COMPARISON OF CULTURE, HISTOPATHOLOGY AND UREASE TESTING FOR THE DIAGNOSIS OF HELICOBACTER PYLORI GASTRITIS AND SUSCEPTIBILITY TO AMOXICILLIN, CLARITHROMYCIN, METRONIDAZOLE AND TETRACYCLINE

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Summary
Gastric biopsy specimens were taken from 737 patients undergoing upper gastrointestinal endoscopy and assessed for Helicobacter pylori infection. The diagnostic utilities of H. pylori culture (733 patients), detection of urease production (724 patients) and histopathological examination (469 patients) were compared. Since each of these techniques may fail to diagnose patients infected with H. pylori, an attempt was made to estimate the true rate of infection using a mathematical approach that combined the results of culture, histopathology and urease testing; 34% of the 733 patients were thought to be infected. Using this figure as a benchmark, the sensitivity, specificity, positive predictive value and negative predictive value of H. pylori culture were 73.2%, 100%, 100% and 86.3%, respectively, compared with histopathology and urease production; 77.0%, 100%, 100% and 82.4%, respectively for histopathology. Thus, histopathological examination was the single most reliable test. A combination of histopathological examination and H. pylori culture diagnosed 99.5% of patients that were estimated to be truly infected. The minimum inhibitory concentrations of a number of antibiotics were measured for 135 isolates of H. pylori. All isolates were susceptible to amoxycillin and tetracycline whereas 5.2% were resistant to clarithromycin and 60% were resistant to metronidazole.

Key words: Helicobacter pylori, diagnosis, urease, histopathology, culture, antibiotic susceptibility.

Accepted 18 December 1997

INTRODUCTION
Since the original observations of Warren and Marshall in 1983,1,2 Helicobacter pylori has become generally accepted as the cause of most duodenal and many gastric ulcers and antimicrobial therapy is now standard treatment for these conditions.3 Likewise, H. pylori infection is associated with chronic atrophic gastritis and possibly non-ulcer dyspepsia and increasingly these patients are being treated with antibiotics.4,5

A diagnosis of H. pylori infection may be inferred from non-invasive breath or serological tests, or may be made more definitively at endoscopy using specimens which are examined histologically, cultured for H. pylori, or assessed for production of urease. The relative merits of these three methods are debated.6-12 The present study was undertaken to compare these three diagnostic modalities.

Furthermore, since successful Helicobacter eradication is likely to require the administration of antibiotics to which the organism is susceptible,13-17 the minimum inhibitory concentrations (MICs) of isolates for amoxycillin, clarithromycin, metronidazole, and tetracycline were determined.

MATERIALS AND METHODS
Patients
Multiple biopsies were taken by a number of gastroenterologists from patients undergoing upper gastrointestinal endoscopy at The Queen Elizabeth Hospital. In many instances, biopsies were taken from both the gastric body and antrum while in the remainder they were obtained from one site or the other.

Urease testing
Urease production was assessed using a commercial kit (CLOtest™, Bentley, Western Australia). It was inoculated with a biopsy specimen, incubated at room temperature, then read after 1 and 24 h for urease production according to the manufacturer’s instructions.

Histological assessment
Gastric biopsies were fixed in formalin, sections prepared and stained with H & E and reported routinely by one of 6 histopathologists with no knowledge of the CLOtest or microbiological results. Their reports were examined retrospectively for notation of infection with Helicobacter-like organisms and for gastritis.

H. pylori culture
Biopsies were transported in a drop of sterile 0.9% saline then plated directly onto chocolate agar and incubated at 35°C in CampyPak Plus™ jars (Becton Dickinson and Company, Cockeysville, MD, USA). Plates were examined after 3 and 6 d. Suspect colonies were Gram-stained and tested for the production of oxidase, catalase and urease (the latter from the CLOtest or, if negative, in a urea broth). Organisms were identified on the basis of typical colonial and Gram stain morphology and if they were positive for oxidase, catalase and urease.

Antibiotic susceptibility testing
Colonies were subcultured on chocolate agar for 3 d then a moistened sweep was taken, immersed in brain heart infusion broth then immediately lawn-plated onto chocolate agar. Plates were incubated again in CampyPak Plus™ jars overnight then harvested to make a 1 McFurland suspension in brain heart infusion broth. This suspension was lawn-plated onto blood Mueller Hinton agar on which Etest® strips (AB Biodisk,
RESULTS

Gastric biopsy specimens were received for culture of *H. pylori* from 737 patients. Four cultures were overgrown with *Proteus* and were discarded from analysis. *H. pylori* was grown in 183 (25.0%) of the remaining 733 specimens. Of the 733 specimens, 724 also had a CLOtest performed; 145 (19.9%) were positive at one hour while 209 (28.9%) were positive after 24 hours.

Four hundred and sixty-nine of the 733 specimens were examined histopathologically; overall, 124 (26.4%) were reported as being positive for *H. pylori*. Three hundred and ninety-three biopsies were taken from the antrum, of which 102 (26.0%) were positive, while 306 were taken from the body with 62 (20.4%) being positive. Biopsies from both the antrum and the body were received for 230 patients. Those from both the antrum and the body were negative in 167 patients (72.6%), both were positive in 39 (17.0%), positive for the antrum only in 18 (7.8%), and positive for the body only for 6 (2.6%).

For this subset of patients, there was 89.6% concordance between the presence of *H. pylori* in the antrum and the body. Of the remaining 10.4%, 18 patients (75%) were positive only in the antrum while organisms were seen only in the body in six subjects (25%). Thirteen of the 18 patients who were positive in the antrum only and three of the six patients who were positive in the body only were also positive by culture and/or urease testing (one hour).

Estimation of the true positivity rate

In order to calculate and compare the sensitivity, specificity, positive predictive value and negative value for each of *H. pylori* culture, the CLOtest, and histopathological examination, it is necessary to have a benchmark against which they can be compared. Unfortunately, there is no simple benchmark and an attempt has been made to estimate the true positivity rate using the following assumptions.

1. All positive cultures of *H. pylori* are probably true. The number of positive cultures = n = 183. Some true infections may fail to grow on culture and will be false-negative results.

2. All biopsies positive by histopathology are probably true. In the subgroup of 469 patients who had both culture and histopathological examination, 41 = p patients were positive histopathologically but negative by culture. In these 469 patients, 117 (25%) were culture-positive. In the subgroup of 264 patients who did not have biopsies, 66 (25%) were also culture-positive. Furthermore, the sex and age distributions of the two subgroups were similar. Thus the two subgroups are probably similar. Therefore the number of histopathology-positive, culture-negative patients in this second subgroup is estimated as q = 41 X (264/469) = 23. Therefore the revised estimate of infected patients taking histopathology results into account = n + p + q = 183 + 41 + 23 = 247.

3. Patients who were CLOtest-positive at 24 hours but negative at one hour and by culture and histopathology are likely to be false positives due to overgrowth of urease-producing organisms. These results were discarded; there were only four such patients in the group of 469 patients who underwent all three investigations. Patients who are CLOtest positive at one hour but negative by culture and by histopathology may be true positives. There were two such patients in this subgroup of 469 patients. Using the same logic as in section (2), the number of patients in the whole group who are likely to be CLOtest (one hour)-positive, culture-negative and histopathology-negative = 2 X ((469 + 264)/469) = 3 = r.

4. The final revised estimate for truly infected patients = n + p + q + r = 183 + 41 + 23 + 3 = 250 and the final estimate for truly uninfected patients = 733 − 250 = 483.

Sensitivity, specificity, and positive and negative predictive values of *H. pylori* culture, CLOtest (one hour) and histopathology

Using this estimate for the true prevalence of infection, the sensitivity, specificity, positive predictive value and negative predictive value of culture for *H. pylori* were calculated as 73.2%, 100%, 100% and 86.3%, respectively (Table 1). The same parameters for the CLOtest at one hour were 58.7%, 100%, 100% and 82.4%, respectively (Table 2). Since one cannot assume that there are no false-positives with the 24 hour CLOtest, it is not possible to perform these calculations for the 24 hour CLOtest result. The sensitivity, specificity, positive predictive value and negative predictive value of histopathology were calculated as 77.0%, 100%, 100% and 89.6%, respectively (Table 3). A comparison of the four parameters for the three tests is shown in Table 4. Histopathological examination was the best diagnostic method, closely followed by culture of the organism, with the CLOtest a relatively poor third. Even so, if histopathological examination alone was relied upon to make the diagnosis, almost one-quarter of cases would have been missed. When both histopathological examination and *H. pylori* culture were used, 99.5% of patients were diagnosed.

### Table 1

<table>
<thead>
<tr>
<th>Culture of <em>H. pylori</em> compared with the estimated true prevalence</th>
<th>Estimated true prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture</td>
<td>Positive</td>
</tr>
<tr>
<td>Culture</td>
<td>183</td>
</tr>
<tr>
<td>Culture</td>
<td>0</td>
</tr>
</tbody>
</table>

### Table 2

<table>
<thead>
<tr>
<th>CLOtest for <em>H. pylori</em> at 1 h compared with the estimated true prevalence</th>
<th>Estimated true prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLOtest</td>
<td>Positive</td>
</tr>
<tr>
<td>CLOtest</td>
<td>145</td>
</tr>
<tr>
<td>CLOtest</td>
<td>0</td>
</tr>
</tbody>
</table>
TABLE 3  Histopathology reports of *H. pylori* compared with the estimated true prevalence

<table>
<thead>
<tr>
<th>Estimated true prevalence</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histopathology Positive</td>
<td>124</td>
<td>0</td>
</tr>
<tr>
<td>Histopathology Negative</td>
<td>36</td>
<td>309</td>
</tr>
</tbody>
</table>

TABLE 4  Comparison of the sensitivity, specificity and positive and negative predictive values of culture for *H. pylori* compared with the CLOtest and histopathological examination of gastric biopsies

<table>
<thead>
<tr>
<th></th>
<th>Culture (%)</th>
<th>CLOtest (1 h) (%)</th>
<th>Histopathology (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>73.2</td>
<td>58.7</td>
<td>77.0</td>
</tr>
<tr>
<td>Specificity</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>PPV</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>NPV</td>
<td>86.3</td>
<td>82.4</td>
<td>89.6</td>
</tr>
</tbody>
</table>

Susceptibility to antibiotics

One hundred and thirty-five isolates were available for susceptibility testing (some of the initial isolates were stored at −80°C and failed to grow on subculture). The MICs of *H. pylori* for amoxycillin are shown in Fig. 1. All isolates were highly susceptible. Similarly, all the isolates were susceptible to tetracycline if an MIC of >1 μg/ml is used as the breakpoint to indicate resistance, although there was a wider spread in the distribution of the MICs as compared with amoxycillin (Fig. 2). When this same breakpoint was applied to clarithromycin, the large majority of isolates were highly susceptible, a few were susceptible with higher MICs, and 5.2% were resistant (Fig. 3). In contrast, when a breakpoint of ≥8 μg/ml was used to indicate resistance to metronidazole, 60% of isolates were resistant, the vast majority having very high MICs (Fig. 4). Fifty-two of the 135 isolates were obtained from females and of these, 65% were resistant to metronidazole compared with 57% of those obtained from males.
advantages. The former include $^{13}$C and $^{14}$C breath tests and the measurements of serum antibody levels against *H. pylori*. These have the advantage of simplicity and cheapness. However, in the case of breath tests, the dividing line between positive and negative values may not be clear-cut, while serology does not differentiate between past and present infection. Moreover, neither investigation permits visualisation of the upper gastrointestinal tract for gross abnormalities. In contrast, endoscopy is an invasive procedure that generally requires intravenous sedation. It allows observation of the gastrointestinal tract as well as the opportunity to take biopsies from the stomach or any obvious lesion for histopathological assessment. In addition, gastric mucosa can be cultured for *H. pylori* and subsequent assessment of antibiotic susceptibility patterns.

The first aim of this study was to compare the relative utilities of three diagnostic modalities in an unselected series of patients who underwent endoscopy. Unfortunately, none of the three techniques used was completely reliable. Excluding the unlikely event of contamination, culture of *H. pylori* from the stomach is definitive proof of infection. Unfortunately, failure to grow *H. pylori* does not exclude infection, as organisms sometimes lose their viability during collection, transport or processing, or occasionally specimens are overgrown with other organisms. Similarly, the CLOtest is relatively non-specific and relies on a pH change brought about by the presence of the enzyme urease. This is dependent upon the number of *H. pylori* present and is a function of time and temperature. Furthermore, there may be contamination with other organisms, particularly *Proteus* species, which may also produce urease. Histopathological examination likewise has its limitations. Small numbers of *Helicobacter* may be missed unless a careful inspection is made. On the other hand, not all the spiral organisms seen in gastric mucus may in fact be *H. pylori*.

These features make it difficult to compare directly the relative efficacies of these three diagnostic techniques. Consequently, an attempt was made to estimate the true prevalence of infection in the population studied as described earlier. If this estimation is accepted as a reasonable approximation of the true prevalence, then the sensitivity, specificity, positive predictive value and negative predictive value of the three techniques can be calculated. It is likely that few, if any, patients will be falsely diagnosed by either culture, histopathological examination or the CLOtest when read at one hour. The most sensitive diagnostic test was histopathological examination, picking up approximately three-quarters of infected patients. This was followed by culture with the CLOtest a relatively poor third. Thijs et al. used a different and, in our opinion, less satisfactory method for defining the benchmark. They regarded a patient with at least two of six tests (culture, polymerase chain reaction test, histological examination, rapid urease test, urea breath test and serology) as being infected. When this gold standard was used, they claimed sensitivities for culture, histology and rapid urease testing of 98.4%, 96.0% and 90.2%, respectively. These results are apparently better than ours but may merely be a function of the true-positive benchmark being set too low. Nevertheless, in our series, when a positive diagnosis was made on the basis of either histopathology or culture, just over 99.5% of patients were diagnosed. Thus, fewer than 0.5% of truly infected patients are likely to be missed if the CLOtest is omitted.

The sensitivity of histopathological examination is influenced by the number of biopsies and the location from which they are taken. It is generally accepted that spiral organisms are more commonly seen in biopsies taken from the antrum than from the body of the stomach. This was confirmed in this series, although it should be noted that in a few patients from whom biopsies were taken from both sites, organisms were seen only in the body. It is likely that the same two factors of number and location influence the likelihood of successfully growing *H. pylori* from infected patients and for having a positive urease test. If the gastroenterologist wishes to minimise the number of biopsies taken, two should be taken from the antrum, one for histopathological examination and one for bacterial culture.

The second aim of this study was to determine the susceptibility of *H. pylori* to a range of antibiotics. Some antibiotic eradication regimens are more successful than others and these are likely to change with the passage of time as antibiotic resistance evolves. It is not possible in a single patient to predict which antibiotics are likely to be effective. Unfortunately, when determining *in vitro* susceptibility to antibiotics, there are no internationally recognised criteria for the definition of susceptibility and resistance of *H. pylori*. Various techniques have been used to measure MICs. We used the E test as have a number of other authors.

With respect to metronidazole, various authors have used breakpoints of $>4$, $>8$ and $>8$ to indicate resistance. We used a breakpoint of $>2$, $>8$ and $>8$ to indicate resistance. Amongst our isolates, there was only a small number of isolates with MICs of 4 or 6 mg/ml. The vast majority of those that were classified as resistant had MICs of $>32$ mg/ml, which is clearly highly resistant. Sixty per cent of our isolates were resistant to metronidazole. This is somewhat higher than in Europe, where rates are generally around 40%, but less than some developing countries where rates of $>70%$ have been found. Our resistance rate may be a reflection of high metronidazole usage in Australia. Metronidazole is one of the drugs in the standard triple regimen currently available on the Australian pharmaceutical benefits schedule. Since over half of the isolates were resistant to metronidazole, this regimen is likely to be inadequate, although this view has been contested.

There is little information available concerning an appropriate breakpoint for amoxycillin; Hachem et al. used a value of $>8$ mg/ml to indicate resistance. When we used a value of only $>1$ mg/ml, all isolates were susceptible, most of them highly so. Nevertheless, there must be some doubt as to the clinical relevance of this *in vitro* observation given that so much amoxycillin is prescribed in the general community yet the prevalence of *H. pylori* infection is so high. Indeed, a formal trial in which amoxycillin was the only antibiotic used (in combination with bismuth) yielded a cure rate of only 50%.

Similarly, there is difficulty in knowing the breakpoint for tetracycline; a value of $>1$ mg/ml was used by Vasquez et al. to indicate resistance. We used a breakpoint of $>1$ mg/ml; all isolates were susceptible although there was a wide range from $<0.016$ to 0.75 mg/ml.

There is some confusion over a breakpoint for clarithromycin. Vasquez et al. used a value of only
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≥ 0.125 µg/ml to indicate resistance whereas other authors used a value of > 2 µg/ml.[22,24,25,33] We used a value of > 1 µg/ml to indicate resistance. Just over 5% of isolates were resistant, with some of them having extremely high MICs. Clarithromycin is being used with increasing frequency in eradication regimens. The occurrence of resistance in some isolates underscores the importance of determining susceptibility if this expensive antibiotic is going to be used.

It is probable that the susceptibility patterns of H. pylori to individual antibiotics will change with the passage of time and increasing numbers of patients with peptic ulcers or H. pylori gastritis being treated with antibiotics. Empirical antibiotic therapy may become less successful and knowledge of the antibiotic susceptibility profile of H. pylori for each infected patient may be helpful in achieving a successful outcome.[14,17,34] This needs to be balanced against the extra costs involved. Excluding the costs of identification and susceptibility testing costs a further 20 dollars or so, most of the expense being in the purchase of labour, which vary according to the salary level of the technical officer, it costs less than one dollar for materials to culture for H. pylori. If the organism is grown, identification and susceptibility testing costs a further 20 dollars or so, most of the expense being in the purchase of Etests.

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References
5. Graham DY. The only good Helicobacter pylori is a dead Helicobacter pylori. Lancet 1997; 350: 70.