to a Hybond-N* (Amersham) nylon membrane by capillary action with 20 X SSC (3 M NaOH, 0.3 M sodium citrate) according to the method of Southern. Following overnight transfer, the DNA was fixed to the membrane with 150 mJ UV light using a UV crosslinker (Bio-Rad, CA, USA) and then the membrane was stored wet in 2 X SSC until required.

2.4.2. Southern transfer of PCR products

PCR products and a 100 bp DNA ladder (Gibco BRL, USA) were separated in a 2% (w/v) agarose gel at 4 – 6 V per cm for 2 to 4 h. The PCR products were visualised under UV light and the position of the markers was indicated by perforating the gel with a sterile pin. The DNA in the agarose gel was denatured, neutralised and transferred to a MagnaGraph (MSI) nylon membrane as described above. The DNA was fixed to the membrane by baking at 85 °C for 2 h.

2.5. Purification of PCR Products from Agarose Gels

PCR amplification products were separated on a 2% agarose gel in 1 X Tris-acetate (TAE) buffer [0.4 M Tris, 1.142 ml (v/v) glacial acetic acid, 0.05 M EDTA, pH 8.0] and visualised under low intensity UV light. Using a sterile scalpel, the desired fragment was
excised from the gel and the DNA was purified using the Gene Clean II kit (Bio 101 Inc., USA) according to the manufacturer's instructions.

2.6. Labelling of DNA Probes

2.6.1. End-labelling of DNA probes with radionucleotide

The oligonucleotide (50 ng) was end-labelled with \([\gamma^{32}\text{P}]\) dATP to a specific activity of >10^6 d.p.m. per \(\mu\text{g}\) using T\(_4\) polynucleotide kinase (Promega, USA) at 37 °C for 45 min. The enzyme reaction was stopped by heating the reaction mixture at 68 °C for 10 min.

2.6.2. End-labelling of DNA probes with Digoxigenin (DIG) ddUTP

The oligonucleotide (100 pmol) was 3'- end-labelled with Digoxigenin-ddUTP using a DIG oligonucleotide 3'-end labelling kit (Boehringer Mannheim GmbH, Germany) according to the manufacturer's instructions.

2.6.3. Random Prime labelling of DNA probes with Digoxigenin

DNA was labelled with Digoxigenin-11-dUTP using the DIG-High Prime reagent (Boehringer Mannheim GmbH, Germany) according to the manufacturer's instructions. The DNA template (16 \(\mu\text{l}\) containing 1 \(\mu\text{g}\) DNA) was denatured by boiling at 100 °C for 10 min and then it was rapidly cooled on ice for 10 min. The DNA template was removed to a clean Eppendorf tube and 4 \(\mu\text{l}\) of DIG-High Prime reagent was added. The mixture was incubated at 37 °C for at least 5 to 12 h depending on the length of the DNA, and the reaction was stopped by the addition of 2 \(\mu\text{l}\) of EDTA (200 mM, pH 8.0) or heating at 65 °C for 10 min.
2.7. DNA-DNA Hybridisation

2.7.1. Hybridisation with radio-labelled probes

PCR products that had been transferred to a nylon membrane by Southern transfer were submerged in 6 X SSC for 30 min and prehybridised for 30 min in 5 X SSPE buffer (0.75 mol NaCl/l, 45 mmol NaH₂PO₄/l and 5 mmol EDTA/l), 0.1% (w/v) SDS, 5 X Denhardt’s solution (Sambrook et al., 1989) and 100 µg of denatured salmon sperm DNA. An end-labelled oligonucleotide probe (5 ng) was added to the prehybridisation solution and membranes were hybridised at 57 °C. After 3 h, membranes were washed once in 6 X SSC for 30 min followed by one wash in 2 X SSC for 20 min at room temperature.

2.7.2. Hybridisation with DIG-labelled probes

Restriction endonuclease-digested *H. pylori* DNA that had been transferred to a nylon membrane by Southern transfer was hybridised with the appropriate DIG-labelled DNA probe. The membrane was pre-hybridised in hybridisation solution [5 X SSC (0.75 mol NaCl/l, 75 mmol sodium citrate/l), 0.1% (w/v) *N*-lauroylsarcosine, 0.02 % (w/v) SDS, 1% blocking reagent (Boehringer Mannheim GmbH, Germany)] for 2 h at 68 °C. The solution was then replaced with fresh hybridisation solution containing the DIG-labelled probe which had been previously heat denatured at 100 °C for 10 min and cooled on ice for 10 min. Hybridisation was continued at 68 °C overnight. The membrane was subsequently washed twice in 2 X SSC with 0.1% for 15 min at room temperature followed by two high-stringency washes in 0.1 X SSC containing 0.1% (w/v) SDS for 15 min.
2.8. Detection of Hybridised Probes

2.8.1. Autoradiography

Nylon membranes that had been hybridised with a radio-labelled probe were exposed to Cronex 10s film (Dupont) using two Quanta rapid intensifying screens (Dupont) for between 4 to 7 h at -70 °C. Films were developed according to the manufacturer's instructions.

2.8.2. Chemiluminescent detection

Nylon membranes that had been hybridised with DIG-labelled probes were developed using CSPD or CDP-Star according to the manufacturer's instructions. Membranes were then exposed to Lumi-Film (Boehringer Mannheim).

2.9. Removal of DNA Probes from Nylon Membranes

DIG-labelled DNA probes were removed from nylon membranes according to manufacturer's instructions. The end-labelled [$\gamma^{32}$P] dATP probes were removed from the nylon membranes by washing them twice in boiled 0.5% (w/v) SDS for 15 min.

2.10. Oligonucleotides and Sequencing of PCR Products

Oligonucleotides used for PCR amplification or as probes for DNA fingerprinting were synthesised by Genosys Biosystems, UK. Automated DNA sequencing of PCR products was performed by the sequencing service at MWG-Biotech UK Ltd., Milton Keynes, UK using a Licor sequencing system.
Chapter 3

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3.1 Introduction

Diagnosis of *Helicobacter pylori* infection is determined either by invasive endoscopy with histological, microbiological and rapid urease test analysis of gastric mucosal biopsies or by non-endoscopic methods, *e.g.*, serology or the urea breath test (see section 1.9). The CLOtest (Marshall *et al.*, 1987b) is a commercially available endoscopy-based urease test that is simple to use, cost-effective and provides a rapid diagnosis of the infection so that *H. pylori* eradication treatment may be prescribed before the patient leaves the endoscopy unit. The CLOtest consists of a gel that contains urea and a pH indicator (phenol red). A biopsy specimen is embedded in the gel and if urease is present, the urea in the surrounding gel is hydrolysed with a concomitant pH change secondary to ammonia production which changes the indicator from yellow to pink.

It is becoming increasingly important to culture *H. pylori* so that *in vitro* antimicrobial susceptibility testing of isolates may be performed. Susceptibility testing determines local antimicrobial resistance rates and guides empirical treatment, and is particularly recommended for second-line treatment where initial treatment has failed (EHP SG, 1997; Malfertheiner *et al.*, 1997). Culture of selected biopsy specimens that are known to be presumptively positive for *H. pylori* by a urease test and susceptibility studies on the isolates recovered might generate both accurate and cost-effective protocols for the diagnosis of *H. pylori* infection in clinical practice and would greatly facilitate patient management.

Each of the biopsy-based diagnostic tests is subject to sampling error and methodological limitations. Histological assessment using a haematoxylin and eosin stain is sensitive but this stain is not specific for *H. pylori*. The urease test and the Gram stain require a high number of organisms to yield a positive result. Culture requires 5 – 7 days but
contamination during isolation compromises sensitivity. The performance of these tests in clinical studies among untreated patients has been extensively documented (see Table 1.2 Schnell and Schubert, 1989; Cutler et al., 1995; Thijs et al., 1996; Andersen et al., 1998; Lerang et al., 1998). However, limited studies have addressed the accuracy of the conventional tests for the assessment of *H. pylori* eradication.

*H. pylori* eradication is routinely assessed four weeks after the end of treatment. This is an arbitrary time interval that would allow replication of the bacteria to numbers that can be detected by the conventional tests. Low numbers of bacteria may still be present at this time and detection of the infection will largely depend on the sensitivity of the routine tests. Thus, it is important to assess the accuracy of conventional tests in post-treatment patients, as the best test for the initial diagnosis of *H. pylori* infection is not necessarily the best test to assess eradication.

Recently, the polymerase chain reaction (PCR) has been employed for the detection of *H. pylori* DNA in clinical samples. Numerous studies have employed the 16S rRNA gene PCR method originally developed by Ho et al. (1991) for the detection of *H. pylori* in faeces, saliva, endoscope flushings and gastric biopsy specimens (Mapstone et al., 1993a; Morera-Brenes et al., 1994; Roosendaal et al., 1994; van Zwet et al., 1994; Namavar et al., 1995; Chong et al., 1996; Lu et al., 1999). However, few studies have assessed this PCR method for the detection of *H. pylori* in post-treatment patients where the sensitivity of conventional diagnostic tests may be compromised in the presence of low bacterial population levels.

The aims of this study were (i) to assess the feasibility of culturing *H. pylori* from biopsies used for the CLOtest from patients who had not previously received *H. pylori*
eradication therapy and (ii) to evaluate a 16S rRNA gene PCR method for the detection of *H. pylori* in paraffin-embedded gastric biopsy specimens in both untreated and post-treatment patients and (iii) to assess the accuracy of conventional tests for the diagnosis of *H. pylori* in these two patient groups.
3.2. Evaluation of Antral Biopsies Used in the Rapid Urease Test (CLOtest) for *H. pylori* Culture

3.2.1. Materials and methods

3.2.1.1. Patient population and study design

Patients attending the Meath and Adelaide Hospitals, Dublin, Ireland for the investigation of dyspepsia were enrolled. Endoscopic procedures were performed by the attending registrar of the respective gastroenterology units. Patients with a history of antibiotic use within the previous month and those taking proton pump inhibitors (PPI’s) or *H₂* receptor antagonists within the previous two weeks were excluded. At endoscopy, six gastric biopsy specimens were collected for *H. pylori* diagnosis in the following order: one antral biopsy for the rapid urease test (CLOtest®, Delta West Ltd, Australia); one antral biopsy for routine microbiology; and two antral and two corporal biopsies for histological assessment of haematoxylin and eosin stained sections. Forty-six consecutive patients with a positive CLOtest were included for the purposes of the study. In addition, 17 separate consecutive patients were selected on the basis of a negative CLOtest.

3.2.1.2. Determination of *H. pylori* status

To assess *H. pylori* urease activity, an antral biopsy specimen was placed into the CLOtest agar gel and inspected every 10 – 15 min, up to a maximum of 180 min, until the agar changed colour to either orange or red. In addition, biopsies from randomly selected negative CLOtest patients, following observation periods of 120 to 180 min, were also included in the study to identify possible false-negative tests. The time taken for the colour change to occur was recorded. The CLOtest biopsy was removed with a sterile needle and placed in a separate vial of nutrient broth and stored at 4 °C with the
routine antral biopsy. Biopsies were transported to the central laboratory and cultured by an experienced microbiologist within five hours.

Culture of both routine and CLOtest antral biopsies was performed by the author. Antral biopsies were inoculated in duplicate onto the surface of fresh blood agar plates (Jones, 1984). Plates were subsequently incubated at 37 °C for 4 – 7 days under microaerophilic conditions by flushing the anaerobic jars with CO₂. *H. pylori* was identified as small translucent colonies that were urease positive (Christensen's agar slopes containing 40% urea; Oxoid Ltd, Basingstoke, England). Culture was recorded as either positive or negative, and the presence of bacterial or fungal contamination was documented.

Gastric biopsies were fixed in buffered formalin, processed and paraffin-embedded sections were stained with haematoxylin and eosin for histological examination (this service was performed by the Department of Histopathology, St. James's Hospital, Dublin, Ireland). Gastritis was graded according to the Sydney classification (Price, 1991) and the presence of *Helicobacter*-like organisms (HLO) was documented.

3.2.1.3. Case definition

A patient was considered to harbour *H. pylori* when culture alone or the combination of the two routine biopsy-based tests, the CLOtest and histology, were positive. When one test other than culture was positive, the *H. pylori* status of the patient was classified as equivocal.

3.2.1.4. Statistical analysis

Proportional data were compared using two-tailed Fisher's Exact test (Bourke *et al.*, 1985).
3.2.2. Results

Forty-six patients were initially enrolled in the study but eight patients were excluded due to the recent use of PPI's or H₁ receptor antagonists. Of the remaining 38 patients (19 male, 19 female, age range 18 – 82 years; mean 46 years), nine (24%) of whom had duodenal ulcer, all were found to harbour \textit{H. pylori} according to the case definition. All of these patients were positive by the CLOtest (mean, 46 min; SEM, 6 min; range 10 – 180 min). Thirty-six patients were found by histological examination to harbour \textit{H. pylori} (see Table 3.1). Gastric atrophy was not observed in the remaining two patients (patients 24 and 33) in whom \textit{H. pylori} was not found by histological examination. The antral biopsy was the best specimen for the histological determination of \textit{H. pylori} infection. However, in three patients (patients 3, 36 and 38) the organism was observed only in the corporal biopsy specimen and in four patients (patients 11, 19, 21 and 28) the corporal biopsy was not available (see Table 3.1). The bacterium was successfully cultured from routine antral biopsies in 34 of these 38 (89%) patients, reflecting the local expertise with this diagnostic technique.

In the case of four routine biopsy specimens (patients 5, 7, 35 and 37; Table 3.1) from which \textit{H. pylori} was not isolated, histological examination did not reveal the presence of gastric atrophy. Contamination may have obscured the growth of \textit{H. pylori} in biopsy specimens from patients 5 and 35, but contamination was not observed in the routine biopsy specimen from patient 37. However, the organism was successfully isolated from the positive CLOtest biopsy of one of these four patients, namely from patient 7.

\textit{H. pylori} was successfully cultured from the biopsy specimen initially used for the CLOtest in 30 of the 38 (79%) patients. This isolation rate was not statistically different from that for the routine biopsies ($P = 0.172$, two-tailed Fisher's Exact test mid $P$).
Table 3.1 Correlation between the speed at which the CLOtest yielded a positive result and results from routine and CLOtest biopsy culture and histological analysis of gastric biopsy specimens from 38 *H. pylori*-positive patients.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>CLOtest</th>
<th>Culture&lt;sup&gt;a&lt;/sup&gt; of routine biopsy</th>
<th>Histological analysis&lt;sup&gt;b&lt;/sup&gt;</th>
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<tbody>
<tr>
<td></td>
<td>Time to positive result (min)</td>
<td></td>
<td>Antrum</td>
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<tr>
<td>1</td>
<td>10</td>
<td>+</td>
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Table 3.1. continued

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<td>180</td>
<td>+</td>
<td>+</td>
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<td>-</td>
</tr>
</tbody>
</table>

* +, *Helicobacter pylori* isolated; -, *Helicobacter pylori* not isolated

b +, *Helicobacter*-like organisms (HLO) observed on histological examination of haematoxylin and eosin stained gastric biopsy sections; -, no HLO observed

c Minimal contamination observed during isolation

d Extensive contamination observed during isolation

na, not available
Figure 3.1. Proportion of CLOtest-positive biopsy specimens from which *H. pylori* was isolated according to the time taken for the CLOtest to yield a positive result. ■, *H. pylori* isolated; □, *H. pylori* not isolated.
Twenty-one (55%) of the 38 CLOtest-positive biopsy specimens yielded a positive urease result at between 10 – 30 min and *H. pylori* was isolated from 16 (76%) of these biopsy specimens (Fig. 3.1). However, *H. pylori* was also isolated from a CLOtest-positive biopsy specimen that had taken >131 min to yield a positive result. In the 34 patients whose routine culture was positive, *H. pylori* was isolated from the positive CLOtest biopsy specimen in 29 (85%) patients (Table 3.1).

Four of the 17 consecutive patients, who were initially selected on the basis of a negative CLOtest, had recently taken PPI's or H₂ receptor antagonists and were excluded from the study. Of the remaining 13 patients with a negative CLOtest, 11 (85%) did not to have *H. pylori* infection, and one patient was of indeterminate infection status (positive histology). Culture of the CLOtest biopsy and the routine biopsy specimens were negative for these 12 patients. The remaining patient did have *H. pylori* infection (positive histology and routine culture), but the organism was not isolated from the CLOtest biopsy that had been removed at 120 min due to extensive contamination.
3.3. Evaluation of a DNA Amplification-Based Technique for H. pylori Diagnosis in Paraffin-Embedded Tissue

3.3.1. Materials and methods

3.3.1.1. Patient population and study design

This retrospective study was performed without prior knowledge of the patients’ H. pylori infection status. Patients attending the Meath and Adelaide Hospitals, Dublin, Ireland underwent endoscopy to obtain biopsies. Endoscopic procedures were performed by the attending registrars of the Gastroenterology units. DNA extraction (see section 3.3.1.3) and PCR (see sections 3.3.1.4 and 3.3.1.5) were performed on sections from consecutive paraffin-embedded histology blocks of gastric biopsies. At the end of the study, patients were divided into two groups according to treatment status; group A consisted of patients undergoing the investigation for dyspepsia who had not received H. pylori eradication treatment whereas group B consisted of post-treatment patients. Patients with gastric carcinoma and patients who had undergone gastric surgery were excluded from the study. The patients in group B received H. pylori eradication treatment that consisted of triple therapy with colloidal bismuth citrate for 4 weeks with metronidazole (400 mg t.i.d) and tetracycline (500 mg q.i.d) for two weeks or dual therapy with a proton pump inhibitor (omeprazole, 20 mg b.i.d) and amoxicillin (1 g b.i.d) for two weeks. At the end of the study, the PCR results were compared to the results of the conventional diagnostic tests.

A patient was considered to be infected with H. pylori when culture alone or a combination of two or more of the conventional tests, namely the CLOtest, Gram stain and histology, were positive. When one conventional test was positive, the patient was considered to be of equivocal H. pylori status. A 13C-urea breath test (13C-UBT) or
endoscopy to obtain biopsies was performed (range, 12 – 16 months; mean, 14.3 months) on consenting patients who were of equivocal *H. pylori* status, irrespective of the PCR results. These patients were considered to harbour *H. pylori* when the follow up $^{13}$C-UBT was positive or when two of the conventional tests performed at the repeat endoscopy were positive.

3.3.1.2. **Assessment of H. pylori status**

At endoscopy, biopsy specimens were collected from each patient to determine their *H. pylori* infection status. To assess *H. pylori* urease activity, an antral biopsy was placed into the CLOtest (Delta West, Western Australia) agar gel and the result was recorded up to 24 h after inoculation. For histological assessment, varying numbers of antral and corporal biopsy specimens were collected from patients (Table 3.2). Histological examination of haematoxylin and eosin stained sections was performed by the Department of Histopathology, St. James’s Hospital, Dublin, Ireland, according to the Sydney classification (Price, 1991). Microscopic examination was performed on a Gram stained antral tissue smear and the presence of curved Gram-negative bacilli was documented. The same biopsy was then inoculated onto a fresh chocolate blood agar plate (performed by the Department of Microbiology, St. James’s Hospital, Dublin, Ireland). Plates were incubated at 37 °C for 7 days under microaerophilic conditions by flushing the anaerobic jars with CO$_2$. *H. pylori* was identified as small translucent colonies that were urease positive.

3.3.1.3. **DNA extraction from paraffin-embedded tissue**

Five 10-$\mu$m sections were cut from consecutive paraffin-embedded gastric biopsy blocks using disposable microtome blades. After sectioning 20 gastric biopsy blocks, liver tissue and wax blocks (negative controls) were cut and processed in parallel to control for
Table 3.2. Numbers of antral and corporal biopsy specimens taken for the histological assessment of *H. pylori* status in untreated (group A) and post-treatment patients (group B) included in the study.

<table>
<thead>
<tr>
<th>Patient group</th>
<th>Numbers of patients from whom the indicated number of antral (a) and corporal (c) biopsies were taken at endoscopy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1a</td>
</tr>
<tr>
<td><strong>Group A</strong></td>
<td>23</td>
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<tr>
<td><strong>Group B</strong></td>
<td>4</td>
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</table>

*Group A, n=49 patients; group B, n=23 patients*
possible contamination during processing. Total DNA (human DNA and *H. pylori* DNA, if present) was extracted with some modifications (Wright and Manos, 1990). Sections were deparaffinised with xylene, rehydrated and incubated overnight with 200 µg proteinase K (Sigma, USA) per ml. DNA was extracted once with phenol:chloroform:isoamyl alcohol [23:1:1 (Sigma, USA)]. The DNA was precipitated with absolute ethanol and pelleted by centrifugation at 14,000 r.p.m. in a bench-top centrifuge. The DNA pellet was washed with 70% ethanol and resuspended in 25 µl of sterile distilled water. Samples were visualised by agarose gel electrophoresis in a 1% agarose gel to check that DNA had been recovered.

### 3.3.1.4. c-myc oncogene PCR

Primers that amplify a 139 bp fragment of the human *c-myc* oncogene (Mabruk *et al.*, 1994) were employed as a positive internal control to identify false-negative results due to the presence of known *Taq* polymerase inhibitors and to ensure that the DNA was of sufficient quality to allow amplification. A 50 µl reaction mix was prepared containing 1.5 mM MgCl₂, 200 µM each dNTP, 3 µM of each primer, reaction buffer (100 mM Tris-HCl, pH 9.0, 500 mM KCl, 1% Triton® X-100) and 5 µl of template. Samples were initially denatured in a thermocycler (Omnigene, Hybaid Ltd, Teddington, Middlesex, UK) at 92 °C for 7 min before adding 2.5 U of *Taq* polymerase (Promega, USA). The DNA was then amplified as follows: 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min for 30 cycles with a final cycle of 72 °C for 10 min (Mabruk *et al.*, 1994). Human DNA was included as a positive control and sterile water was used as a negative control in all reactions. PCR products were visualised on a 2% agarose gel (see section 2.3).
3.3.1.5. 16S rRNA gene PCR

*H. pylori* DNA was detected by a PCR technique using 16S rRNA gene primers (Ho *et al.*, 1991), namely, Hp1 and Hp2 (Hp1; 5'-CTG GAG AGA CTA AGC CCT CC-3' and Hp2; 5'-ATT ACT GAC GCT GAT TGT GC-3') corresponding to nucleotide positions 834 – 853 and 744 – 763, respectively, of the 16S rRNA gene of *E. coli*. A 50 µl reaction mixture was prepared containing reaction buffer [500 mM KCl, 100 mM Tris-HCl (pH 9.0), 1% Triton X-100], 1.5 mM MgCl₂, 200 µM each dNTP, 50 pmol of each primer and 1.25 U of *Taq* polymerase (Promega, USA). The PCR mixture was overlaid with mineral oil and exposed to high intensity UV light for 10 min before adding 5 µl of template. Samples were amplified (Omnigene, Hybaid Ltd, Teddington, Middlesex, UK) as follows: 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s for 40 cycles followed by a final cycle at 72 °C for 10 min. PCR products were visualised on a 2% agarose gel. A sample was considered *H. pylori*-positive if a 109 bp PCR product was amplified. In all set of reactions, DNA of a *H. pylori* reference strain, NCTC 11638, was used as a positive control and distilled water was used a negative control. The recommendations of Kwok and Higuchi (1989) were followed.

3.3.1.6. Southern blot analysis

To confirm the specificity of the PCR product, Southern blot analysis was performed on a representative sample of 16S rRNA gene amplified products using a 19 bp end-labelled [γ-3²P] dATP probe, pHp [5'-CAT CCA TCG TTT AGG GCG TG-3' (Ho *et al.*, 1991)], which is complementary to the internal sequence of the Hp1-Hp2 PCR product. The DNA was transferred to a nylon membrane (see section 2.4.2) and pre-hybridised for 30 min at 57 °C in 20 ml of 5 X SSPE [0.75 mol NaCl/l, 45 mmol NaH₂PO₄/l and 5 mmol EDTA/l (pH 7.4), 0.1% (w/v) SDS, 5 X Denhardt's solution and 100 µg denatured salmon sperm DNA]. The probe pHp was end-labelled with [γ-3²P]dATP using T₄

70
polynucleotide kinase (see section 2.6.1). It was added to the pre-hybridisation solution and hybridisation was allowed to proceed for 3 h at 57 °C. The membranes were washed once in 6 X SSC (1 X SSC; 0.15 mol NaCl/l, 15 mmol sodium citrate/l) for 30 min at room temperature and subsequently washed in 2 X SSC for 20 min at room temperature. Membranes were exposed to Cronex 10s film with two Quanta rapid intensifying screens (Dupont) at -70 °C overnight (see section 2.8.1).

3.3.1.7. Evaluation of primer specificity

The 16S rRNA gene primer specificity was assessed with DNA from *Helicobacter pylori* reference strains NCTC 11637 and NCTC 11639, *Helicobacter mustelae* NCTC 12032, *Helicobacter acinonyx* (kindly supplied by Dr Jim Fox, MIT, Ma, USA), *Wolinella succinogenes* NCTC 11488, and clinical isolates of *Campylobacter jejuni*, *Campylobacter coli*, *Escherichia coli*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter cloacae* and *Serratia marsescens* (supplied by the Department of Clinical Microbiology, St. James's Hospital, Dublin). The DNA was extracted using a Wizard genomic DNA extraction kit (Promega, USA) according to manufacturer's instructions. The quantity of DNA recovered was determined by agarose gel electrophoresis by visual comparison with a low molecular mass DNA ladder (Gibco BRL). The DNA (8 ng) was amplified using the 16S rRNA gene PCR method (see section 3.3.1.5) and the Hp1-Hp2 PCR products were transferred to a nylon membrane by Southern blotting (see section 2.4.1). The membrane was pre-hybridised for 2 h at 65 °C as previously described (see section 2.7.2). The solution was then replaced with fresh hybridisation solution containing a Digoxigenin end-labelled (see section 2.6.2) pH probe and hybridisation was allowed to continue at 65 °C overnight. The membranes were developed according to manufacturer's instructions.
To further assess primer specificity, five endometrial histology specimens (control samples from another patient population) were included in the study. DNA samples were prepared (see section 3.3.1.3) and amplified by *c-myc* oncogene PCR and 16S rRNA gene PCR as previously described (see sections 3.3.3.4 and 3.3.1.5).

3.3.1.8. *Sensitivity of the 16S rRNA gene PCR method*

To accurately assess the sensitivity of the 16S rRNA gene PCR method, *H. pylori* reference strain NCTC 11638 was embedded in agar, fixed, processed, paraffin-embedded and the DNA extracted (see section 3.3.1.3) to a concentration that could be reliably determined spectrophotometrically, *i.e.*, within the $A_{260\ nm}$ range 0.1 - 1 (Sauer *et al.*, 1998).

3.3.1.9. *Follow-up $^{13}$C-UBT*

At the end of the study, the PCR results were compared to the routine conventional diagnostic tests. A $^{13}$C-UBT was performed by the staff of the Dept. of Gastroenterology, Meath Hospital, Dublin, Ireland on patients who were of equivocal *H. pylori* status, *i.e.*, only one conventional diagnostic test was positive. $^{13}$C-UBT was performed on consenting patients using the modified European standard $^{13}$C-UBT protocol (Logan *et al.*, 1991). A patient was considered *H. pylori*-positive when the urease activity increased breath $^{13}$CO$_2$ by an excess of >5 per ml after subtraction of the pre-$[^{13}\text{C}]$ breath sample.

3.3.1.10. *Data analysis*

The sensitivity, specificity, positive and negative predictive values were calculated in accordance with Bourke *et al.* (1985). The sensitivity of a test was defined by “the number of true-positives as a percentage of the total with the disease” whereas the
specificity of a test was defined as “the number of true-negatives divided by the total without the disease” (Bourke et al., 1985). The predictive value of a test was the proportion of true cases among all those with a positive test result. The diagnostic accuracy of a test was defined as the sum of the number of true-positives and true-negatives divided by the total number of subjects expressed as a percentage.
3.3.2. Results

3.3.2.1. Optimisation of the 16S rRNA gene PCR method

A preliminary experiment was performed to determine the optimum size of DNA fragment that could be amplified from paraffin-embedded antral biopsy specimens. PCR was performed using *c-myc* oncogene primers (see section 3.3.1.4) that generate a 139 bp PCR product. In addition, two primer sets were included that amplify a 200 bp and a 600 bp fragment of the human β-globin gene. The results showed that a PCR product of ≥ 200 bp could not be reliably amplified from paraffin-embedded tissue samples. This result was expected since the fixative, the length of time a sample is maintained in a fixative and the age of the specimen are factors that are known to affect the efficiency of subsequent DNA amplification (Coates et al., 1991; Greer et al., 1991a; Greer et al., 1991b). Therefore, *H. pylori* primers, namely Hp1 and Hp2, were chosen that generate an amplification product of 109 bp. The 16S rRNA gene PCR technique was optimised and a 1.5 mM MgCl₂ concentration was chosen that yielded a PCR product of the correct size and good visual intensity. To ensure that the test DNA sample volume employed in the PCR was sufficient to detect low-level *H. pylori* infection, DNA was extracted from sections from paraffin-embedded gastric biopsy specimens from patients who harboured a high-level *H. pylori* infection such that all of the conventional diagnostic tests were positive and from patients who harboured a low-level *H. pylori* infection where only two of the conventional tests were positive. A sample volume of 5 µl was sufficient to detect low-level *H. pylori* infection and this volume was used in all subsequent PCR reactions. The sensitivity of the 16S rRNA gene PCR method was 0.5 ng (see Fig. 3.2).

3.3.2.2. c-myc oncogene PCR from paraffin-embedded histology blocks

c-myc oncogene PCR and the 16S rRNA gene PCR methods were performed on the DNA extracted from 72 paraffin-embedded histology blocks of antral biopsy specimens.
Figure 3.2. Sensitivity of 16S rRNA gene PCR.

*H. pylori* reference strain NCTC 11638 was suspended in agar and a 1 cm³ block of agar was fixed in formalin and paraffin-embedded. The DNA was extracted from paraffin-embedded sections and quantitated. Agarose gel electrophoresis showing PCR products from standard reactions with the following amount of *H. pylori* DNA; Lane 2, 50 ng; lane 3, 5 ng; lane 4, 0.5 ng; lane 5, 0.05 ng; lane 6, 5 pg; lane 7, 0.5 pg; lane 8, 0.05 pg; lane 9, 5 fg; lane 10, sterile water negative control. Molecular size (bp) markers are indicated.
Figure 3.3. *c-myc* oncogene PCR results from various clinical samples.

Agarose gel electrophoresis of positive and negative *c-myc* oncogene PCR products; lane 1, 100 bp molecular size markers; lanes 2-13 clinical specimens; lane 14, liver sample positive control; lane 15, sterile water negative control. These samples are unrelated to those shown in Fig. 3.4 and Fig. 3.5.
Representative agarose gels of clinical samples are shown in Figs. 3.3 and 3.4. The samples shown in each figure are generated from two separate experiments where different clinical samples were included in each experiment. The presence of a 139 bp amplification product of the \textit{c-myc} oncogene PCR method assured that possible \textit{Taq} polymerase inhibitors were not present in the sample and that the DNA was of sufficient quality to allow amplification of a PCR product of this size (Fig. 3.3).

3.3.2.3. 16S rRNA gene PCR from paraffin-embedded histology blocks

Amplification products of 139 bp size generated by the \textit{c-myc} oncogene PCR method confirmed that the DNA extracted from paraffin-embedded gastric biopsy specimens was of good quality. These DNA samples were then amplified by the 16S rRNA gene PCR method. The generation of a 109 bp PCR product indicated the presence of \textit{H. pylori} DNA. The 16S rRNA gene PCR method using DNA extracted from paraffin-embedded tissue samples resulted in the appearance of intense 'primer-dimer' amplification products of approx. 30 bp (Figs 3.2 and 3.4). These artefacts are known to occur if the sample material is poor. They are generated in the early stages of the reaction when low temperatures promote the non-specific annealing of the primers with each other resulting in primer oligomerisation. Once formed, these non-specific products are amplified throughout the remaining PCR cycles (Coates \textit{et al.}, 1991). Further experiments towards the completion of this work using a different \textit{Taq} polymerase (AmpliTaq Gold, Perkin-Elmer Applied Biosystems, USA), that is only active at higher temperatures, demonstrated that these 'primer-dimer' amplification products could be avoided. Southern blot hybridisation with the pHp probe was performed on a representative number of PCR products to confirm the PCR product specificity (Fig. 3.5).
Figure 3.4. 16S rRNA gene PCR results from various clinical samples.

Agarose gel showing positive and negative 16S rRNA gene Hp1-Hp2 PCR products; lane 1, 100 bp molecular size markers; lane 2, sample from wax sections cut between processing of gastric biopsy specimens; lane 3-10, samples from gastric biopsy specimens; lanes 11-13, *H. pylori* reference strain NCTC 11638 DNA from artificially embedded agar samples; lane 14, sterile water negative control.
Figure 3.5. Southern blot hybridisation of positive and negative 16S rRNA gene PCR products.

Positive and negative 16S rRNA gene PCR products were transferred to a nylon membrane by Southern blotting and hybridised with a 19 bp pHp probe complementary to the internal sequence of the Hp1 and Hp2 109 bp PCR product. Lane 1, *H. pylori* reference strain NCTC 11638 positive control; lanes 2-14 gastric biopsy samples. These samples are unrelated to those shown in Fig. 3.3 and Fig. 3.4.
Comparison of the 16S rRNA gene PCR method with conventional diagnostic tests

Forty-nine consecutive untreated patients under investigation for symptoms of dyspepsia (group A; 20 male, 29 female, age range 17 – 88 years; mean 43.3 years) and 23 consecutive post-treatment patients (group B; 17 male, 6 female, age range 19 – 72 years, mean 41 years) were included in the study. The results of the routine tests and 16S rRNA gene PCR are shown in Table 3.3. In group A, 19 of 49 (39%) patients were found to harbour *H. pylori* according to the case definition. Of these 19 infected patients, all were correctly diagnosed by 16S rRNA gene PCR (100% sensitivity). Thirty patients did not harbour *H. pylori*, including two patients who were of equivocal *H. pylori* status. Eight of these latter patients were positive by PCR (73% specificity) including one equivocal patient who was positive by histology alone. The CLOtest had not been performed on an antral biopsy specimen from this patient. This patient was found to harbour *H. pylori* as assessed by the 13C-UBT (excess 14.7 13CO2 per ml) 15 months after the initial endoscopy. The remaining equivocal patient (positive Gram stain), who had a negative CLOtest and PCR result, was also negative by the 13C-UBT when assessed at 16 months after the initial endoscopy.

In group B, of the 4 patients (17%) who were found to harbour *H. pylori* infection, all were positive by the PCR technique (100% sensitivity). However, in the 19 patients who had *H. pylori* eradicated, twelve of these patients were positive by PCR (37% specificity) including six equivocal patients who were positive by only one conventional test (three positive by the CLOtest, two by Gram stain, and one by histology). Three of these equivocal patients had received additional *H. pylori* eradication treatment since the initial endoscopy and were therefore excluded from further analysis. One equivocal patient refused to participate. In the case of the two remaining equivocal patients (one positive
Table 3.3. Comparison of results of conventional tests and 16S rRNA gene PCR for the detection of *H. pylori* infection in untreated patients (group A) and post-treatment patients (group B).

<table>
<thead>
<tr>
<th>Infection status</th>
<th>CLOtest</th>
<th>Culture</th>
<th>Gram stain</th>
<th>Histology</th>
<th>PCR</th>
</tr>
</thead>
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<td><strong>Group A</strong></td>
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</tr>
<tr>
<td><em>H. pylori</em> present (n=19)</td>
<td>17</td>
<td>2</td>
<td>9</td>
<td>10</td>
<td>16</td>
</tr>
<tr>
<td><em>H. pylori</em> absent (n=30)</td>
<td>0</td>
<td>23(^a)</td>
<td>0</td>
<td>30</td>
<td>1</td>
</tr>
<tr>
<td><strong>Sensitivity</strong></td>
<td>89%</td>
<td>47%</td>
<td>84%</td>
<td>89%</td>
<td>100%</td>
</tr>
<tr>
<td><strong>Specificity</strong></td>
<td>100%</td>
<td>100%</td>
<td>96%</td>
<td>96%</td>
<td>73%</td>
</tr>
<tr>
<td><strong>Group B</strong></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td><em>H. pylori</em> present (n=4)</td>
<td>4</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td><em>H. pylori</em> absent (n=19)</td>
<td>3</td>
<td>13(^c)</td>
<td>0</td>
<td>19</td>
<td>2</td>
</tr>
<tr>
<td><strong>Sensitivity</strong></td>
<td>100%</td>
<td>25%</td>
<td>50%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td><strong>Specificity</strong></td>
<td>81%</td>
<td>100%</td>
<td>89%</td>
<td>94%</td>
<td>37%</td>
</tr>
</tbody>
</table>

\(^a\) CLOtest not available for seven patients
\(^b\) Gram stain not available for one patient
\(^c\) CLOtest not available for three patients
by CLOtest, one by Gram stain), they were found to be uninfected by the $^{13}$C-UBT or a repeat gastroscopy with biopsies at the follow-up stage.

The predictive values of the 16S rRNA gene PCR technique and the routine biopsy-based tests are shown in Table 3.4. The 16S rRNA gene PCR technique had low positive predictive value for the diagnosis of *H. pylori* infection in untreated patients whereas it had high negative predictive value for the assessment of *H. pylori* eradication in post-treatment patients.

3.3.2.5. Performance of conventional tests for *H. pylori* diagnosis

Among the untreated patients in group A, the most sensitive diagnostic tests for the detection of *H. pylori* infection were the CLOtest (89%) and histology (89%) [see Table 3.3]. However, the finding that one equivocal patient (positive histology) harboured *H. pylori* infection by the $^{13}$C-UBT at the follow-up stage indicates that this was in fact a true-positive result and hence, the sensitivity of histology is 94%. The sensitivity of Gram stain was 84%. The diagnostic test with the lowest sensitivity for the detection of *H. pylori* infection was culture (47%). The CLOtest and histology were the best tests (with the greatest combined sensitivity and specificity) for the accurate diagnosis of *H. pylori* infection.

Among the patients in group B, the most specific diagnostic test for the detection of *H. pylori* infection in post-treatment patients was culture (100%) followed by histology (94%) and the Gram stain (89%) [see Table 3.3]. The CLOtest and histology appeared to be more sensitive for the diagnosis of *H. pylori* in treated patients than in untreated patients while the sensitivity of culture (25%) and the Gram stain (50%) was greatly
Table 3.4. The positive and negative predictive values as determined for four routine biopsy-based tests and 16S rRNA gene PCR technique among untreated patients (group A) and patients who had received *H. pylori* eradication treatment (group B).

<table>
<thead>
<tr>
<th>Predictive value</th>
<th>CLOtest</th>
<th>Culture</th>
<th>Gram stain</th>
<th>Histology</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>100%</td>
<td>100%</td>
<td>94%</td>
<td>94%</td>
<td>70%</td>
</tr>
<tr>
<td>Negative</td>
<td>92%</td>
<td>75%</td>
<td>90%</td>
<td>93%</td>
<td>100%</td>
</tr>
<tr>
<td><strong>Group B</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>57%</td>
<td>100%</td>
<td>50%</td>
<td>80%</td>
<td>25%</td>
</tr>
<tr>
<td>Negative</td>
<td>100%</td>
<td>86%</td>
<td>89%</td>
<td>100%</td>
<td>100%</td>
</tr>
</tbody>
</table>
reduced. Histology was the best single diagnostic test for the accurate assessment of *H. pylori* eradication.

The predictive values of the routine biopsy-based tests are shown in Table 3.4. The positive predictive values obtained with the four biopsy-based tests in untreated patients were high (≥ 94%) but the highest positive predictive values were obtained with culture and the CLOtest. Among post-treatment patients, the tests with the highest negative predictive values were histology and the CLOtest.

### 3.3.2.6. Evaluation of primer specificity

Due to the high number of discrepant PCR results, the specificity of the Hp1-Hp2 primer set was evaluated using a number of closely related *Helicobacter* species and a selection of Gram-negative bacteria. The 16S rRNA gene PCR method generated the expected 109 bp PCR product from both *H. pylori* reference strains. However, PCR products of 109 bp size were also generated from *H. acinonyx, H. mustelae* and *C. coli* samples (Fig. 3.6a). The bands representing 109 bp generated from *H. mustelae* and *C. coli* are not visible in Fig. 3.6a. A very faint non-specific band of between 650 and 850 bp size was generated in the samples from *C. coli* and *Ent. cloacae*. A non-specific band of between 400 and 500 bp size was also generated from *K. oxytoca*. These bands are not visible in Fig. 3.6a. DNA hybridisation with the pHp probe that is complementary to the internal sequence of the 109 bp PCR product generated a strong hybridisation signal of the expected size for both *H. pylori* reference strains and *H. acinonyx*. A very faint signal that is not visible in Fig. 3.6b was also observed with *H. mustelae* and *C. coli*.

Assessment of endometrial samples was included in the study to determine whether the Hp1-Hp2 primer set could amplify human DNA. The 16S rRNA gene PCR method
Figure 3.6. Assessment of 16S rRNA gene Hp1 and Hp2 primer specificity.

(a) Agarose gel electrophoresis of 16S rRNA gene PCR products from DNA samples of the following; lane 2, *H. pylori* reference strain NCTC 11637; lane 3, *H. pylori* reference strain NCTC 11639; lane 4, *Wolinella succinogenes* NCTC 11488; lane 5, *Helicobacter acinonyx*; lane 6, *Helicobacter mustelae* NCTC 12032; lane 7, *Campylobacter jejuni*; lane 8, *Campylobacter coli*; lane 9, *Proteus mirabilis*; lane 10, *Klebsiella pneumoniae*; lane 11, *Klebsiella oxytoca*; lane 12, *Eschericia coli*; lane 13, *Enterobacter cloacae*. A very faint non-specific band of between 650 and 850 bp size was observed in the samples of *C. coli* and *Ent. cloacae* and a band of between 400 and 500 bp size was observed in the *K. oxytoca* sample. These bands are not visible in the gel photograph. Lanes 1 and 14 contain molecular size markers. (b) Southern blot hybridisation of positive and negative 16S rRNA gene PCR products from (a) using a 19 bp DIG-labelled pH probe complementary to the internal sequence of the 109 bp PCR product.
generated the 109 bp PCR product from two of these five endometrial samples (data not shown).
3.4. Discussion

3.4.1. Evaluation of antral biopsies used in the rapid urease test (CLOtest) for *H. pylori* culture

In this study, *H. pylori* was isolated from routine biopsy specimens in 34 of 38 *H. pylori*-infected patients. Extensive plate contamination during isolation may have obscured the growth of *H. pylori* in one of these biopsy specimens. Therefore, the use of a selective medium, in conjunction with a non-selective medium, for the primary isolation of *H. pylori* might improve isolation rates (Tee *et al.*, 1991; Piccolomini *et al.*, 1997a).

However, the organism was successfully isolated from the positive CLOtest biopsy from patient 7 when culture of the bacterium from the routine biopsy was unsuccessful. This may be explained by sampling error due to the non-uniform distribution of *H. pylori* in the gastric mucosa (Bayerdörffer *et al.*, 1989) and the presence of low numbers of bacteria in the routine biopsy specimen (Xia *et al.*, 1994a). It is noteworthy that the absence of gastric atrophy in gastric mucosal biopsy specimens from these four patients supports the hypothesis that sampling error was responsible for the unsuccessful isolation of *H. pylori* in these patients rather than thinning of the gastric mucosa and the concomitant loss of *H. pylori* from the atrophic area. The absence of atrophy in gastric mucosal biopsy specimens from the two CLOtest-positive, routine culture-positive patients in whom the infection was not histologically detected (patients 24 and 33) also implies that sampling error is responsible for this discrepancy.

*H. pylori* was successfully isolated from the positive CLOtest biopsy specimen in 29 of 34 patients whose routine culture was positive. The compromised sensitivity observed when positive CLOtest biopsy specimens are used for culture may be due to the inhibitory effect of the bacteriostatic preservative, namely, methyl hydroxybenzoate (0.2%), and urea (2%) on bacterial viability (Soltesz *et al.*, 1992). Contamination
obscured the growth of *H. pylori* in only one positive CLOtest biopsy specimen (patient 11). Herein and in other studies (Rautelin *et al*., 1997) it was observed that bacterial colonies isolated from rapid urease test specimens were slower to appear thus necessitating prolonged incubation. The presence of a bacteriostatic compound in the CLOtest may also account for the differences in recovery rates observed in the present study (79%) compared to the 88% recovery rate reported by Rautelin *et al.* (1997) who evaluated Jatrox urease tubes, as a transport medium for gastric biopsy specimens (Rautelin *et al*., 1997).

Culture of CLOtest-negative biopsies was performed to identify false-negative CLOtests. In this study, one patient was identified who did harbour *H. pylori* infection as determined by histology and routine culture, although the organism was not isolated from the CLOtest biopsy that had been removed at 120 min because of extensive plate contamination. Since the sensitivity of the CLOtest increases with time [75% at 2 h compared with 90% at 3 h (Marshall *et al*., 1987b)], it may be argued that this CLOtest would have been positive at a later time if the biopsy had not been removed prematurely.

In the gastroenterology units culture of gastric mucosal biopsies is performed routinely on all patients with upper gastrointestinal symptoms to determine their *H. pylori* infection status. This results in expensive, time-consuming attempted culture of the organism in an ever increasing population of *H. pylori*-negative individuals. In the present study optimal isolation rates were achieved with routine biopsies rather than biopsies obtained from positive CLOtests. Therefore, from a practical viewpoint, it may be more cost-effective to store the routine microbiology biopsy at 4 °C until the CLOtest yields a positive result (allowing a 3 h observation period as previously described) before sending the routine biopsy for culture. In the Meath and Adelaide Hospitals, 33% of
CLO tests from a routine endoscopy list were positive at the time this study was conducted. Thus, culture of selected biopsy specimens that are known to be positive for the organism would result in an estimated annual cost-saving of £6,515 ($10,600). While this approach would ultimately lead to more cost-effective management of *H. pylori* infection, further studies with a larger sample size are advocated.

### 3.4.2. Evaluation of a DNA amplification-based technique for *H. pylori* diagnosis in paraffin-embedded tissue

In this study a 16S rRNA gene PCR technique was evaluated for the diagnosis of *H. pylori* infection in both untreated and post-treatment patients. The 16S rRNA gene PCR method correctly identified patients with proven *H. pylori* infection in both patient populations. However, the technique performed poorly in patients who did not have the infection according to the strict case definition (negative by all four conventional tests), producing a high number of discrepant PCR results.

The finding of a high number of discrepant PCR results in patients who did not harbour *H. pylori* infection is in accordance with the data of two published studies that also evaluated a 16S rRNA gene PCR using the primers designed by Ho *et al.* (1991). Morera-Brenes *et al.* (1994) found that 16S rRNA gene PCR analysis of paraffin-embedded specimens in untreated patients had a sensitivity of 98% but a specificity of 33%. Lu *et al.* (1999) evaluated five PCR methods, including a 'semi-nested' 16S rRNA gene PCR method, for the detection of *H. pylori* DNA in gastric biopsy specimens from untreated patients. In their study, 12 of 25 culture-negative biopsy specimens were positive by the 16S rRNA gene PCR method whereas all 25 were negative by three other PCR methods, resulting in a low negative predictive value (46%) for the 16S rRNA gene PCR method.
In the present study, an evaluation of primer specificity found that the 16S rRNA gene primers were not specific for *H. pylori* and this may explain the high frequency of false-positive PCR results and the poor correlation observed with conventional routine tests. The 16S rRNA gene primers were originally designed by comparison of the nucleotide sequences of the 16S rRNA genes of *E. coli*, *Wolinella succinogenes*, *Helicobacter* spp and *Campylobacter* spp, and the primer sequences were derived from the regions exhibiting the least sequence similarity among the sequences examined (Ho et al., 1991). These primers appeared to be specific for *H. pylori* when analysed with other bacteria (Ho et al., 1991; Mapstone et al., 1993b). However, in a study aimed to characterise spiral bacteria obtained from gastric biopsy samples of rhesus monkeys, Drazek et al. (1994) found that the nucleotide sequence corresponding to positions 711 – 873 of the *E. coli* 16S rRNA gene (which includes the region from positions 744 – 853 that is amplified by the primers Hpl and Hp2) was not useful for distinguishing between species within the genus *Helicobacter*. The DNA similarity in this region was found to range from 94.2% to 99.4% among human *H. pylori* isolates, from 95.9% to 99.1% between a strain isolated from a rhesus monkey and four *H. pylori* strains and from 92.4% to 99.1% between a rhesus monkey strain and other *Helicobacter* spp. Therefore, the high DNA sequence similarity observed in this region among *Helicobacter* species may explain the amplification of the 109 bp PCR product from *H. mustelae* and *H. acinonyx*. Furthermore, the present study showed that non-specific bands were generated from the DNA specimens of *C. coli*, *Ent. cloacae* and *K. oxytoca*. Non-specific bands have been reported by Mapstone et al. (1993b), although the reasons for their presence has not been addressed.
Chong et al. (1996) reported that the 16S rRNA gene primers generated a 109 bp PCR product from human tissue including three liver biopsy specimens, four foetal liver specimens, five leukocyte samples and one human cell line. In the present study, the 109 bp PCR product was amplified from two of five uterine endometrial samples. Amplification of a 109 bp fragment from endometrial samples that are not known to harbour *H. pylori* organisms suggests that these unexpected positive results may be due to the non-specific amplification of human genomic DNA by the Hp1-Hp2 primers used in the 16S rRNA gene PCR method. However, the 109 bp fragment was not amplified from liver biopsy samples that were cut between clinical biopsy specimens to control for cross contamination during processing.

The high number of discrepant PCR results may be caused by the detection of non-viable organisms in post-treatment patient samples, although this does not explain the high positive rate observed in the untreated patient group. Recent reports have shown that cross infection by means of endoscopes considered to be adequately disinfected and iatrogenic transmission by endoscopy are possible (Langenberg et al., 1990; Miyaji et al., 1995; van der Hulst et al., 1996). *H. pylori* DNA has been detected from endoscope (Roosendaal et al., 1994) and biopsy forcep samples (Mapstone et al., 1993b) and may be a source of contamination in the PCR reactions. However, these two studies also used the primers described by Ho et al. (1991) and therefore, the validity of these results is questionable. Although laboratory contamination of samples can not be excluded as a possible cause of some of the discrepant positive PCR results, numerous measures were employed to control for contamination at each stage in the technique and good laboratory technique was executed at all times.
3.4.3. Evaluation of conventional biopsy-based tests for H. pylori diagnosis in untreated and post-treatment patients

The accuracy of conventional biopsy-based tests for the diagnosis of H. pylori infection in two different patient populations was assessed. The CLOtest was the best conventional test for the accurate diagnosis of H. pylori infection (95% diagnostic accuracy). However, this may be misleading because the results from seven patients were not available for analysis. The high sensitivity observed with the CLOtest in this study is in agreement with other published studies that have prospectively evaluated the accuracy of conventional tests for the diagnosis of H. pylori infection, without selecting a single test as the gold standard. Cutler et al. (1995) found that the CLOtest was a highly sensitive (89.6%) and specific (100%) diagnostic test for the detection of the infection in untreated patients.

The finding that the positive predictive value of the CLOtest was significantly lower in post-treatment patients questions the usefulness of this test for H. pylori diagnosis in this patient population. The observed increase in the sensitivity of the CLOtest after treatment was unexpected. Laine et al. (1998) found that the CLOtest sensitivity was lower in patients who had received eradication therapy. In addition, the CLOtest sensitivity was lower after the use of more effective treatment (dual therapy > monotherapy). Conversely, in one of the few published studies that aimed to evaluate the accuracy of routine tests for the assessment of H. pylori infection status in post-treatment patients, Rollán et al. (1997) found that the CLOtest achieved 91.7% sensitivity and 100% specificity when multiple antral biopsy specimens were placed into the same test well. However, the use of multiple biopsy specimens, rather than one, for the CLOtest is not a standard clinical practice. Moreover, other studies have shown conflicting results. Laine et al. (1996b) found that there was no significant difference in
the sensitivity of the CLO test when either one or two biopsy specimens were used in the same test well.

Histology achieved comparable diagnostic accuracy (93%), compared with the CLO test, for the diagnosis of *H. pylori* infection in untreated patients. Moreover, histology was the best single test for the accurate assessment of *H. pylori* eradication (95% diagnostic accuracy). The increased sensitivity observed may be due to the increased number of gastric biopsy specimens obtained for histological examination. For example, histological examination of antral and corporal biopsies was performed in 12 out of 49 (24%) of untreated patients compared with 12 out of 23 (52%) of treated patients (Table 3.2). However, only four patients with persistent *H. pylori* infection (group B, Table 3.3) were analysed and hence, the results may not accurately reflect the true sensitivity of this test for *H. pylori* diagnosis in treated patients. The limitations associated with the histological assessment of gastric biopsy specimens, e.g., sampling error, pathologist inexperience (Maconi et al., 1999) and biopsy orientation and/or processing, are well known and are further accentuated in post-treatment patients where the infection is at a low-level and may be scanty. Although this study did not identify any false-negative cases, one false-positive case was identified. In this patient, moderate acute and chronic inflammation was observed in the antral biopsy specimen and histological examination of the corporal biopsy specimen documented the presence of *Helicobacter*-like organisms. The *H. pylori* status of this patient could not be further investigated as *H. pylori* eradication treatment was prescribed. It is possible that misinterpretation by the investigating pathologist may be responsible for this result.

In this prospective study culture, performed by a routine microbiological laboratory, was the least sensitive routine test in the two patient populations. While this test is highly
specific, failure to isolate the bacterium does not necessarily confirm that the patient is uninfected. The poor sensitivity of this test may be due to a number of factors, e.g., bacterial and/or fungal contamination during isolation due to the sole use of a non-selective medium for *H. pylori* culture and possibly technician inexperience. However, high sensitivity has been achieved in certain centres with a special interest in the method. Lerang *et al.* (1998) reported that culture had a high sensitivity (93%) when two antral biopsy specimens were used for culture while Thijs *et al.* (1996) found that culture sensitivity was 98.4% when only one antral biopsy specimen was used. Indeed, these isolation rates are on a par with those achieved in section 3.2.2. by the author.

In summary, the 16S rRNA gene primers employed in this study are not specific for *H. pylori* and preclude their use for the detection of *H. pylori* in clinical specimens. The findings of the study shows that the best test employed for the primary diagnosis of *H. pylori* infection is not necessarily optimum for the assessment of *H. pylori* eradication. The CLOtest and histology were the most accurate tests presently employed for *H. pylori* diagnosis in untreated patients but histology had better diagnostic accuracy for the assessment of *H. pylori* eradication. However, no single routine test is ideal for the detection of *H. pylori* infection for a variety of different reasons. Hence, a combination of two tests with acceptable sensitivity and specificity is therefore recommended for accurate *H. pylori* diagnosis.
Chapter 4

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4.4.2. Direct comparison of the agar dilution method, E-test and the disc-diffusion test for susceptibility testing of *H. pylori* ........................................... 103
4.1. Introduction

Metronidazole (or tinidazole), clarithromycin and amoxicillin are frequently included in \textit{H. pylori} PPI-based treatment regimens (see section 1.10.2). In the current climate where the increasing use of these antimicrobials for the treatment of non-\textit{H. pylori}-related diseases, \textit{e.g.}, gynaecological and respiratory tract infections, culture and susceptibility testing of \textit{H. pylori} has become an important requisite to monitor resistance rates in the local population to guide empirical treatment. Moreover, the success rates of eradication treatment in clinical practice are often compromised by a number of factors, namely, sub-optimal treatment regimens, poor patient compliance and underlying primary resistance to metronidazole, clarithromycin and more recently, amoxicillin. Therefore, culture and susceptibility testing is necessary to assess resistance rates in patients who fail first-line treatment so that an appropriate second-line treatment regimen may be prescribed (EHPSG, 1997).

Presently there is no single method generally accepted for susceptibility testing of \textit{H. pylori} (see section 1.13) and standardisation of the methodology is now required to allow the direct comparison of resistance levels between centres. The agar dilution method is considered to be the reference method, although the labour intensive nature of this procedure is such that it is unsuitable for the routine testing of individual isolates. The Epsilometer (E) test is an alternative susceptibility testing method which offers a quantitative approach, is less labour intensive, and is suitable for testing a broad number of antimicrobials against a small number of \textit{H. pylori} isolates. The plastic-coated E-test strip containing a preformed antimicrobial gradient on one side and a concentration scale on the other is placed on to the agar inoculated with a homogeneous bacterial suspension. After incubation, the MIC is taken as the point where the elliptical zone of growth inhibition intersects the strip. Qualitative susceptibility results are obtained using the
The aims of the study were (i) to assess the prevalence of metronidazole, clarithromycin and amoxicillin resistance among clinical isolates of *H. pylori* by the E-test susceptibility method, and (ii) to evaluate the E-test and disc-diffusion methods for clarithromycin susceptibility testing of *H. pylori* by comparison with the reference agar dilution method.
4.2. Prevalence of Primary Metronidazole, Clarithromycin and Amoxicillin Resistance in *H. pylori*

4.2.1. Materials and methods

4.2.1.1. Study design

The study was performed as part of a European multi-centre *in vitro* antimicrobial resistance survey in *H. pylori*. Non-duplicate clinical isolates of *H. pylori* were prospectively collected over a six-month period between January and July 1998. The MIC values of the isolates were determined for metronidazole, clarithromycin and amoxicillin using the E-test method according to a standard procedure. Standardisation of the method involved a preliminary study to determine the optimum testing conditions (size of the bacterial inoculum, culture medium, duration of incubation) which yielded the best concordance of MIC values between the E-test and the reference agar dilution method.

4.2.1.2. Patients and *H. pylori* isolates

Fifty-four consecutive non-duplicate clinical isolates of *H. pylori*, obtained from gastric antral biopsies from patients who underwent endoscopy for the investigation of dyspepsia, were collected. *H. pylori* isolates from three patients were excluded from the study as these patients had been previously treated with metronidazole or a macrolide (erythromycin, clarithromycin, roxithromycin, azithromycin) either for treatment of *H. pylori* or other infections. *H. pylori* isolates were identified by Gram stain and by the urease reaction and stored at -70 °C until required.
4.2.1.3. Determination of the Minimum Inhibitory Concentration (MIC) by the E-test method

The E-test MIC determination of *H. pylori* isolates to metronidazole, clarithromycin and amoxicillin was performed on Mueller Hinton agar (Oxoid Ltd., UK) supplemented with 10% horse blood and the pH of the medium adjusted between 7.2 and 7.4 when necessary. This medium was recommended for susceptibility testing as results of the preliminary evaluation phase found that this agar was less prone to variations from one manufacturer/lot to another. Plates were used within 14 days of their preparation.

Colonies from a two-day old, blood agar plate culture were harvested into 2 ml of nutrient broth equivalent to McFarland turbidity standard (bioMérieux, France) No. 4 (approx. 1.2 x 10^9 CFU/ml). Prior to inoculation the shape and motility of the organisms were assessed by Gram stain and phase contrast microscopy. The bacterial suspension was discarded if more than 25% of the bacteria were rounded (coccoid) non-motile forms. Pre-warmed agar plates were inoculated by confluent swabbing of the surface with 0.25 ml of the bacterial suspension and after 10 min an E-test strip (AB Biodisk, Solna, Sweden) for each antimicrobial (concentration range 0.016 to 256 μg/ml consisting of 24 x 0.5 log₂ increments) was placed on each plate which was then incubated for 3 days at 37 °C under microaerophilic conditions using CampyGen gas packs (Oxoid Ltd., UK). The MIC value was recorded at the intersection between the edge of the inhibition (elliptical) zone and the concentration on the E-test strip. Isolated colonies growing inside the inhibition zone were documented and for metronidazole, strains showing the presence of such isolated colonies were reported as resistant regardless of the size of the zone of inhibition. The breakpoint MIC value for metronidazole was defined as ≥ 8 μg/ml, while the MIC value that defined
clarithromycin and amoxicillin resistance was $\geq 2\mu g/ml$ (Xia et al, 1994b; Xia et al, 1996a).

4.2.1.4. Quality control strains

The reproducibility of the E-test method was assessed by determining the MIC's for a set of three quality control strains. *H. pylori* reference strains CCUG 38770, 38771 and 38772 were obtained from the Culture Collection at the University of Göteborg and the strains were sent as lyophilised vials to each test centre. The MIC values of these reference strains were determined for amoxicillin, clarithromycin and metronidazole on three separate occasions, *i.e.*, at the beginning of the study, at half-way through the study, and at the end of the study. During the whole study period and between the three control test runs, the quality control strains were kept viable by freezing them at -70 °C. Repeated subculture was avoided to prevent the emergence of possible strain modifications.

4.2.1.5. Statistical methods

The differences in prevalences of metronidazole-resistant *H. pylori* among different groups of patients were assessed with the Chi-square test with Yate's correction, where applicable (Bourke et al., 1985).
4.2.2. Results

The distribution of MIC's of the *H. pylori* isolates obtained from 51 consecutive Irish patients (31 male, 20 female; age range 18 to 88 yrs, mean 48 yrs) to metronidazole, clarithromycin and amoxicillin are shown in Fig. 4.1. The MIC values for amoxicillin ranged from < 0.016 to 0.5 μg/ml and resistance to this antimicrobial was not observed (Fig. 4.1c). The MIC's of metronidazole for the *H. pylori* isolates ranged from 0.023 to > 256 μg/ml (Fig. 4.1a). Of these isolates 15 (29.4%) were resistant and 36 (70.5%) were susceptible. The incidence of metronidazole resistance in *H. pylori* did not differ significantly between men (8/31, 25.8%) and women [(7/20, 35%), *P* > 0.5]. However, metronidazole resistance was high among the 50 – 59 and 60 – 69 age groups but was not observed among patients under the age of 20 years (Table 4.1). The MIC's of clarithromycin for the *H. pylori* isolates ranged from < 0.016 to > 256 μg/ml and demonstrated a bimodal distribution (see Fig. 4.1b). Two (3.9%) *H. pylori* isolates were resistant to clarithromycin and 49 were susceptible. These resistant *H. pylori* isolates had been recovered from 2 female patients (ages 80 yrs and 66 yrs) and were also resistant to metronidazole, *i.e.* dual resistance.

The reproducibility of the E-test method was evaluated by determining the MIC values for *H. pylori* reference strains CCUG 38770, 38771 and 38772 at three different time points during the study period, namely, at the beginning of the study, at half-way through the study and at the end of the study (see Table 4.2). Consistent MIC values were obtained for amoxicillin with the three *H. pylori* reference strains tested throughout the study period. For metronidazole, susceptibility testing of *H. pylori* reference strains CCUG 38770 and 38771 yielded reproducible MIC values on the three testing occasions. However, the MIC value obtained for *H. pylori* reference strain CCUG 38772 at the end
(a) Metronidazole MIC (µg/ml)

(b) Clarithromycin MIC (µg/ml)
Figure 4.1. The E-test MIC values of metronidazole, clarithromycin and amoxicillin against 51 strains of *H. pylori*.

The MIC values for 51 clinical isolates of *H. pylori* to (a) metronidazole, (b) clarithromycin and (c) amoxicillin as determined by the E-test method.
Table 4.1. Incidence of metronidazole-resistant *H. pylori* among patients of different age groups.

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>No. of patients</th>
<th>Metronidazole phenotype (no. of isolates)</th>
<th>Incidence of resistant isolates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Susceptible</td>
<td>Resistant</td>
<td></td>
</tr>
<tr>
<td>&lt; 20</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>20 – 29</td>
<td>4</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>25.0</td>
</tr>
<tr>
<td>30 – 39</td>
<td>7</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>14.2</td>
</tr>
<tr>
<td>40 – 49</td>
<td>15</td>
<td>11</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>26.6</td>
</tr>
<tr>
<td>50 – 59</td>
<td>12</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>50.0</td>
</tr>
<tr>
<td>60 – 69</td>
<td>5</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>40.0</td>
</tr>
<tr>
<td>≥ 70</td>
<td>6</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>16.0</td>
</tr>
</tbody>
</table>
Table 4.2. The MIC’s for metronidazole, clarithromycin and amoxicillin against three *H. pylori* reference strains obtained by the E-test at the beginning of the study, at half-way through the study and at the end of the study period.

<table>
<thead>
<tr>
<th>Reference strain</th>
<th>Metronidazole</th>
<th>Clarithromycin</th>
<th>Amoxicillin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC (µg/ml)</td>
<td>MIC (µg/ml)</td>
<td>MIC (µg/ml)</td>
</tr>
<tr>
<td></td>
<td>Beginning</td>
<td>Middle</td>
<td>End</td>
</tr>
<tr>
<td>CCUG 38770</td>
<td>&gt; 256</td>
<td>&gt; 256</td>
<td>&gt; 256</td>
</tr>
<tr>
<td>CCUG 38771</td>
<td>2.0</td>
<td>2.0</td>
<td>1.0</td>
</tr>
<tr>
<td>CCUG 38772</td>
<td>&gt; 256</td>
<td>&gt; 256</td>
<td>32</td>
</tr>
</tbody>
</table>


of the study was > 3 log₂ dilution steps lower than the MIC’s obtained on the previous two testing occasions.

For clarithromycin, consistent MIC’s were obtained at the beginning and at the half-way time points for *H. pylori* reference strains CCUG 38770 and 38772 but the MIC’s obtained at the end of the study period were greater by 3 log₂ and 4 log₂ dilution steps, respectively, than the MIC’s obtained on the earlier testing occasions. Susceptibility testing of *H. pylori* reference strain CCUG 38771 demonstrated that while consistent MIC’s were obtained at the beginning and at the end of the study, the MIC’s obtained at the half-way stage were lower by 1.5 log₂ dilution steps.
4.3. Direct Comparison of the Agar Dilution Method, E-Test and the Disc-Diffusion Test for Susceptibility Testing of Selected Pre- and Post-Treatment *H. pylori* Isolates to Clarithromycin

4.3.1. Materials and methods

4.3.1.1. Patient population and *H. pylori* isolates

Patients presenting at the Meath and Adelaide Hospitals, Dublin, Ireland with symptoms of dyspepsia underwent endoscopy to obtain biopsies to assess their *H. pylori* status. One antral biopsy was used for the rapid urease test (CLOtest, Delta West, Australia), one antral and one corporal biopsy were taken for histological examination, and one antral biopsy was taken for culture and Gram stain of a tissue smear. Histological and microbiological analyses were performed by the staff of the Departments of Histology and Microbiology, St. James’s Hospital, Dublin, Ireland.

Infected patients received *H. pylori* eradication treatment regimens consisting of a one or two-week triple therapy regimen with (i) a proton-pump inhibitor [PPI, omeprazole (20 mg b.i.d)], clarithromycin (500 mg b.i.d) and metronidazole (400 mg t.i.d) or (ii) a PPI, clarithromycin and amoxicillin (1 g b.i.d) or (iii) a two-week dual therapy with a PPI (20 mg b.i.d) and clarithromycin (500 mg t.i.d) or (iv) a two-week regimen of colloidal bismuth citrate, metronidazole (400 mg t.i.d) and tetracycline (500 mg q.i.d). Endoscopy was performed four weeks after completion of treatment to attain biopsies for the assessment of *H. pylori* eradication. An additional antral biopsy was taken at this visit and sent to the Sir Patrick Dunn Microbiology Laboratory, St. James’s Hospital, Dublin for culture. In the instances where patients failed treatment, they were re-treated with a similar or different regimen depending on the results of the disc-diffusion susceptibility test, and eradication was assessed in the same way.
*H. pylori* isolates which had been previously stored (see section 2.1.1) were selected from seven patients (patients A-G) for whom pre- and post-treatment isolates were available and whose pre-treatment isolate(s) were susceptible to clarithromycin, as determined by the disc-diffusion test, and whose post-treatment isolate(s) were resistant to clarithromycin after failing a clarithromycin-based *H. pylori* eradication regimen. Patient hospital charts were reviewed to confirm the treatment status of each patient at the time of isolation of each *H. pylori* strain.

### 4.3.1.2. MIC determination by the agar dilution method

Primary *H. pylori* isolates (six pre-treatment and 22 post-treatment) were recovered from -70 °C storage (see section 2.1.1). Isolates were subcultured twice on blood agar and a Gram stain was performed to exclude cultures exhibiting > 25% coccoid forms. MIC determination was performed in duplicate according to NCCLS recommendations (NCCLS, 1991). Clarithromycin antibiotic powder (with stated potency) was supplied by Abbott Laboratories Ltd., UK. Wilkins Chalgren agar (Oxoid Ltd., Basingstoke, UK) supplemented with 5% heated horse blood was prepared in duplicate containing clarithromycin in concentrations ranging from 0.007 to 128 μg/ml. Each isolate was suspended in nutrient broth to yield a turbidity equivalent to McFarland No. 0.5 (approx. 10⁶ CFU/ml). The suspensions were further diluted 1:10 in fresh nutrient broth and an inoculum of 10⁵ CFU per spot was delivered on to the agar surface with a multipoint inoculator (Biddulph Co. Ltd., Manchester, UK). Plates were incubated for 3 days at 37 °C using CampyGen gas packs (Oxoid Ltd., UK). *Haemophilus influenzae* NCTC 10479, *Staphylococcus aureus* ATCC 29213 and *Staphylococcus aureus* strain Oxford NCTC 6571 were used to control for intra-assay variation. *H. pylori* reference strains NCTC 11637 and NCTC 11638 were included as growth controls. The MIC was defined as the lowest concentration of antimicrobial at which there was no visible growth of
bacteria on the agar. The breakpoint MIC value for clarithromycin resistance was defined as $\geq 2 \, \mu g/ml$.

4.3.1.3. E-test method

The E-test was performed as previously described (see section 4.2.1.3).

4.3.1.4. Disc-diffusion test

The disc-diffusion test was performed on \textit{H. pylori} isolates prior to July 1995 by Dr. Huaxiang Xia, St. James's Hospital, Dublin, Ireland. Susceptibilities to clarithromycin were determined according to the methods previously described (Xia et al., 1996a). Isolates were suspended in nutrient broth (Oxoid Ltd., UK) to yield a turbidity of McFarland No. 3 (approx. $9 \times 10^8$ CFU/ml) and the suspension was then inoculated in duplicate onto fresh blood agar plates consisting of Columbia agar base supplemented with 7% horse blood. Susceptibilities to clarithromycin were determined by applying a 15 $\mu g$ clarithromycin paper disc onto the surface of the inoculated plates. Plates were incubated at 37 °C for 3 – 5 days under microaerophilic conditions by flushing the jars with CO$_2$ and the diameters of inhibition zones were subsequently measured for each isolate. For clarithromycin, a previously determined 30 mm breakpoint zone diameter was employed to distinguish between clarithromycin-susceptible and -resistant \textit{H. pylori} isolates (Xia et al., 1996a).

4.3.1.5. Statistical analysis

In order to directly compare MIC values obtained with the agar dilution method, E-test MIC's that fell between two-fold dilution steps were rounded up to the higher concentration. In order to assess the correlation between the MIC values obtained by these two methods, the distribution of the differences in the MIC values obtained by each
method was analysed and these differences were expressed in terms of the number of
two-fold dilutions. The percentage of isolates which yielded values within the limits of
accuracy of the standard tests (± 1 log₂ dilution) was calculated. Since the concentration
ranges differed between the two methods, i.e., the agar dilution method MIC
concentration range was 0.007 to 128 µg/ml while the E-test MIC range was 0.016 to 256
µg/ml, the isolates whose MIC values were outside the range of the tests, e.g., an MIC >
128 or > 256, were excluded from this analysis.
4.3.2. Results

4.3.2.1. Patient population

Seven patients (4 male, 3 female, age range 31 – 61 years; mean 44 years, patients A-G) were included in the study and their clinical details are summarised in Table 4.3. At initial endoscopy, three patients had duodenal ulcer, one patient had duodenitis, two patients had reflux oesophagitis of varying degrees of severity and in one patient the stomach was within normal limits. Review of patient records confirmed that the initial isolates from patients A, B, C, F and G were pre-treatment isolates that had been recovered before the patients had received *H. pylori* eradication treatment. All of the isolates from patients D and E were post-treatment isolates. In total, six pre-treatment isolates and 22 post-treatment isolates were available for analysis.

4.3.2.2. Comparison of the E-test and the disc-diffusion test with the reference agar dilution method

Susceptibility testing of the 28 *H. pylori* isolates to clarithromycin was performed by the agar dilution, E-test and disc-diffusion methods and the results are shown in Table 4.4. Susceptibility testing by either the agar dilution or the E-test method could not be determined for two strains due to unsuccessful recovery from frozen stocks. Clarithromycin susceptibility testing by the reference agar dilution method was performed on 27 *H. pylori* isolates. Of the 12 isolates recovered from patients who had not received *H. pylori* eradication treatment or who had received treatment with a regimen that did not contain clarithromycin, the MIC's ranged from 0.03 to 0.125 μg/ml. However, MIC's of 4 to > 128 μg/ml were obtained with the 15 *H. pylori* strains recovered from patients who had received clarithromycin as a component in the treatment regimen.
Table 4.3. Clinical details and treatment status/regimen of each of the seven patients at the time each *H. pylori* isolate was obtained.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age</th>
<th>OGD date&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Time (months) between samplings&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Clinical pathology&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Treatment status/regimen&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Isolate no.&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>31</td>
<td>05.10.94</td>
<td></td>
<td>DU</td>
<td>pre-Rx</td>
<td>A1a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>02.12.94</td>
<td>2 m</td>
<td>post PPI+C</td>
<td>PPI-l-C-i-M</td>
<td>A2a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>02.12.94</td>
<td></td>
<td>post PPI+C</td>
<td>D2b</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>61</td>
<td>12.03.93</td>
<td></td>
<td>WNL</td>
<td>pre-Rx</td>
<td>B1a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>02.07.93</td>
<td>4 m</td>
<td>post-B+M+T</td>
<td>B2a</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>10.12.93</td>
<td></td>
<td>post-PPI+C</td>
<td>B2b</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>46</td>
<td>30.03.93</td>
<td></td>
<td>DU</td>
<td>pre-Rx</td>
<td>C1a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>09.06.93</td>
<td>2 m</td>
<td>post PPI+C</td>
<td>C2a</td>
<td></td>
</tr>
<tr>
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<td></td>
<td>15.06.95</td>
<td></td>
<td>PPI only</td>
<td>C2b</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>15.06.95</td>
<td></td>
<td>PPI only</td>
<td>C2c</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>38</td>
<td>16.09.94</td>
<td></td>
<td>DI</td>
<td>post B+M+T/</td>
<td>D2a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16.09.94</td>
<td></td>
<td>post B+M+T/</td>
<td>D2b</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>13.02.95</td>
<td>5 m</td>
<td>post B+M+T</td>
<td>D2c</td>
<td></td>
</tr>
<tr>
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<td></td>
<td>13.03.95</td>
<td>1 m</td>
<td>post PPI+C</td>
<td>D2d</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>23.03.95</td>
<td></td>
<td>post PPI+C</td>
<td>D2e</td>
<td></td>
</tr>
<tr>
<td></td>
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<td></td>
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<td>----</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>32</td>
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<td>post B+M+T</td>
<td>E2a</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
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<td>21 m</td>
<td>post PPI+C+M</td>
<td>E2b</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>14.02.95</td>
<td></td>
<td>post PPI+C+M</td>
<td>E2c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>51</td>
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<td>DU</td>
<td>pre-Rx</td>
<td>F1a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>18.04.94</td>
<td></td>
<td>pre-Rx</td>
<td>F1b</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>15.06.94</td>
<td>2 m</td>
<td>post PPI+C</td>
<td>F2a</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>15.06.94</td>
<td></td>
<td>post PPI+C</td>
<td>F2b</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>15.06.94</td>
<td></td>
<td>post PPI+C</td>
<td>F2c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>53</td>
<td>07.04.95</td>
<td>GORD I</td>
<td>pre-Rx</td>
<td>G1a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>02.06.95</td>
<td>2 m</td>
<td>post PPI+C+M</td>
<td>G2a</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>27.07.95</td>
<td>1 m</td>
<td>post PPI+C</td>
<td>G2b</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>20.09.96</td>
<td>13 m</td>
<td>GORD II</td>
<td>post PPI+A+C</td>
<td>G2c</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20.09.96</td>
<td></td>
<td>post PPI+A+C</td>
<td>G2d</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* OGD, oesophago-gastroduodenal endoscopy
* Time elapsed between biopsy samplings to the nearest month
* Clinical pathology observed at endoscopy: DU, duodenal ulcer; WNL, within normal limits; DI duodenitis; GORD, gastro-oesophageal reflux disease grade 1 (I) and grade 2 (II)
* Rx, treatment; PPI, proton-pump inhibitor; C, clarithromycin; M, metronidazole; B, colloidal bismuth citrate; T, tetracycline; A, amoxicillin
* Isolate nomenclature describes the treatment status of the patients; the designation 1 indicates a pre-treatment isolate and the designation 2 indicates a post-treatment isolate. Isolates are then sequentially labelled a, b, c, etc.
* This patient had received B+M+T two years previously
Table 4.4. Treatment status/regimen of each of the seven patients at the time of isolation of each *H. pylori* strain and susceptibility testing of 28 *H. pylori* isolates to clarithromycin by the reference agar dilution method, E-test method and the disc-diffusion test.

<table>
<thead>
<tr>
<th>Patient isolate</th>
<th>OGD date</th>
<th>Rx status/regimen</th>
<th>Clarithromycin MIC (µg/ml)</th>
<th>Disc-diffusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Agar dilution</td>
<td>E-test</td>
</tr>
<tr>
<td>A1a</td>
<td>05.10.94</td>
<td>pre-Rx</td>
<td>0.06</td>
<td>0.064</td>
</tr>
<tr>
<td>A2a</td>
<td>02.12.94</td>
<td>post PPI+C+M</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td>A2b</td>
<td>02.12.94</td>
<td>post PPI+C+M</td>
<td>32</td>
<td>64</td>
</tr>
<tr>
<td>B1a</td>
<td>12.03.93</td>
<td>pre-Rx</td>
<td>0.06</td>
<td>0.064</td>
</tr>
<tr>
<td>B2a</td>
<td>02.07.93</td>
<td>post B+M+T</td>
<td>0.06</td>
<td>0.064</td>
</tr>
<tr>
<td>B2b</td>
<td>10.12.93</td>
<td>post PPI+C</td>
<td>64</td>
<td>&gt;256</td>
</tr>
<tr>
<td>C1a</td>
<td>30.03.93</td>
<td>pre-Rx</td>
<td>0.125</td>
<td>0.125</td>
</tr>
<tr>
<td>C2a</td>
<td>09.06.93</td>
<td>post PPI+C</td>
<td>128</td>
<td>&gt;256</td>
</tr>
<tr>
<td>C2b</td>
<td>15.06.95</td>
<td>PPI only</td>
<td>128</td>
<td>&gt;256</td>
</tr>
<tr>
<td>C2c</td>
<td>15.06.95</td>
<td>PPI only</td>
<td>&gt;128</td>
<td>&gt;256</td>
</tr>
<tr>
<td>D2a</td>
<td>16.09.94</td>
<td>post B+M+T</td>
<td>0.06</td>
<td>0.064</td>
</tr>
<tr>
<td>D2b</td>
<td>16.09.94</td>
<td>post B+M+T</td>
<td>0.06</td>
<td>0.032</td>
</tr>
<tr>
<td>D2c</td>
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<td>post B+M+T</td>
<td>0.125</td>
<td>0.064</td>
</tr>
<tr>
<td>D2d</td>
<td>13.02.95</td>
<td>post B+M+T</td>
<td>0.125</td>
<td>0.064</td>
</tr>
<tr>
<td>D2e</td>
<td>23.03.95</td>
<td>post PPI+C+M</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>E2a</td>
<td>23.04.93</td>
<td>post B+M+T</td>
<td>0.125</td>
<td>0.032</td>
</tr>
<tr>
<td>E2b</td>
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<td>post PPI+C+M</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td>E2c</td>
<td>14.02.95</td>
<td>post PPI+C+M</td>
<td>16</td>
<td>nd</td>
</tr>
<tr>
<td>F1a</td>
<td>18.04.94</td>
<td>pre-Rx</td>
<td>0.03</td>
<td>0.016</td>
</tr>
<tr>
<td>F1b</td>
<td>18.04.94</td>
<td>pre-Rx</td>
<td>0.03</td>
<td>0.064</td>
</tr>
<tr>
<td>F2a</td>
<td>15.06.94</td>
<td>post PPI+C</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>F2b</td>
<td>15.06.94</td>
<td>post PPI+C</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td>F2c</td>
<td>15.06.94</td>
<td>post PPI+C</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td>G1a</td>
<td>07.04.95</td>
<td>pre-Rx</td>
<td>0.06</td>
<td>0.064</td>
</tr>
<tr>
<td>G2a</td>
<td>02.06.95</td>
<td>post PPI+C+M</td>
<td>32</td>
<td>128</td>
</tr>
<tr>
<td>G2b</td>
<td>27.07.95</td>
<td>post PPI+C</td>
<td>32</td>
<td>&gt;256</td>
</tr>
<tr>
<td>G2c</td>
<td>20.09.96</td>
<td>post PPI+A+C</td>
<td>32</td>
<td>64</td>
</tr>
<tr>
<td>G2d</td>
<td>20.09.96</td>
<td>post PPI+A+C</td>
<td>nd</td>
<td>64</td>
</tr>
</tbody>
</table>

^a OGD, oesophago-gastroduodenal endoscopy

^b Rx, treatment; see Table 4.3. footnote for description

^c Breakpoint for clarithromycin susceptibility, MIC < 2 µg/ml

^d S, susceptible; R, resistant

^e Patient had received B+M+T two years previously

nd, not determined
Table 4.5. Distribution of differences of two-fold dilutions in the MIC values of clarithromycin for 26 *H. pylori* isolates, as determined by the agar dilution and E-test methods.

<table>
<thead>
<tr>
<th>Antimicrobial (no. of strains)</th>
<th>No. of strains for which MIC's differed by the indicated number of log₂ dilutions</th>
<th>Results out of range</th>
<th>Percentage correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&gt; +2</td>
<td>+2</td>
<td>+1</td>
</tr>
<tr>
<td>Clarithromycin (21)</td>
<td>0</td>
<td>1</td>
<td>6</td>
</tr>
</tbody>
</table>

*a Correlation of *H. pylori* strains with MIC’s within the concentration range of the agar dilution method. The total number of strains tested equals the in-range and out-of-range results.

*b +, means higher MIC’s with the reference agar dilution method; -, means higher MIC’s with the E-test method.

c Either one or both of the agar dilution and E-test MIC results were outside the highest antimicrobial concentration range.

*d The percentage of *H. pylori* isolates (in range) within \( \pm 1 \) log₂ dilution limits of accuracy.
Susceptibility testing results as determined by the reference agar dilution and E-test methods were available for 26 isolates of *H. pylori*. The correlation between the MIC's determined by these two methods is shown in Table 4.5. The correlation between the two methods was possible for only 21 of 26 (81%) of the isolates since with the remaining isolates one or both MIC values were outside the highest antimicrobial concentration. The agreement between the two methods was high (90% within $\pm 1 \log_2$ dilution step) and the correlation between $\pm 2 \log_2$ two-fold dilutions was 100%. The MIC's obtained with the E-test tended to be lower than those obtained by the reference agar dilution method. No major error (resistant by the E-test and susceptible by the reference method) or very major error (susceptible by the E-test and resistant by the reference method) was found between the E-test and agar dilution MIC’s for the 26 *H. pylori* isolates.

Comparison of the disc-diffusion test with the reference agar dilution method was possible for 27 *H. pylori* strains (Table 4.4). By applying a breakpoint MIC of $\geq 2 \mu g/ml$ to define clarithromycin resistance, the disc-diffusion test correctly categorised all (100%) of the *H. pylori* strains.
4.4. Discussion

4.4.1. Prevalence of primary metronidazole, clarithromycin and amoxicillin resistance in H. pylori

The susceptibility results of this study showed that, although a low proportion (4%) of \( H. \) pylori strains demonstrated resistance to clarithromycin, metronidazole resistance was high (29%). The prevalence of clarithromycin resistance among \( H. \) pylori strains obtained in this study is lower than the rates observed in other European countries, e.g., France (10%), Belgium (11%), Italy (10%), (Jovene et al., 1999), but higher than Switzerland (0%) and The Netherlands (3%) (Mégraud, 1994; Mégraud, 1998). However, the high prevalence (10 – 50%) of metronidazole resistance observed among European centres (Glupczynski, 1992) and in the present study is a cause for concern and may be increasing due to the use of nitroimidazoles for other infections. Indeed, the present study found that the incidence of metronidazole resistance was higher among isolates from patients aged between 50 – 69 years. This may reflect the previous use of this antimicrobial for the treatment of gastrointestinal or gynaecological infections. The finding that the incidence of metronidazole resistance was not significantly different between men and women was unexpected since a higher prevalence of metronidazole resistance in women has been reported elsewhere (Glupczynski, 1992; Banatvala et al., 1994; Midolo et al., 1996b). This may be due to the low number of \( H. \) pylori strains analysed in the study and this association may become clearer when results are compiled from the other 19 European test centres.

The susceptibility results of this study, compared with earlier studies in Dublin using \( H. \) pylori strains isolated in this laboratory, showed decreased resistance rates to clarithromycin and metronidazole (Xia et al., 1996a; Buckley et al., 1997; Lee et al., 1999). The decreased primary resistance rates obtained for these agents may be due to
the smaller number of *H. pylori* isolates tested and the different methods employed for susceptibility testing, *i.e.*, E-test compared to the disc-diffusion test. Nonetheless, comparison of primary resistance rates since 1995 to 1999 suggests that metronidazole resistance remains high (mean 33%), clarithromycin resistance is low (mean 4%), and prevalence rates do not appear to be increasing.

The present study assessed the reproducibility of the E-test by determining the MIC’s for a set of three *H. pylori* reference strains for amoxicillin, clarithromycin and metronidazole on three separate occasions. Reproducible MIC’s were obtained for all three *H. pylori* reference strains for amoxicillin throughout the study. Better reproducibility was observed at the beginning and at the half-way stage for all three strains against clarithromycin and metronidazole. However, poor reproducibility was observed at the completion of the study as higher MIC’s of $\geq 1.5 \log_2$ dilutions were obtained for all of the strains against clarithromycin. The factors affecting the reproducibility of susceptibility testing, *e.g.*, medium, pH, inoculum and incubation time, are well known (Berger *et al.*, 1993; Henriksen *et al.*, 1996; Hartzen *et al.*, 1997). Although this study attempted to control for these variables, it is possible that the poor reproducibility observed in some instances with the E-test may have been due to either small fluctuations in the inoculum density or the stability of the E-test strips.

Primary metronidazole resistance (Thijs *et al.*, 1997; Buckley *et al.*, 1997) and clarithromycin resistance (Wurzer *et al.*, 1997) are important factors that reduce the clinical efficacy of *H. pylori* eradication therapies. In the present study, primary amoxicillin resistance was not encountered and until recently, resistance to this antimicrobial had not been demonstrated (Dore *et al.*, 1997; van Zwet *et al.*, 1998). Furthermore, two *H. pylori* isolates demonstrated dual resistance to metronidazole and
clarithromycin. Dual resistance and, more importantly, the high rate of acquisition of secondary clarithromycin resistance among patients who fail first-line eradication treatment (Buckley et al., 1997; Teare et al., 1999; Lee et al., 1999) is an increasing problem facing the clinical management of *H. pylori* infection. There is concern that the selection of resistant strains may become widespread in the population which would arise if ineffective treatment regimens are prescribed (Penston and Mistry, 1996).

This study was conducted as part of the first multi-centre European study in which *H. pylori* resistance to clarithromycin and amoxicillin was assessed with a well defined *in vitro* testing procedure. The low number of *H. pylori* strains analysed in the Irish component of this European study requires that the results should be interpreted with caution. The low prevalence of primary clarithromycin resistance suggests that routine susceptibility testing of pre-treatment *H. pylori* isolates is not cost-effective. The high incidence of metronidazole resistance among patients between the ages of 50 to 69 years suggests that treatment with a regimen containing this agent is not appropriate in elderly patients. Routine susceptibility testing of *H. pylori* strains from patients who fail *H. pylori* eradication treatment is advisable.

4.4.2. Direct comparison of the agar dilution method, *E*-test and the disc-diffusion test for susceptibility testing of *H. pylori*

The ideal routine test for the determination of antimicrobial susceptibility should be easy to perform, accurate, reproducible and cost-effective. The agar dilution method is considered the reference susceptibility method. However, it is labour intensive, expensive and is not suitable for testing a small number of individual isolates. Therefore, other susceptibility methods, e.g., the disc-diffusion test and more recently the E-test, are available which are rapid, less labour intensive and more applicable for routine use.
Numerous studies have evaluated the E-test as an antimicrobial susceptibility testing method for a broad range of Gram-negative bacteria (Brown and Brown, 1991; Baker et al., 1991) and fastidious organisms (Jorgensen et al., 1991; Herra et al., 1995). However, few studies have evaluated the E-test method for the susceptibility testing of *H. pylori* to clarithromycin. Two such studies have found a good correlation between the MIC's determined by the E-test and either the agar dilution or broth microdilution reference methods with 94.1% and 88.5% agreement, respectively, within the ± 1 log₂ dilution limit of accuracy (Hachem et al., 1996; Piccolomini et al., 1997b). Similarly, ≥ 90% agreement has been reported in studies assessing the correlation between the E-test and agar dilution methods for erythromycin (Glupczynski et al., 1991; Cederbrant et al., 1993).

The present study evaluated the E-test using a high percentage of resistant isolates to verify the ability of this test to detect resistance. Analysis of the MIC values in this study found that the E-test yielded very high agreement (90.4%) when compared with the reference agar dilution method against selected *H. pylori* strains. All strains with documented resistance to clarithromycin were detected by the E-test. This result was expected as clarithromycin exhibits a bimodal distribution of MIC values and strains are easily divided into susceptible and resistant populations. The study also found that the disc-diffusion test yielded excellent agreement (100%) when compared with the reference method.

Susceptibility testing to antimicrobial agents that are components of combination treatment regimens is warranted in patients in whom eradication therapy for *H. pylori* infection fails so that an effective second-line treatment regimen may be prescribed. Since the MIC values between clarithromycin-susceptible and -resistant populations is so
distinct, any of the *in vitro* susceptibility testing methods is applicable. Indeed, the accuracy of the E-test MIC values found in this study suggests that this method may be suitable as a routine susceptibility method. However, while the E-test is rapid and easy to perform, the high cost of the test may preclude its use as a routine method. Accurate results were also obtained with the disc-diffusion test which is a more cost-effective method and provides a qualitative result that is sufficient for the clinical management of *H. pylori* infection.
Chapter 5

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5.1. Introduction

It is now standard clinical practice to eradicate *H. pylori* infection in patients who present with low-grade MALT lymphoma of the B-cell type or peptic ulcer (NIH, 1994; EHPSG, 1997). However, the indications for treatment are expanding to other patients with *H. pylori*-associated diseases such as reflux oesophagitis and non-ulcer dyspepsia. Presently, the recommended treatment consists of a one-week proton pump inhibitor (PPI)-based ‘triple therapy’ consisting of a PPI and two of the following antimicrobials, namely, a macrolide (clarithromycin), a nitroimidazole (either metronidazole or tinidazole) and amoxicillin (EHPSG, 1997; Lee and O'Morain, 1997). Effective *H. pylori* eradication treatment prevents ulcer recurrence (Coghlan et al., 1987) and leads to duodenal ulcer cure (Rauws and Tytgat, 1990; Patchett et al., 1992; Forbes et al., 1994). Although eradication rates of > 90% are achieved in many multi-centre studies (Lind et al., 1996; Lind et al., 1999), the actual success rates in clinical practice are often compromised by sub-optimal treatment regimens (Penston and Mistry, 1996), poor patient compliance (Graham et al., 1992) and an underlying primary resistance to nitroimidazoles and macrolides (Mégraud, 1997a). Consequently, treatment failure is associated with resistance to one or both of the antimicrobials used in the regimen (Wurzer et al., 1997; Lind et al., 1999).

*H. pylori* recurrence may be due to the presence of the original strain which was only transiently suppressed (recrudescence), or it may be due to true reinfection with the same strain or a different strain (see section 1.12). Studies have shown that the vast majority of ‘reinfections’ occur within the first year after *H. pylori* eradication treatment (Bell et al., 1993a), and are, among other factors, directly related to the effectiveness of the treatment regimen (see section 1.12.1, Xia, et al., 1995b; Bell et al., 1996). Molecular genotyping of *H. pylori* isolates recovered from patients before and after treatment has
shown that these early 'reinfections' are in fact recrudescence of the original bacterium (Langenberg et al., 1986; Xia et al., 1995a; van der Hulst et al., 1997). It would appear therefore, that the low-level infection was undetected by conventional diagnostic tests at assessment four weeks after the patient had completed eradication treatment, resulting in recrudescence in the ensuing months following treatment. Therefore, if reinfection is strictly defined as the recurrence of the infection one year after treatment where complete eradication of *H. pylori* had been accomplished following initial treatment, then statistics show that the true reinfection rate is low and is estimated to be approximately 1% per year (Bell et al., 1993a; Borody et al., 1994; Bell and Powell, 1996; Abu-Mahfouz et al., 1997).

This study was conducted to determine whether recurrence of *H. pylori* was due to reinfection or recrudescence by molecular characterisation of the pre-treatment and recurrent isolates from patients, who had been treated with eradication therapy, using three fingerprinting techniques, namely, random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR), repeat sequence oligonucleotide analysis and *cagA* gene RFLP analysis. Furthermore, with reference to the susceptibility testing data of these isolates (Chapter 4), this study aimed to investigate whether acquired antimicrobial resistance was due to *in vivo* strain selection.
5.2. Materials and Methods

5.2.1. Patient population and H. pylori isolates

DNA fingerprinting studies were performed on H. pylori isolates that had been recovered from antral biopsy specimens obtained before (six isolates) and after (22 isolates) eradication treatment (see sections 4.3.1.1 and 4.3.2.1). A review of patient charts confirmed that the initial H. pylori isolates recovered from five patients (patients A, B, C, F and G) were pre-treatment isolates. However, the initial H. pylori isolates recovered from two patients (patients D and E) were post-treatment isolates.

5.2.2. MIC determination of H. pylori isolates to clarithromycin

In vitro susceptibility testing of H. pylori isolates to clarithromycin was performed by the agar dilution method as previously described (section 4.3.1.2). The MIC values of H. pylori isolates to clarithromycin are shown in Table 4.4. Pre-treatment H. pylori isolates and isolates recovered from patients who had not received a clarithromycin-based eradication regimen were susceptible to clarithromycin (MIC < 2 μg/ml). Conversely, H. pylori isolates recovered from patients who had failed eradication treatment with either a dual or triple clarithromycin-based treatment regimen had acquired resistance to clarithromycin.

5.2.3. RAPD-PCR fingerprinting

Optimisation of the RAPD-PCR conditions had been determined previously (Marshall, 1996b) and these conditions were employed in the present study. H. pylori DNA was extracted and quantitated as previously described (section 2.2.1). RAPD-PCR fingerprinting was performed using a combination of three primer sets (Akopyanz et al., 1992) namely, primers 1281 and 1283, D11344 and 1281 and primers 1254 and D8635 (Table 5.1). For reproducibility, the same primer batches were used in all of the RAPD-
### Table 5.1. Oligonucleotide primers used for RAPD-PCR

<table>
<thead>
<tr>
<th>Primer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Primer sequence (5'–3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>1254</td>
<td>CCGCAGCCAA</td>
</tr>
<tr>
<td>D11344</td>
<td>AGTGAATTTCGCGGTGAGATGCCA</td>
</tr>
<tr>
<td>1281</td>
<td>AACGCGCAAC</td>
</tr>
<tr>
<td>1283</td>
<td>GCGATCCCCCA</td>
</tr>
<tr>
<td>D8635</td>
<td>GAGCGGCCAAGGGAGCAGAC</td>
</tr>
</tbody>
</table>

<sup>a</sup> Primer nomenclature according to Akopyanz et al., 1992
PCR reactions. A 25-μl reaction mix was prepared containing 3 mM MgCl₂, 20 pmol of each primer, 2.5 U of Taq DNA polymerase (Promega, WI, USA) and 250 μM each of dCTP, dGTP, dATP, dTTP (Promega, WI, USA) in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 1% (v/v) Triton X-100. The mixture was overlaid with 30 μl of mineral oil and 100 ng of H. pylori DNA was then added to the reaction mix. The cycling programme (PTC-100; MJ Research Inc.) consisted of 4 cycles of 94 °C for 5 min, 36 °C for 5 min and 72 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 36 °C for 1 min and 72 °C for 2 min with a final cycle of 72 °C for 10 min. DNA from H. pylori reference strain NCTC 11638 was used as a positive control and sterile distilled water was used as template for the negative control in all reactions. PCR products and a 1 kb DNA molecular weight marker (Gibco, BRL) were run at 70 V for 5 h on a 2% (w/v) agarose gel containing ethidium bromide. The gels were photographed under ultraviolet light.

5.2.4. Hybridisation band analysis with a repeat sequence oligonucleotide (GTG)₅

Oligonucleotide hybridisation was performed according to the method of Marshall et al. (1996a) with some modifications. The methodology was adapted for the use of a non-radioactive, chemiluminescent detection system (see section 2.7.2). DNA from each of the patient isolates and H. pylori reference strain NCTC 11638 was extracted and the amount of DNA recovered was quantitated (see section 2.2.1). Total genomic DNA (1.5 – 3.0 μg) was digested overnight at 37 °C with 10 U of HindIII restriction enzyme (Boehringer Mannheim GmbH, Germany) according to the manufacturer’s instructions. Digested DNA was separated on a 1% agarose gel (see section 2.3) and transferred to a nylon membrane as previously described (see section 2.4). The (pre)hybridisation temperature for the (GTG)₅ oligonucleotide probe was calculated according to the method of Thein and Wallace (1986). Nylon membranes were pre-hybridised at 45 °C for 2 h in hybridisation bottles containing high SDS prehybridisation solution [7% (w/v) SDS, 50%
formamide, 5 X SSC (750 mM NaCl, 75 mM sodium citrate), 2% blocking reagent (Boehringer Mannheim), 50 mM NaH₂PO₄ (pH 7.0), 0.1% (w/v) N-lauroylsarcosine) in a rotary hybridisation oven. Following pre-hybridisation, a fresh hybridisation solution containing a commercially purchased Digoxigenin-labelled (GTG)₅ oligonucleotide probe (Genosys, UK) was added and the membrane was incubated at 45 °C overnight. Filters were washed twice at room temperature with 6 X SSC, 0.1% SDS buffer for 15 min and then washed twice with 2 X SSC, 0.1% SDS buffer for 15 min. Membranes were developed according to the manufacturer’s instructions using the chemiluminescent detection system (see section 2.8.2).

5.2.5. Hybridisation with a cytotoxin-associated gene A (cagA) gene probe

Filters were washed to remove bound probe (see section 2.9). Plasmid pMC3 which contains a 3.6 kb fragment of the cagA gene of *H. pylori* strain 84 – 183 (Tummuru *et al.*, 1993) was HindIII-digested and the 3.6 kb fragment was agarose-gel-purified (see section 2.5) and Digoxigenin-labelled (see section 2.6.3). Filters were re-hybridised at 68 °C with this probe as previously described (see section 2.7.2). *H. pylori* reference strain NCTC 11638 which harbours the cagA gene was used as a positive control.

5.2.6. Data analysis

Band profiles generated using the repeat sequence oligonucleotide (GTG)₅, the cagA gene and RAPD-PCR were visually compared. RAPD-PCR profiles generated from *H. pylori* isolates from the same individual that differed by ≤ 3 bands were classified as subtypes of a predominant strain. However, RAPD-PCR profiles generated from *H. pylori* isolates from the same individual that differed by > 3 bands were classified as a different strain.
5.3. Results

5.3.1. RAPD-PCR analysis of *H. pylori* isolates

RAPD-PCR fingerprinting was performed on the 28 *H. pylori* isolates from seven patients using a combination of three primer sets in order to sample a larger area of the *H. pylori* genome. RAPD-PCR yielded amplimer profiles ranging from 9 – 17 bands of between 200 bp and > 3 kb. RAPD-PCR reproducibility was demonstrated by the consistency of profiles generated with the *H. pylori* reference strain. Common amplimer bands were shared between profiles of isolates from different patients with all primer combinations. *H. pylori* isolates from the same patient yielded RAPD-PCR profiles that were very similar or identical to each other in six patients (patients A, C-G) with the three primer sets tested (Figs. 5.1 – 5.3).

Minor differences were observed in the individual RAPD-PCR profiles of isolates from the same patient as demonstrated by the presence or absence of certain bands. This was observed in the case of individual isolates from two patients (patients A and E). In the case of isolates from patient A, a band of approximately 1800 bp (compare lanes 2-4 in Figs. 5.1a and 5.2a) was present in the pre-treatment isolate, A1a, but absent in the post-treatment isolates whereas the converse occurred for a band of approximately 1500 bp with primers D11344 and 1281 (compare lanes 3-5 in Fig. 5.3a). In patient E, a band of approximately 550 bp was present in the post-treatment isolate E2a, but absent in the remaining isolates whereas bands of approximately 1100 bp and 1500 bp were absent in the post-treatment isolate, E2a, but present in the remaining isolates (compare lanes 2-4 in Fig. 5.1c).

The isolates from patient B were less similar to each other than those from patients A and C-G. The individual RAPD-PCR profiles generated from the three *H. pylori* isolates...
Figure 5.1. RAPD-PCR profiles, generated using an equimolar combination of primers 1281 and 1283, from *H. pylori* isolates recovered from pre- and post-treatment biopsy specimens from patients A-G.

Agarose gels showing RAPD-PCR profiles of *H. pylori* isolates recovered before and after eradication treatment (see Table 4.3 for nomenclature). (a) Isolates recovered from patients A-C; (b) Isolates recovered from patients D and G; and (c) Isolates recovered from patients E and F. DNA from *H. pylori* reference strain NCTC 11638 (R) and a negative control (N) were included in all reactions. Molecular size markers (bp) are indicated.
Figure 5.2. RAPD-PCR profiles, generated using an equimolar combination of primers 1254 and D8635 and primers D11344 and 1281, from *H. pylori* isolates recovered from pre- and post-treatment gastric biopsies from patients A-G.

Agarose gels showing RAPD-PCR profiles of *H. pylori* isolates recovered from patients before and after eradication treatment. (a) Isolates recovered from patients A, B and D; (b) Isolates recovered from patients C and F; (c) Lanes 2-6, isolates from patient G using primers D11344 and 1281; lanes 7-14, isolates recovered from patients G and E using primers 1254 and D8635. DNA from *H. pylori* reference strain NCTC 11638 (R) and a negative control (N) were included in all reactions. Molecular size markers (bp) are indicated.
Figure 5.3. RAPD-PCR profiles generated using primers D11344 and 1281 from *H. pylori* isolates recovered from patients before and after eradication treatment from patients A-F.

Agarose gels showing RAPD-PCR profiles of *H. pylori* isolates from (a) Patients A-C and (b) Patients D-F. DNA from *H. pylori* reference strain NCTC 11638 (R) and a negative control (N) were included in all reactions. Molecular size markers (bp) are indicated.
from patient B were similar to each other with primer sets D11344 and 1281 (compare lanes 6-8 in Fig. 5.3a). However, the profile of the pre-treatment isolate, B1a, more closely resembled that of the second post-treatment isolate, isolate B2b, with the primer sets 1281 and 1283 (compare lanes 5-7 in Fig. 5.1a) than the profile generated with the first post-treatment isolate, isolate B2a. In contrast, the profiles of the post-treatment isolates, isolates B2a and B2b, more closely resembled each other with primer sets 1254 and D11344 (compare lanes 5-7 in Fig. 5.2a) than the profile generated with the pre-treatment isolate, isolate B1a.

5.3.2. Repeat sequence oligonucleotide analysis of H. pylori isolates

DNA hybridisation band analysis with the repeat sequence oligonucleotide (GTG), generated complicated hybridisation band patterns with 26 to 33 bands of between < 564 bp to 21.2 kb. Although the direct visual comparison of profiles was difficult, similar DNA profiles were generated from the individual isolates from patients A, C-G (Fig. 5.4). The DNA profiles generated from the three isolates recovered from patient B were similar to each other but some band differences were apparent in isolate B2a (Fig. 5.4a).

5.3.3. Screening of digested genomic DNA for the cagA gene

5.3.3.1. cagA gene status

Hybridisation of HindIII-digested H. pylori DNA with the cagA gene probe generated simple hybridisation band patterns of between 275 bp and > 4.0 kbp size (Fig. 5.5). The cagA gene was present in 25 (89%) of the H. pylori isolates. All of the isolates from five patients (patients A, C-F) were cagA-positive while two patients (patients B and G) harboured H. pylori isolates that were cagA-positive and cagA-negative (Fig. 5.5a and c). Of the isolates recovered from patient G, one pre-treatment and one post-treatment isolate were cagA-negative while the remaining post-treatment isolates were cagA-
Figure 5.4. DNA fingerprint profiles, generated by (GTG)$_5$ repeat sequence oligonucleotide hybridisation, from pre- and post-treatment *H. pylori* isolates recovered from patients A-G.

*HindIII*-digested *H. pylori* DNA was transferred to a nylon membrane by Southern blotting and the resulting band profiles were generated by hybridisation with a DIG-labelled (GTG)$_5$ repeat sequence oligonucleotide. Band profiles of *H. pylori*: (a) Isolates recovered from patient B; (b) Isolates from patient E; (c) Isolates from patients A, C and D; and (d) Isolates from patients F and G.
positive. This \textit{cagA}-positive strain persisted > 15 months after initial eradication treatment (see Table 4.3. for details). Of the isolates recovered from patient B, the pre-treatment and the following post-treatment isolate possessed the \textit{cagA} gene but the \textit{cagA} gene was absent in the final post-treatment isolate (Fig. 5.5a).

5.3.3.2. \textit{cagA} gene RFLP

\textit{CagA} gene RFLP patterns were compared among individual \textit{H. pylori} isolates from the same patient. In four patients (patients A, C, D and F), the \textit{cagA} gene hybridisation band patterns generated from individual \textit{H. pylori} isolates from the same patient were identical (Fig. 5.5). However, the \textit{cagA} gene band patterns obtained from individual patients were different from each other. The post-treatment isolates recovered from patient E demonstrated slight variation within the \textit{cagA} gene as noted by the upper band in the pattern produced with isolate E2a compared with the other isolates, E2b and E2c (compare lanes 9-11 in Fig. 5.5d).

Of the three isolates from patient B, who harboured \textit{H. pylori} strains of mixed \textit{cagA} status, the two \textit{cagA}-positive isolates generated RFLP band patterns that were different from each other (Fig. 5.5a). In contrast, comparison of \textit{cagA} gene RFLP patterns among individual isolates recovered from patient G, who also harboured a mixed \textit{H. pylori} population of \textit{cagA}-positive and \textit{cagA}-negative strains, revealed identical band patterns in all of the post-treatment isolates that possessed the \textit{cagA} gene (Fig. 5.5c).
Figure 5.5. *cagA* gene hybridisation band patterns generated from *H. pylori* isolates recovered from pre- and post-treatment gastric biopsies from patients A-G.

Nylon membranes with *HindIII*-digested *H. pylori* DNA were hybridised with a 3.6 kbp DIG-labelled fragment of the *cagA* gene. Hybridisation band patterns generated from isolates from (a) Patient B; (b) Patient D; (c) Patients F and G and (d) Patients A, C and E. *H. pylori* reference strain NCTC 11638 (R) that possesses the *cagA* gene was included as a positive control. Artefacts are indicated by black arrows. Molecular size (kbp and bp) markers are indicated.

<table>
<thead>
<tr>
<th>kbp</th>
<th>Patient A</th>
<th>Patient C</th>
<th>Patient E</th>
<th>bp</th>
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<tbody>
<tr>
<td>M</td>
<td>1a</td>
<td>2a</td>
<td>1a</td>
<td>R</td>
</tr>
<tr>
<td>3.53</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>1.58</td>
<td>-</td>
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<td>0.94</td>
<td>-</td>
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<td>0.56</td>
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</tr>
<tr>
<td>1650</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>
5.4. Discussion

A review of patient charts confirmed that five patients (patients A, B, C, F and G) had not received *H. pylori* eradication treatment. Accordingly, the genotyping data generated from these patient’s isolates could be employed to determine whether *H. pylori* recurrence in these patients was due to recrudescence of a temporarily suppressed infection or reinfection with a different strain.

RFLP analysis of the *cagA* gene demonstrated that isolates recovered before and after treatment were identical in four of these five patients (A, C, F and G), while RAPD-PCR analysis and the DNA band patterns generated with the repeat sequence oligonucleotide yielded similar profiles in all of the isolates from the same four patients. Taken together these molecular fingerprinting data confirm that patients A, C, F and G suffered relapse of infection with their original strains, *i.e.* recrudescence rather than reinfection.

However, RAPD-PCR analysis of the *H. pylori* isolates from one patient (patient B) gave results indicating somewhat less identity with different primer combinations; all of the three isolates from this patient were quite similar to each other with one of the primer combinations, but a greater similarity was observed between the pre-treatment and the second post-treatment isolate with the other primer sets. The *cagA* gene RFLP analysis mirrored these results. The latter finding was also obtained with the repeat sequence oligonucleotide analysis. This patient is probably infected with two different subtypes of the same strain that have undergone microevolution *in vivo* (Marshall, 1996b; Marshall *et al.*, 1998) and recrudescence of the original subtype was observed eight months after initial eradication treatment.
Collectively, the data suggest that recurrence of the infection is largely due to recrudescence of the original strain or a subtype variant rather than reinfection with a new strain. This is not a surprising finding since the majority of patients were found to harbour the infection by conventional diagnostic tests (i.e. culture) at the follow-up endoscopy (mean 2.1 months after completing *H. pylori* eradication treatment). Furthermore, in the present study, similar RAPD-PCR profiles and repeat sequence hybridisation band patterns were observed in clarithromycin-susceptible and -resistant isolates recovered from the same patient. Therefore, acquired resistance to clarithromycin in these patients is most likely due to *in vivo* strain selection rather than reinfection with a different clarithromycin-resistant strain.

The variation between hybridisation band profiles among individual patients observed with the repeat sequence oligonucleotide and the *cagA* gene RFLP analysis confirm the genetic variability exhibited by *H. pylori* *in vivo* in individual patients. The RAPD-PCR profile differences noted between individual isolates from the same patient may represent mutations or *in vivo* rearrangements. Indeed, variations either within the *cagA* gene or in *cagA* gene status may account for the differences observed in the RAPD-PCR profiles. The finding that consistent RAPD-PCR profiles were generated among the isolates from patient G, who harboured a mixed population of *cagA*-positive and *cagA*-negative strains, rules out the possibility that the differences seen in RAPD-PCR profiles in the individual isolates from patients E and B are due to the variation within the *cagA* gene itself. Hence, the likely explanation for the differences in RAPD-PCR fingerprints may be the presence or absence of plasmids (Oudbier *et al.*, 1990; Cellini *et al.*, 1996). Alternatively, minor RAPD-PCR band differences may be due to the coexistence of different subpopulations of bacteria which may have been detected if multiple pre-treatment biopsies were available for genotyping analysis (Couthino, 1993).
In the present study, it may be argued that the genotyping profiles represent the combined DNA fingerprints of more than one *H. pylori* strain harboured by individual patients since the nature of the *H. pylori* collection was such that a multiple colony sweep of bacteria was used for genotyping analysis. However, RAPD-PCR analysis of single colonies recovered from Irish patients has demonstrated that a homogenous *H. pylori* population exists at one site in the stomach (Marshall et al., 1995) and hence the fingerprinting profiles shown here are probably representative of an homogenous population of bacteria. The latter study and other studies of the same nature (Taylor et al., 1995) have found that patients can be infected with more than one *H. pylori* strain. Unfortunately, only one antral biopsy was available for culture at the pre-treatment visit and the inclusion of multiple biopsies from different gastric sites and even genotyping of single colonies could possibly have identified different *H. pylori* strains or subtypes. Indeed, RAPD-PCR genotyping studies of single colonies recovered from multiple biopsies from the antral and corporal sites have found that patients may be infected with up to six *H. pylori* variants or subtypes (Jorgensen et al., 1996).

caga gene analysis found that two patients harboured a mixed *H. pylori* population of caga-positive and caga-negative strains, a phenomenon that has been reported by others (Fantry et al., 1996; van der Ende et al., 1996; Figura et al., 1998). Moreover, Enroth et al. (1999) found that in 5 patients, the caga gene status varied between single colonies despite the finding that the AP-PCR banding patterns generated from these colonies were identical. In one patient (patient G), the two initial *H. pylori* isolates were caga-negative but the gene was then detected in the isolates recovered from subsequent biopsy specimens 15 months after initial eradication treatment. This patient was probably initially infected with a caga-positive variant but this population was not detected due to the limited number of pre-treatment biopsies analysed in this study. Conversely,
genotyping analysis of the isolates from patient B demonstrated recrudescence of the original strain approximately eight months after initial eradication treatment and that the \textit{cagA} gene had been lost during the intervening months. Chromosomal excision has been proposed as a mechanism that can lead to the loss of the \textit{cag-PAI}. Kersulyte \textit{et al.} (1999) demonstrated that \textit{cagA}-negative strains probably arise from a \textit{cagA}-positive strain as the result of transfer of DNA containing an ‘empty site’ allele from a \textit{cagA}-negative strain and homologous recombination rather than excision of the \textit{cag-PAI} without DNA transfer.

In conclusion, the findings of the present study show that \textit{H. pylori} recurrence in patients who have received eradication treatment is due to recrudescence of the original infection rather than reinfection with a different strain. Clarithromycin resistance is due to \textit{in vivo} strain selection of a resistant population rather than reinfection with a different resistant strain. Patients may be infected with more than one strain/subtype at one site in the stomach and a mixed infection of \textit{cagA}-positive and \textit{cagA}-negative strains can exist in the same patient.
Chapter 6

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6.1. Introduction

Clarithromycin, an acid stable new generation macrolide, is an important component of triple therapy treatment regimens currently employed for *H. pylori* eradication. The level of primary clarithromycin resistance among different countries is estimated to be between 1 to 10% and in Ireland, surveillance studies have found that the primary level of clarithromycin resistance is 3.9% (see Chapter 4). Clarithromycin resistance is an important cause of treatment failure (see section 1.10.2). Furthermore, clarithromycin resistance develops rapidly *in vivo* and patients who fail eradication treatment often harbour strains that have acquired resistance to clarithromycin. Moreover, coupled with a high level of metronidazole resistance, first line eradication treatment with a one week treatment regimen of omeprazole, metronidazole and clarithromycin has shown that secondary clarithromycin resistance was acquired in 58% of strains in cases of treatment failure (Buckley *et al.*, 1997).

The mechanism of action of macrolides is mediated by the reversible binding of these compounds to the peptidyltransferase region of the 23S rRNA (see section 1.14.4.2). Binding prevents the release of tRNA after peptide bond formation and ultimately inhibits protein synthesis. Using the methodology originally employed to detect clarithromycin resistance in *Mycobacterium intracellulare* (Meier *et al.*, 1994), Versalovic *et al.* (1996) reported that point mutations in the peptidyltransferase region (domain V) of the 23S rRNA gene confer clarithromycin resistance in *H. pylori* (Versalovic *et al.*, 1996). In *H. pylori*, altered target site resistance is mediated by three known point mutations of the 23S rRNA gene at positions 2143 and 2144 (cognate to positions 2058 and 2059 in *E. coli*), namely, an A-to-G transition mutation at position 2143 or 2144 (Versalovic *et al.*, 1996) or an A-to-C transversion mutation at position 2143 (Stone *et al.*, 1996). The point mutations of the 23S rRNA gene region associated

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with clarithromycin resistance have been confirmed by a number of centres worldwide (Debets-Ossenkopp et al., 1996; Stone et al., 1997; Occhialini et al., 1997; Szczebara et al., 1997; Hultén et al., 1997; Sevin et al., 1998; Maeda et al., 1998; García-Arata et al., 1999). Taylor et al. (1997) have reassigned the former 2058 and 2059 mutations as positions 2142 and 2143 based on their exact positions in the 23S rRNA gene in *H. pylori*.

The aims of this study were (i) to investigate the mechanism, frequency and stability of point mutations in the 23S rRNA gene that confer clarithromycin resistance among paired Irish *H. pylori* isolates, (ii) to determine the relationship of these mutations with the level of clarithromycin resistance, (iii) to investigate cross-resistance among macrolides and (iv) to determine the possible relationship between the type of point mutations observed and treatment regimen.
6.2. Materials and Methods

6.2.1. Patient population and \textit{H. pylori} isolates

Seven patients (4 male, 3 female, age range 31 – 61 years; mean 44 years) were included in the study. The same 28 \textit{H. pylori} isolates that had been recovered from sequential antral biopsy specimens from each patient, as used in Chapters 4 and 5 (see sections 4.3.1.1, 4.3.2.1, and 5.3), were analysed. The relevant data from Chapter 4 is summarised in Table 6.1.

6.2.2. Susceptibility testing and MIC determination

6.2.2.1. MIC determination of \textit{H. pylori} isolates to clarithromycin and azithromycin

\textit{In vitro} susceptibility testing of \textit{H. pylori} isolates to clarithromycin and azithromycin was performed by the agar dilution method according to NCCLS recommendations (see section 4.3.1.2). Azithromycin antibiotic powder, with stated potency, was supplied by Pfizer Ltd., UK.

6.2.2.2. Susceptibility of \textit{H. pylori} isolates to clarithromycin and metronidazole by the disc-diffusion test

The disc-diffusion test was performed on all of the \textit{H. pylori} isolates to determine their susceptibility to clarithromycin and metronidazole. Susceptibilities to clarithromycin (see section 4.3.1.4) and metronidazole were performed on \textit{H. pylori} isolates prior to July 1995 by Dr. Huaxiang Xia, St. James's Hospital, Dublin, Ireland. Susceptibilities to metronidazole were performed according to the methods previously described (Xia \textit{et al.}, 1994b) by applying 5 \(\mu\)g metronidazole paper discs onto the surface of the inoculated agar plates. After incubation, isolates with a zone diameter of < 20 mm were defined as resistant (Xia \textit{et al.}, 1994b).
6.2.3. Amplification of the 23S rRNA gene of *H. pylori* by PCR

Amplification of a 425 bp fragment of the 23S rRNA gene was performed using the primers described by Occhialini *et al.* (1997), namely, the forward primer FMHP1, extending from position 1820 to 1839 (5'-CCA CAG CGA TGT GGT CTC AG-3') and the reverse primer, FMHP2 from position 2244 to 2225 (5'-CTC CAT AAG AGC CAA AGC CC-3'). A 100 µl reaction mix was prepared containing 1.5 mM MgCl₂, 20 pmol of each primer, 200 µM each of dCTP, dGTP, dATP, dTTP (Promega, WI, USA) and 2 U of Taq DNA polymerase (Promega, WI, USA) in 10 mM Tris-HCl (pH 8.3), 50 mM KCl and 1% (v/v) Triton X-100. The mixture was overlaid with 50 µl of mineral oil and 10 ng of template was added to the reaction mix. The cycling programme [PTC-100 thermocycler (MJ Research Inc.)] consisted of 1 cycle of 94 °C for 10 min, followed by 30 cycles of 94 °C for 60 s, 57 °C for 60 s and 72 °C for 60 s, and a final cycle of 72 °C for 10 min. Sterile water was included as a negative control in all reactions. PCR products and a 1 kb plus molecular weight DNA marker (Gibco, BRL) were run at 70 V for 2 h on a 2% agarose gel, containing ethidium bromide and photographed under ultraviolet light. Samples were sequenced by MWG-Biotech U.K. Ltd., Milton Keynes, U.K.

6.2.4. Cloning of 23S rRNA gene products

The 425 bp PCR product of the 23S rRNA gene was cloned into *E. coli* (TOP10 One Shot™) using the TOPO TA dual promotor cloning kit (Invitrogen BV, The Netherlands) according to the manufacturer's instructions. PCR products were cloned into the pCR II-TOPO vector and transformed into competent *E. coli*. The genotype of the *E. coli* strain used for cloning and blue/white screening of transformed cells was as follows: F⁻ mcrA Δ (mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 deoR araD139 Δ(ara-leu) 7697 galU galK rpsL (Str^R) endA1 nupG. After blue-white screening of recombinants
(Sambrook et al, 1989), plasmid DNA was purified as previously described (see section 2.2.2) and recombinants were identified by EcoRI restriction digestion at 37 °C for 1 h followed by agarose gel electrophoresis. Positive E. coli clones were inoculated into 3 ml L-broth medium supplemented with 50 µg ampicillin per ml and grown overnight as previously described (see section 2.2.2). A 0.85 ml aliquot of the bacterial suspension was added to a freezer vial containing 0.15 ml of sterile glycerol, mixed thoroughly and stored at -80 °C until required. Recombinant plasmid DNA was sequenced by MWG-Biotech U.K. Ltd., Milton Keynes, U.K.

6.2.5. Sequence analysis

The nucleotide sequences of PCR products (both strands) and of the recombinant clones were compared to three known 23S rRNA gene sequences deposited in the Genbank and EMBL databases using the GeneJockey computer package.

6.2.6. 23S rRNA gene RFLP analysis

RFLP analysis with the restriction enzymes BsaI and BbsI was performed to detect the restriction sites created by the 2143 A-to-G mutation and 2142 A-to-G mutation, respectively. H. pylori genomic DNA (see section 2.2.1) was digested overnight with either BbsI at 37 °C or BsaI at 56 °C (New England Biolabs Ltd., U.K.) according to the manufacturer's instructions. DNA digests were separated at 30 V overnight on a 0.7% agarose gel containing ethidium bromide and photographed under UV light. The DNA was then transferred to a nylon membrane by Southern blotting (see section 2.4). The recombinant plasmid clone containing the 425 bp fragment of the 23S rRNA gene was linearised with the restriction enzyme HindIII and the plasmid DNA was labelled with Digoxigenin by the random prime method (see section 2.6.3). Membranes were
hybridised at 68 °C overnight (see section 2.7.2) with the Digoxigenin-labelled probe. Membranes were developed according to manufacturer's instructions.
6.3. Results

6.3.1. Susceptibility testing and MIC determination

6.3.1.1. Susceptibility testing of \textit{H. pylori} strains by the disc-diffusion test

The disc-diffusion method of susceptibility testing for metronidazole and clarithromycin was performed on 28 \textit{H. pylori} isolates recovered from patients A-G (see Table 6.1). For metronidazole, 24 isolates were classified as resistant and three isolates were susceptible. Susceptibility testing of the isolate G2a from patient G produced different zone diameter results when the disc-diffusion test was performed on two separate occasions, \textit{i.e.}, the isolate was categorised as susceptible on one occasion and resistant when the test was repeated. This indicates that this patient harboured a mixed population of metronidazole-susceptible and -resistant colonies. However, the finding that the following isolate (isolate G2b) was susceptible to metronidazole and that subsequent \textit{H. pylori} isolates (G2c and G2d) were metronidazole-resistant after the patient had received an additional treatment regimen that did not contain metronidazole indicates that resistant colonies were not detected by the disc-diffusion test at the time isolate G2a was originally tested.

Susceptibility testing of the \textit{H. pylori} isolates to clarithromycin by the disc-diffusion method revealed that 15 isolates were resistant to clarithromycin and 12 isolates were susceptible. Susceptibility testing of isolate G2a from patient G demonstrated that the isolate was categorised as susceptible on one occasion (result of Dr. Huaxiang Xia) and resistant when the disc-diffusion test was repeated by the author. Therefore, susceptibility testing of this \textit{H. pylori} isolate demonstrated that this patient, while harbouring a population of strains with different metronidazole susceptibilities, also harboured a heterogeneous population of clarithromycin-resistant and susceptible strains.
Table 6.1. Clarithromycin, azithromycin and metronidazole susceptibilities and 23S rRNA gene point mutations detected in pre- and post-treatment *H. pylori* isolates from seven patients.

<table>
<thead>
<tr>
<th>Patient isolate</th>
<th>OGD date&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Rx status/ regimen&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Susceptibility by disc-diffusion test&lt;sup&gt;c&lt;/sup&gt;</th>
<th>MIC (µg/ml)&lt;sup&gt;d&lt;/sup&gt;</th>
<th>23S rRNA domain V&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1a</td>
<td>05.10.94</td>
<td>pre-Rx</td>
<td>S</td>
<td>0.06</td>
<td>nd</td>
</tr>
<tr>
<td>A2a</td>
<td>02.12.94</td>
<td>post PPI+C+M</td>
<td>R</td>
<td>32</td>
<td>&gt; 128</td>
</tr>
<tr>
<td>A2b</td>
<td>02.12.94</td>
<td>post PPI+C+M</td>
<td>R</td>
<td>32</td>
<td>&gt; 128</td>
</tr>
<tr>
<td>B1a</td>
<td>12.03.93</td>
<td>pre-Rx</td>
<td>S</td>
<td>0.06</td>
<td>nd</td>
</tr>
<tr>
<td>B2a</td>
<td>02.07.93</td>
<td>post B+M+T</td>
<td>S</td>
<td>0.06</td>
<td>nd</td>
</tr>
<tr>
<td>B2b</td>
<td>10.12.93</td>
<td>post PPI+C</td>
<td>R</td>
<td>64</td>
<td>&gt; 128</td>
</tr>
<tr>
<td>C1a</td>
<td>30.03.93</td>
<td>pre-Rx</td>
<td>S</td>
<td>0.125</td>
<td>nd</td>
</tr>
<tr>
<td>C2a</td>
<td>09.06.93</td>
<td>post PPI+C</td>
<td>R</td>
<td>128</td>
<td>&gt; 128</td>
</tr>
<tr>
<td>C2b</td>
<td>15.06.95</td>
<td>PPI only</td>
<td>R</td>
<td>128</td>
<td>&gt; 128</td>
</tr>
<tr>
<td>C2c</td>
<td>15.06.95</td>
<td>PPI only</td>
<td>R</td>
<td>&gt; 128</td>
<td>&gt; 128</td>
</tr>
<tr>
<td>D1a</td>
<td>16.09.94</td>
<td>pre-Rx</td>
<td>S</td>
<td>0.06</td>
<td>nd</td>
</tr>
<tr>
<td>D1b</td>
<td>16.09.94</td>
<td>pre-Rx</td>
<td>S</td>
<td>0.06</td>
<td>nd</td>
</tr>
<tr>
<td>D2a</td>
<td>13.02.95</td>
<td>post B+M+T</td>
<td>S</td>
<td>0.125</td>
<td>nd</td>
</tr>
<tr>
<td>D2b</td>
<td>13.02.95</td>
<td>post B+M+T</td>
<td>S</td>
<td>0.125</td>
<td>nd</td>
</tr>
<tr>
<td>D2c</td>
<td>23.03.95</td>
<td>post PPI+C+M</td>
<td>R</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>Sample</td>
<td>Date</td>
<td>Antimicrobial Therapy</td>
<td>Susceptibility</td>
<td>MIC</td>
<td>Resistance</td>
</tr>
<tr>
<td>---------</td>
<td>---------</td>
<td>------------------------</td>
<td>-----------------</td>
<td>------</td>
<td>-------------</td>
</tr>
<tr>
<td>E2a</td>
<td>23.04.93</td>
<td>post B+M+T</td>
<td>S</td>
<td>R</td>
<td>0.125</td>
</tr>
<tr>
<td>E2b</td>
<td>14.02.95</td>
<td>post PPI+C+M</td>
<td>R</td>
<td>R</td>
<td>16</td>
</tr>
<tr>
<td>E2c</td>
<td>14.02.95</td>
<td>post PPI+C+M</td>
<td>R</td>
<td>R</td>
<td>16</td>
</tr>
<tr>
<td>F1a</td>
<td>18.04.94</td>
<td>pre-Rx</td>
<td>S</td>
<td>R</td>
<td>0.03</td>
</tr>
<tr>
<td>F1b</td>
<td>18.04.94</td>
<td>pre-Rx</td>
<td>S</td>
<td>R</td>
<td>0.03</td>
</tr>
<tr>
<td>F2a</td>
<td>15.06.94</td>
<td>post PPI+C</td>
<td>R</td>
<td>R</td>
<td>16</td>
</tr>
<tr>
<td>F2b</td>
<td>15.06.94</td>
<td>post PPI+C</td>
<td>R</td>
<td>R</td>
<td>32</td>
</tr>
<tr>
<td>F2c</td>
<td>15.06.94</td>
<td>post PPI+C</td>
<td>R</td>
<td>R</td>
<td>32</td>
</tr>
<tr>
<td>G1a</td>
<td>07.04.95</td>
<td>pre-Rx</td>
<td>S</td>
<td>S</td>
<td>0.06</td>
</tr>
<tr>
<td>G2a</td>
<td>02.06.95</td>
<td>post PPI+C+M</td>
<td>S/R</td>
<td>S/R</td>
<td>0.125/32</td>
</tr>
<tr>
<td>G2b</td>
<td>27.07.95</td>
<td>post PPI+C</td>
<td>R</td>
<td>S</td>
<td>32</td>
</tr>
<tr>
<td>G2c</td>
<td>20.09.96</td>
<td>post PPI+A+C</td>
<td>R</td>
<td>R</td>
<td>32</td>
</tr>
<tr>
<td>G2d</td>
<td>20.09.96</td>
<td>post PPI+A+C</td>
<td>R</td>
<td>R</td>
<td>nd</td>
</tr>
</tbody>
</table>

\(^a\) OGD, oesophago-gastroduodenoscopy

\(^b\) Rx, treatment; PPI, proton-pump inhibitor; C, clarithromycin; M, metronidazole; B, bismuth; T, tetracycline; A, amoxicillin

\(^c\) Clr, clarithromycin; Mtz, metronidazole; S, susceptible; R, resistant; S/R susceptible and resistant, respectively, on 2 separate occasions

\(^d\) Clr, clarithromycin; Azith, azithromycin; Breakpoint for Clr and Azith resistance, MIC ≥ 2 µg/ml and ≥ 8 µg/ml, respectively

\(^e\) A-to-G mutation at position 2143, A-to-G mutation at position 2142, A-to-G mutation at position 2182

\(^f\) The mutation was detected from a single colony growing at 2.5 µg/ml within the elliptical zone of the E-test

\(\text{nd, not determined}\)
6.3.1.2. MIC’s of H. pylori strains to clarithromycin and azithromycin

The MIC's of the *H. pylori* isolates to clarithromycin and azithromycin recovered from patients A-G are shown in Table 6.1. Of the 28 *H. pylori* isolates examined, 12 isolates that were obtained from patients prior to treatment or from patients who had failed eradication with a non-clarithromycin-based treatment regimen were susceptible to clarithromycin (MIC, 0.03 to 0.125 µg/ml). The clarithromycin MIC's for the isolate(s) recovered from patients who had failed eradication treatment with a regimen that included clarithromycin were considerably higher (MIC, 4 to > 128 µg/ml). *H. pylori* isolates that were resistant to clarithromycin demonstrated cross resistance to azithromycin (MIC 8 to > 128 µg/ml).

Susceptibility testing of *H. pylori* isolate G2a to clarithromycin and azithromycin was performed on numerous occasions by the agar dilution method. The MIC values for clarithromycin and azithromycin were 0.125 and 32 µg/ml and 0.25 and >128 µg/ml, respectively, when this isolate was tested on two separate occasions. These results confirmed that this isolate represented a mixed population of strains with respect to clarithromycin susceptibility (see section 6.3.2.1). Susceptibility testing of this isolate to clarithromycin was then performed by the E-test method in an attempt to isolate resistant colonies. As expected, different MIC values (0.032, 0.125 and 128 µg/ml) were obtained when this isolate was tested on three separate occasions. However, a resistant colony was observed within the elliptical zone of inhibition when the plate was re-incubated for a further 1-2 days.

6.3.2. Optimisation of the 23S rRNA gene PCR method

Initial PCR and cycle sequencing (Applied Biosystems, Foster City, CA) studies using the method described by Versalovic *et al.* (1996) were unsuccessful and an alternative
PCR method, described herein, was employed (Occhialini et al., 1997). Initial PCR experiments were performed using two high fidelity DNA polymerases, namely, Pwo and Expand (Boehringer Mannheim GmbH, Germany), to ensure that any point mutations observed were not due to errors in transcription by the enzyme. However, these experiments were not reproducible for all of the isolates (see Fig. 6.1). The PCR method was subsequently optimised at a high annealing temperature using Taq DNA polymerase.

To assess the accuracy of Taq DNA polymerase for point mutation analysis, PCR was performed in duplicate with one clarithromycin-susceptible isolate (F1a) and one clarithromycin-resistant isolate (F2c) and the PCR products were sequenced. The nucleotide sequences obtained from duplicate PCR samples for each isolate were identical. Furthermore, the sequence obtained from the clarithromycin-susceptible isolate was identical to three 23S rRNA gene sequences deposited in the Genbank database (GenBank accession No. U27270, Tomb et al., 1997). The nucleotide sequence obtained from the clarithromycin-resistant isolate, F2c, differed from the Genbank sequences by a single base substitution A-to-G at position 2143. Therefore, these preliminary experiments confirmed that the Taq polymerase enzyme was reliable for point mutation analysis and consequently, the 23S rRNA genes of the remaining isolates were amplified with this enzyme. A 10 ng DNA template concentration and a 1.5 mM MgCl₂ concentration were chosen that yielded PCR products of the correct size and good visual intensity (Fig. 6.2).

6.3.3. Detection of 23S rRNA gene point mutations

The DNA from a total of 24 H. pylori isolates was amplified by 23S rRNA gene PCR and the nucleotide sequence was determined for both DNA strands of each 425 bp PCR
Figure 6.1. 23S rRNA gene PCR using three different DNA polymerases.

Agarose gel electrophoresis of 23S rRNA gene PCR products from four *H. pylori* isolates using the following DNA polymerases; lanes 2-5, *Pwo* high fidelity polymerase, isolates 95-148, G2d, G2a and A1a, respectively; lanes 6-9, Expand high fidelity polymerase, isolates 95-148, G2d, G2a and A1a, respectively, and lanes 10-13, *Taq* DNA polymerase, isolates 95-148, G2d, G2a and A1a, respectively. Sterile distilled (N) water was included as a negative control. Molecular size (bp) markers are indicated.
Figure 6.2. 23S rRNA gene PCR of two *H. pylori* isolates using *Taq* DNA polymerase.

The 23S rRNA gene PCR was optimised with *Taq* DNA polymerase to yield a specific 425 bp size PCR product of good visual intensity. Lane 1, 1 kb molecular DNA size ladder; lane 2, *H. pylori* isolate C2b, lane 3, *H. pylori* isolate G2d; lane 4, sterile distilled water was used as a negative control.
The A-to-G point mutations at positions 2142 and 2143 were detected in the nucleotide sequences derived from both DNA strands in all of the isolates that were resistant to clarithromycin, with the exception of isolate D2c (see Table 6.1). Moreover, these mutations were not observed in the nucleotide sequences from isolates that were susceptible to clarithromycin. The 2142 A-to-G mutation was present in four isolates from two patients (B and C) and the 2143 A-to-G mutation was present in 11 isolates from four patients (A, E-G). The 2142 (formerly 2143) A-to-C transversion mutation described in the literature (Stone et al., 1996) was not observed.

PCR and sequencing studies of the 23S rRNA gene region of isolate G2a demonstrated the presence of a mixed population of wild-type colonies and mutant colonies with the latter possessing the A-to-G 2143 mutation. Initial PCR and sequence analysis of the DNA from this isolate consistently yielded wild-type sequence. However, the A-to-G 2143 mutation was only detected from the clarithromycin-resistant variant of isolate G2a that grew within the elliptical zone of the E-test (see Fig. 6.3). These results are in accordance with the observed variable in vitro susceptibility data and further demonstrate that this isolate comprises of a heterogeneous bacterial population with respect to clarithromycin susceptibility.

Interestingly, sequencing analysis did not detect the A-to-G 2142 and 2143 point mutations in the 23S rRNA gene region of the H. pylori isolate D2c (patient D) that demonstrated low-level resistance to clarithromycin (MIC 4 μg/ml, MIC to azithromycin 8 μg/ml). However, a T-to-C silent mutation at position 2182 was detected in the 23S rRNA gene of this isolate (Fig. 6.3), on two separate occasions, and it was also detected in the 23S rRNA genes of two clarithromycin-susceptible isolates (D1a and D2a) from this patient. Furthermore, this mutation was also detected in the 23S rRNA gene
U27270 ccacagcgat gttgccctcag caaagagtcc ctcccgactg tttaccaaaa 1869
C2a ag caaagagtcc ctcccgactg tttaccaaaa
G2a* ggtctcag caaagagtcc ctcccgactg tttaccaaaa
D2c teag caaagagtcc ctcccgactg tttaccaaaa

U27270 acacagcact ttgccaactc gtaagaggaa gtataaggtg tgacgcctgc 1919
C2a acacagcact ttgccaactc gtaagaggaa gtataaggtg tgacgcctgc
G2a* acacagcact ttgccaactc gtaagaggaa gtataaggtg tgacgcctgc
D2c acacagcact ttgccaactc gtaagaggaa gtataaggtg tgacgcctgc

U27270 ccggtgctcg aaggttaaga ggatgcgtca gtcgcaagat gaagcgttga 1969
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G2a* ccggtgctcg aaggttaaga ggatgcgtca gtcgcaagat gaagcgttga
D2c ccggtgctcg aaggttaaga ggatgcgtca gtcgcaagat gaagcgttga

U27270 attgaagccc gatgtaaacgg cggccgtaac tataacggtc ctaaggtagc 2019
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D2c gggagctgtc tcaaccagag attcagtgaa attgtagtgg aggtgaaaat

U27270 tcctcctacc cgcggcaaga cggGaagacc cggGagacc cggGagacc 2142
C2a tcctcctacc cgcggcaaga cggGaagacc cggGagacc cggGagacc
G2a* tcctcctacc cgcggcaaga cggGaagacc cggGagacc cggGagacc
D2c tcctcctacc cgcggcaaga cggGaagacc cggGagacc cggGagacc

2142

U27270 tcctcctacc cgcggcaaga cggGaagacc cggGagacc tcctcctacc 2169
C2a tcctcctacc cgcggcaaga cggGaagacc cggGagacc tcctcctacc
G2a* tcctcctacc cgcggcaaga cggGaagacc cggGagacc tcctcctacc
D2c tcctcctacc cgcggcaaga cggGaagacc cggGagacc tcctcctacc
The nucleotide sequences were derived from 425 bp PCR products of the 23S rRNA genes of isolates C2a, G2a* (asterisk denotes that the sequence was derived from the clarithromycin-resistant colony that grew within the elliptical zone of the E-test) and D2c. The blue underlined nucleotide sequence corresponds to the primer binding sites. The base pair numbers are according to the published sequence of the 23S rRNA DNA gene of *H. pylori* (GenBank accession no. U27270) while the arrow (‡) above the nucleotide sequence indicates the base pair number according to Taylor *et al.*, 1997. Nucleotide differences between isolates are highlighted in red capitals. Isolate C2a possessed the A-to-G 2142 point mutation, isolate G2a* the A-to-G 2143 point mutation and isolate D2c the T-to-C 2182 silent mutation.

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Figure 6.3. *Comparison of the nucleotide sequences of a 425 bp region of the 23S rRNA genes of three clarithromycin-resistant H. pylori isolates.*
sequence obtained from the clarithromycin-susceptible isolate, isolate B2a, recovered from patient B. This patient had completed *H. pylori* eradication treatment with a regimen consisting of colloidal bismuth citrate, metronidazole and tetracycline at the time of isolation of this strain. The patient was then treated with a PPI and clarithromycin in an attempt to eradicate the persistent infection but this was unsuccessful and consequently, the isolate recovered (isolate B2b) had acquired resistance to clarithromycin. PCR and sequencing analysis showed that an A-to-G 2142 point mutation was present in the 23S rRNA gene region of this isolate.

### 6.3.4. Stability of point mutations and correlation with the level of clarithromycin resistance, metronidazole resistance and treatment regimen

The same point mutation was observed in the peptidyltransferase region of the 23S rRNA gene among clarithromycin-resistant *H. pylori* isolates recovered from sequential antral biopsy specimens from individual patients. Furthermore, of the isolates from patient C, the same mutation was observed in identical sequential isolates (based on DNA fingerprinting studies, see Chapter 5) that had been collected over a two-year period. There was an association between the type of point mutation observed and the level of clarithromycin resistance in post-treatment isolates. The 2142 mutation was detected in isolates with an MIC of $\geq 64$ $\mu$g/ml and the 2143 mutation was found in isolates with an MIC of 16 – 32 $\mu$g/ml. There was some indication of a link between the type of treatment regimen employed for *H. pylori* eradication and the *in vitro* level of resistance. An MIC $\geq 64$ $\mu$g/ml was observed in 2 of 3 patients (patients B, C and F) who had received a dual therapy regimen consisting of a proton pump inhibitor and clarithromycin. There was no association between the type of point mutation observed and metronidazole susceptibility.
6.3.5. 23S rRNA gene RFLP analysis

RFLP analysis was performed to confirm the sequencing results. Cloning of a 425 bp PCR fragment of the 23S rRNA gene from isolate G2d was successful (Fig. 6.4). However, 23S rRNA gene RFLP analysis with this clone could not be optimised due to methodological problems. Therefore, a 425 bp fragment of the 23S rRNA gene was amplified from *H. pylori* isolate A2a, agarose gel purified (see section 2.5) and Digoxigenin-labelled by the random prime method (see section 2.6.3). Membranes containing *Bbs*I-digested or double *Bbs*I- and *Hind*III-digested *H. pylori* DNA were hybridised at 68 °C overnight with the Digoxigenin-labelled PCR fragment and these were developed according to the manufacturer's instructions.

RFLP analysis of a small selection of the patient isolates is shown in Fig. 6.5. Comparison of the RFLP profiles of *Bsa*I and *Hind*III double digestion samples of *H. pylori* DNA from isolates B1a and B2b showed that three additional hybridisation bands were generated in the clarithromycin-resistant isolate (compare lanes 10 and 11 in Fig. 6.5a) suggesting the presence of an additional *Bbs*I restriction site. These 23S rRNA gene RFLP results are in agreement with the sequencing data, as the A-to-G 2142 mutation creates a *Bbs*I restriction site and this mutation was detected in the 23S rRNA gene nucleotide sequences of clarithromycin-resistant isolates from this patient (see Table 6.1). Similarly, RFLP analysis was performed on *Bbs*I-digested *H. pylori* DNA from the isolates recovered from patients C and F (Fig. 6.5b). The A-to-G 2142 mutation, by creating a restriction site, resulted in the digestion by *Bbs*I of the DNA from clarithromycin-resistant isolates from patient C to generate three to four additional bands (compare lanes 2, 3 and 4, Fig. 6.5b). Furthermore, RFLP band profiles generated from clarithromycin-susceptible and -resistant isolates obtained from patient F were very similar to each other and consisted of four predominant bands. The absence of additional
Figure 6.4. Cloning of a 425 bp PCR fragment of the 23S rRNA gene into the EcoRI restriction site of plasmid vector pCR II-TOPO.

Agarose gel electrophoresis demonstrating the following: Lane 1, a 425 bp PCR product of the 23S rRNA gene from *H. pylori* isolate G2d; lane 2, 1kb molecular DNA size ladder; lane 3, purified preparation of the plasmid vector pCR II-TOPO; lane 4, EcoRI restriction digestion of the plasmid vector; lane 5, purified preparation of the plasmid vector pCR II-TOPO containing the cloned 425 bp insert; lane 6, EcoRI restriction digestion of the plasmid vector pCR II-TOPO containing the cloned 425 bp insert and lane 7, 1 kb molecular DNA size ladder. Molecular size (bp) markers are indicated.
<table>
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![Image of gel electrophoresis with markers and sample gels.](image)

- Lanes 1-7: BbsI digestion
- Lanes 8-14: BbsI/HindIII digestion

Markers: 2000, 1650, 1000, 850
Figure 6.5. RFLP analysis of BbsI-digested and BbsI/HindIII-double digested DNA from selected *H. pylori* isolates recovered from patients A-C and F-G using a DIG-labelled 425 bp 23S rRNA gene PCR product.

*BbsI-* and *BbsI/HindIII-*digested *H. pylori* DNA was transferred to a nylon membrane by Southern blotting and the resulting band profiles were generated by hybridisation with a DIG-labelled 425 bp PCR product of the 23S rRNA gene. Band profiles generated from *H. pylori* isolates from (a) Patients (Pt) A, B and G. Lanes 1-8, *BbsI*-digested DNA and lanes 9-14, *BbsI/HindIII*-digested DNA. (b) Band profiles generated from *BbsI*-digested DNA from *H. pylori* isolates recovered from patients C and F. Molecular size (bp) markers are indicated.
bands among these clarithromycin-resistant isolates indicates that an additional \textit{BbsI} restriction site, and hence the 2142 mutation, was not present. Nucleotide sequence analysis showed that these resistant isolates in fact possessed the A-to-G 2143 mutation (see Table 6.1).
6.4. Discussion

The findings of the present study confirm that A-to-G point mutations at positions 2142 and 2143 in domain V of the peptidyltransferase region of the 23S rRNA gene are associated with clarithromycin resistance in the majority of Irish *H. pylori* isolates. The same mutation was observed in the peptidyltransferase region of the 23S rRNA gene among clarithromycin-resistant isolates recovered from sequential antral biopsy specimens from individual patients. Moreover, in one patient (patient C), the same mutation was observed in identical sequential isolates that had been collected over a two-year period. This strongly indicates that clarithromycin resistance is stable *in vivo* since no reversion of the resistance phenotype was observed in the absence of selective antimicrobial pressure. Furthermore, genotyping analysis of the *H. pylori* isolates recovered from patients A-G showed that, in the majority of patients, isolates recovered from the same patient were identical or very similar to each other (see Chapter 5). Therefore, clarithromycin resistance has developed *in vivo* and is the result of the selection of variants of the infecting strain rather than infection with an exogenous resistant strain.

The majority of clarithromycin-resistant isolates possessed the A-to-G 2143 mutation. The predominance of the 2143 mutation has been reported in other studies (Maeda *et al.*, 1998; Sevin *et al.*, 1998; García-Arata *et al.*, 1999). However, this distribution is not observed in American centres where a higher proportion of clarithromycin-resistant isolates possessing the 2142 mutation exists (Versalovic *et al.*, 1997; Stone *et al.*, 1997). The A-to-C 2142 transversion mutation described in the literature was not observed (Stone *et al.*, 1996). One explanation for the predominance of the A-to-G mutants has been provided by Wang *et al.* (1999) who showed that clarithromycin-resistant mutants possessing the A-to-G 2142 demonstrated a competitive growth advantage over other

The present study identified one patient (patient D) in whom the low-level clarithromycin-resistant isolate did not possess either of the known point mutations associated with clarithromycin resistance. Sequencing studies did identify a silent T-to-C mutation at position 2182 of the transpeptidase region of the 23S rRNA gene in all of the isolates that were sequenced from this patient. Maeda et al. (1998) have reported the existence of mutant strains with clarithromycin resistance and wild-type 23S rRNA. In the present study, it is possible that the presence of mutations at position 2611, as described for *E. coli* (Douthwaite, 1992; Vannuffel et al., 1992), may be responsible for clarithromycin resistance since sequencing studies were only performed on a section (domain V) of the 23S rRNA gene. Alternatively, another mechanism of clarithromycin resistance may be involved.

*H. pylori* possesses two copies of the 23S rRNA gene (Bukanov and Berg, 1994; Taylor et al., 1997; Tomb et al., 1997). Some studies have reported that a small proportion (7%) of clarithromycin-resistant *H. pylori* isolates are heterozygous for the mutation, *i.e.* these isolates harbour a mutation in only one of the two copies of the 23S rRNA gene (Versalovic et al., 1996; Hultén et al., 1997). However, a mutation in only one of the 23S rRNA genes is sufficient to confer clarithromycin resistance. The presence of a single allele may confer only a low-level of resistance (Weisblum, 1998). Therefore, it is possible that the low-level clarithromycin-resistant isolate, isolate D2c, is heterozygous for the mutation and that sequence analysis only detected the wild-type allele.
An alternative explanation for the finding that point mutations associated with clarithromycin resistance were not detected in the low-level clarithromycin-resistant isolate D2c may be the presence of a mixed population of susceptible and resistant variants. In this case, sequence analysis may have only detected the wild-type susceptible population and not the resistant variants, especially if the mutant population was present in low numbers. This was the case with isolate G2a where the A-to-G 2143 point mutation could only be detected from a variant that grew within the elliptical zone of the E-test. Indeed, Debets-Ossenkopp et al. (1998) found that intermediate MIC’s were the result of heterogeneous cell populations; susceptible colonies had an MIC < 0.016 μg/ml and possessed wild-type 23S rRNA gene sequence whereas colonies for which the MIC’s to clarithromycin were high had the mutant sequence.

The MIC values of *H. pylori* isolates to clarithromycin showed a bimodal distribution which clearly separated them into susceptible and resistant categories. Clarithromycin-resistant isolates demonstrated cross-resistance to azithromycin. Although a small number of resistant isolates were included in this study, the 2142 mutation was found to be associated with a higher MIC level (≥ 64 μg/ml) whilst the 2143 mutation was associated with lower clarithromycin MIC levels of 16 – 32 μg/ml. This association has been reported in other studies (Hultén et al., 1997; Versalovic et al., 1997; Debets-Ossenkopp et al., 1998).

In conclusion, the present study has shown that A-to-G point mutations at positions 2142 and 2143 in the peptidyltransferase region of the 23S rRNA gene of *H. pylori* are stable and, in the majority of cases, these mutations are associated with clarithromycin resistance among Irish isolates. The A-to-G 2143 mutation is more frequently observed and isolates with this mutation appear to have lower MIC’s to clarithromycin. Since the
known mutations were not detected in one resistant isolate, another mechanism of clarithromycin resistance may be involved.
Chapter 7

General Discussion
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7.1. *H. pylori* Diagnosis

At present, no single diagnostic test is available that offers supreme sensitivity and specificity and hence, there is a continual search for a single ‘gold standard’ test for *H. pylori* diagnosis. An effective means for the selection of presumptive *H. pylori*-positive patients in an ever increasing population of *H. pylori*-negative individuals would offer a considerable practical and cost-savings procedure for diagnosis of this infection. A selection procedure using the CLOtest was evaluated in patients who had not previously received *H. pylori* eradication treatment. It would be worthwhile to repeat the study in a larger number of patients (e.g. n=200). This approach may be more beneficial for the selection of infected patients who have failed eradication therapy so that culture and susceptibility testing may be performed on the isolate recovered. This approach assumes that the sensitivity of the CLOtest for the diagnosis of *H. pylori* infection is equivalent in both untreated and post-treatment patients. However, the data from the few clinical studies that have assessed the performance of the CLOtest in treated patients are inconsistent. It is clear, therefore, that studies are needed to determine the accuracy of diagnostic tests for the assessment of *H. pylori* eradication so that more practical approaches to the diagnosis of this infection in clinical practice may be sought.

In clinical practice, the current consensus of opinion favours the ‘test and treat’ option (see Fig 7.1). In this case, the non-invasive UBT is a suitable screening method and may eliminate the need for some endoscopic procedures, particularly in younger patients who present with dyspepsia. A new non-invasive diagnostic test based on the direct detection of *H. pylori* antigen in the stool specimens of patients with active infection has produced promising results in one large multi-centre study (Vaira *et al.*, 1999). This rapid and cost-effective test may be used as an alternative screening method and may obviate the
Patients with Dyspepsia

> 50 years, weight loss, dysphagia

< 50 yrs, no alarm symptoms

Endoscopy

- PCR – no application
- Selection of positive CLOtests – promising

H. pylori Diagnosis

- UBT/H. pylori stool antigen – ?application

H. pylori -ve

H. pylori +ve

H. pylori Eradication Treatment

Assessment of H. pylori Status

- PCR – equivocal
- Selection of positive CLOtests – equivocal

H. pylori +ve

Failure

Success

Susceptibility Testing

- PCR-based susceptibility testing – promising but limited application

Figure 7.1. Flow diagram for the management of patients with dyspepsia.
need for some endoscopic procedures. It may also be a suitable alternative for monitoring treatment in the future.

In the developed world, the prevalence of *H. pylori* infection is decreasing and consequently, this affects the predictive value of a particular diagnostic test. For example, if the prevalence of *H. pylori* infection in a population with dyspepsia is 40% but only one in 10 *H. pylori*-infected individuals has an ulcer, then the positive predictive value of the test for ulcer disease (with 95% sensitivity and specificity) is 45%, compared with 90% if four in 10 *H. pylori*-infected dyspepsia patients have peptic ulcer disease. Therefore, the proportion of non *H. pylori*-associated peptic ulcer disease in developed countries is bound to rise and hence, the suitability of this ‘test and treat’ strategy may need to be revised.

The PCR technique is considered to be a highly sensitive method that may be particularly suitable for *H. pylori* diagnosis in post-treatment patients where the sensitivity of the conventional tests may be compromised. However, the present study highlights the importance of the evaluation of new methods with conventional tests that are already used for diagnosis of *H. pylori* infection. PCR may have an application for the diagnosis of the infection in the post-treatment patient population since recent studies that have evaluated different primer sets have provided more promising results (Lu et al., 1999). However, it would seem that the non-invasive UBT may be a more favourable method for the assessment of *H. pylori* status in treated patients (see Fig 7.1). Despite the fact that it is relatively inexpensive but not widely available on-site in all gastroenterology units, it has the distinct advantage that a positive result indicates the presence of a current *H. pylori* infection, unlike the PCR methods that detect viable and non-viable bacterial cells.
7.2. Antimicrobial Resistance in *H. pylori*

The present study confirmed that target site modification by point mutations in the peptidyltransferase region of the 23S rRNA of *H. pylori* is the mechanism responsible for clarithromycin resistance in the majority of *H. pylori* isolates. Recent studies have described the use of PCR-based molecular methods for the direct detection of macrolide resistance in gastric biopsy specimens which offers a rapid means of detecting resistance (Björkholm *et al.*, 1998; Marais *et al.*, 1999). However, the disadvantages associated with the detection of resistance determinants by molecular methods are that they require knowledge of the resistance mechanism involved, are associated with high costs and are labour intensive. Conversely, phenotypic susceptibility testing is widely used, cost-effective and can be applied to other antimicrobial agents used in *H. pylori* eradication regimens, namely, metronidazole and amoxicillin. Hence, it is likely that phenotypic susceptibility testing will remain the gold standard method at present. While a point mutation in the 23S rRNA gene is so far the only mechanism of clarithromycin resistance that has been elucidated, it is possible that other mechanisms of macrolide resistance may exist in *H. pylori*. Supporting this, the current investigation detected the presence of silent mutations within the 23S rRNA nucleotide sequence of clinical *H. pylori* isolates that may represent spontaneous mutation events within the bacterial population *in vivo*. It is noteworthy that these mutations were present in both wild-type isolates and, more interestingly, a *H. pylori* isolate with low-level resistance to clarithromycin, the domain V 23S rRNA region of which did not possess the well characterised point mutations that are known to confer clarithromycin resistance in this bacterium.

The silent mutation observed in these isolates may merely represent a spontaneous mutation and may reflect the continual microevolution of *H. pylori in vivo*. It is possible that this mutation may lead to a minor alteration in the ribosomal structure and may
predispose the bacterium to ribosomal modification at other sites. Whether there is any advantage to this mutation is unclear.

Genomic sequencing studies have identified putative ATP-binding cassettes and multidrug efflux transporter genes within the *H. pylori* genome (Tomb *et al.*, 1997). The presence of these genes suggest that an efflux mechanism conferring macrolide resistance may exist in *H. pylori*. Inducible erythromycin resistance, encoded by the *msrA* gene, has been identified in a plasmid of *Staphylococcus epidermidis* (Ross *et al.*, 1990). The MsrA protein appears to act independently of a transport-related protein and may specifically act upon other transmembrane efflux complexes of staphylococcal cells. Furthermore, the active efflux of [14C] erythromycin from the cells of *S. aureus* possessing the *msrA* gene has also been reported.

The presence of putative methylase genes in the *H. pylori* genome suggests that a mechanism of target modification of domain V region of the 23S rRNA may be mediated by methyltransferase enzymes. However, the studies by Hultén *et al.* (1997) and Debets-Ossenkopp *et al.* (1996) have shown that a mechanism of drug inactivation via the production of an extracellular or rRNA methylase does not exist in *H. pylori*. It is clear that further research into the mechanisms of macrolide resistance is required.

Taylor *et al.* (1997) have demonstrated that clarithromycin resistance determinants could be transferred from a resistant donor strain to a susceptible recipient strain by natural transformation and therefore genetic transfer of resistance between *H. pylori* strains *in vivo* is theoretically possible. There is concern that the increased use of antimicrobial agents for the treatment of *H. pylori* infection and the frequent development of secondary
resistance to these agents in *H. pylori* may promote the spread of multiple resistance in the population. Surveillance studies are now required to monitor *H. pylori* resistance patterns. The importance of this is brought to the fore in the light of recent reports describing the emergence of stable resistance to amoxicillin, an antibiotic that is a frequent component of *H. pylori* treatment regimens (van Zwet *et al.*, 1998).

### 7.3. Susceptibility Testing and Genotyping

To date, there is no standard method of susceptibility testing for *H. pylori* and, moreover, the breakpoint MIC's for clarithromycin and amoxicillin have not been established. It is clear that further research is required to accurately monitor *H. pylori* resistance patterns at a local level and between different centres.

Further research to elucidate the mechanisms involved in nitroimidazole, macrolide and β-lactam resistance is advocated. This may lead to the development of PCR-based methods for the detection of antimicrobial resistance directly from gastric biopsy specimens and would offer a rapid means of susceptibility testing of *H. pylori*.

Genotyping studies may provide important insights into the virulence of bacterial strains and their involvement in the clinical outcome of disease in the host. Patients may harbour a mixed population of *cagA*-positive and *cagA*-negative strains and it is possible that their relative proportions may be a factor in determining the severity of infection or alternatively, other virulence genes that are co-expressed with CagA may be more directly involved in disease outcome than CagA itself.
Clinical research on peptic ulcer disease has proceeded at such a rapid pace that at present *H. pylori* infection can be successfully eradicated in the majority of patients but, as yet, the actual route of transmission of this bacterium is currently unknown. This emphasises the problem of the lack of knowledge of fundamental areas of *H. pylori* biology. Highly discriminatory molecular fingerprinting techniques could be applied to establish the source of the recurrence of *H. pylori* infection. This may be particularly applicable for extensive family studies that may identify a possible common source of the infection. However, vaccination may provide an effective means of reducing carriage and transmission of *H. pylori* infection.
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