An investigation of Helicobacter pylori using culture, histopathological and serological examination methods and its antimicrobial sensitivities

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ABSTRACT

Objectives: In this study, the determination of Helicobacter pylori (H. pylori) by culture, histopathological and serological methods in cases of endoscopically diagnosed as duodenitis and duodenal ulcer (DU), a comparison of their relative advantages, and its antibiotic sensitivities were investigated.

Methods: Helicobacter pylori was investigated using 3 methods (culture, histopathological and serological examination) in 50 patients (25 diagnosed with duodenitis and 25 with DU) at the Department of Gastroenterology, Istanbul Haydarpasa Numune Hospital, Turkey between December 2000 and February 2001. An investigation into its antibiotic sensitivities to amoxicillin, clarithromycin, metronidazole and azithromycin by disc diffusion methods and to amoxicillin and clarithromycin by E-test were investigated.

Results: Helicobacter pylori bacteria were observed in Gram stained preparates prepared from biopsy material in 34 out of 50 patients (68%), and were able to be produced in active culture in all these cases. Histopathological examination revealed the presence of H. pylori in 80% cases of DU and 60% cases of duodenitis; anti-CagA(IgG) was positively determined in 88% DU cases and in 60% duodenitis cases. There was a significant difference between the 2 groups in terms of diagnosis by histopathological and serological methods. The difference between the 2 groups produced in active culture in 84% cases of DU cases and 52% of duodenitis was statistically significant (p=0.0322). Using the E-test and disc diffusion methods, 8.8% of the strains that reproduced in culture were resistant to clarithromycin. All strains were determined to be sensitive to amoxicillin: 17.6% of the strains were determined to be resistant to metronidazole, 11.7% to azithromycin.

Conclusion: It was observed that Gram staining is a rapid and reliable method of pre-diagnosis for H. pylori; that histopathological examination methods are of considerable importance in diagnosis; and that the investigation of the positivity of anti-CagA(IgG) will be a guide in the identification of virulent strains in particular. In addition, it was also concluded that since serological examination does not require invasive measures, this will pose an advantage. The culture method can be applied with the aim of diagnosis in cases identified as DU using endoscopy, and that in cases resistant to treatment it can be applied for the purpose of determining antimicrobial sensitivity. E-test and disc diffusion methods exhibited a rather good correlation, for which reason the disc diffusion method can be used in the determination of antimicrobial sensitivity in H. pylori strains.

Following the definition of Helicobacter pylori (H. pylori) as a result of intense research into peptic ulcer pathogenesis, the agent was isolated from stomach mucosa biopsy specimens from individuals with chronic active gastric and peptic ulcers. In later studies, it was determined that with the eradication of H. pylori some 90-95% of duodenal ulcer (DU) cases and 70% of gastric ulcer cases healed completely. Tests used today need endoscopic examination for histopathological examination, bacteria culture, tissue urea test, DNA probe and polymerase chain reaction (PCR) tests; no need for invasive interventions with the urea breath test, serological tests, stomach fluid examination with PCR and the investigation of N15-labelled ammonia in urine. The eradication of H. pylori is rather difficult, and usually requires the combined use of more than one drug, and there is still no agent that has been shown to be 100% effective. The drugs used in treatment are proton pump inhibitors or ranitidine, bismuth compounds, clarithromycin, amoxicillin, metronidazole, azithromycin and tetracycline. The treatment recommended today is for a proton pump inhibitor accompanied by 2 antibiotics (amoxicillin, clarithromycin, metronidazole). The results have been very successful, with an approximately 90% eradication rate. This rate has been reported to be 48% in dual therapy and 18% with single-agent treatment. However, the major factor affecting this treatment is variations in antibiotic resistance patterns. From the clinical point of view, resistance to the nitroimidazoles and macrolide group antibiotics in many treatment regimes is important.

Methods. A total of 50 patients, 25 diagnosed with duodenitis and 25 with DU at the Department of Gastroenterohepatology, Istanbul Haydarpasa Numune Hospital, Turkey, between December 2000 and February 2001, were included in the study. Cases which had used antibiotics within the preceding month, or had used non-steroid anti-inflammatory agents before their complaints began, and cases with illnesses which could not tolerate upper gastrointestinal system endoscopy were excluded from the study.

Using sterile biopsy forceps, a total of 4 biopsy specimens: one from the corpus, 2 from the antrum, and one from the duodenum quadrant, as well as serum specimens were taken from all patients. One biopsy specimen from the antrum was placed into brain-heart infusion medium and then was sown into the Pylori agar (Biomérieux, Marcy 1Etoile, France) within 30 minutes. After incubation in a microaerophilic environment (GENbag microaer bag from Biomérieux) at 37°C for 5-7 days, urease, catalase and oxidase tests were performed from 1 mm diameter transparent colonies. The culture was considered as positive with the positivity of all 3 tests and with the presence of Gram negative, coccoid, spiral or gull wing shaped bacteria in the Gram staining. Bacterial suspensions from the bacteria which reproduced in culture were prepared with sterile salt water and a density of 0.5x10^9 cfu/mL (McFarland 3-4). The prepared bacterial suspensions were inoculated into 3 culture media with a sterile ecuvion. Amoxicillin (10 µg), clarithromycin (15 µg), metronidazole (5 µg) and azithromycin (15 µg) (Oxoid) antibiotic discs were placed on one of the slides.

E-test strips (AB biodisc) for amoxicillin and clarithromycin were placed on the other 2 culture media with an applicator, in such a way that the minimal inhibitory concentration (MIC) reading scale would be on the top, at an angle of approximately 90 degrees. The inoculum was incubated at 37°C in a microaerophilic environment for 5-7 days. The lowest value at which reproduction was not observed in the elliptical inhibition zone was determined as the MIC value. ATCC 43504 was used as the control strain for both methods. The minimal acceptable inhibitor concentration level for this strain was 0.015-0.12 for amoxicillin and 0.015-0.12 for clarithromycin. And in disc diffusion method, isolates showing scattered colonies within the zones of inhibition were recorded as resistant. Strains with an inhibition zone of 15 mm, 40 mm, 20 mm and 25 mm or more were considered as susceptible for metronidazole, amoxicillin, clarithromycin and azithromycin.

The other biopsy specimens were sent to the pathology laboratory with 10% buffered formalin solution. The preparations readied with hematoxylin-eosin and modified Giemsa stains were examined and classified according to the Sydney classifications. Once the cases were completed, the serum specimens stored at -70°C were brought to room temperature and antiCagA (IgG) (Equiper) were investigated using the enzyme-linked immunosorbent assay (ELISA) method. In both groups values above 5.00 Uarb/mL were regarded as positive.

Statistical analysis was performed using Fisher’s exact test.

Results. Helicobacter pylori is investigated using culture, histopathological and serological examination methods in patients, which endoscopically diagnosed with duodenitis and DU. Antibiotic sensitivity in cases of reproduction determined in culture was measured. The ages of the 50 patients in the study ranged from 19-75, with an average of 49 years. Patients in our study were endoscopically divided into 2 groups, DU and duodenitis. Of the DU group 17 (68%) were men and 8 (32%) women, in duodenit group 6 (24%) were men and 19 (76%) women. Helicobacter pylori was positively identified in Gram stained
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Figure 1 - Gram stained preparate from biopsy material of patients with a) duodenal ulcer and b) duodenitis.

preparates from biopsy material in 34 (68%) of the 50 patients. Reproduction in culture was determined in all the Gram stain positive cases. *Helicobacter pylori* was positively identified in cultures in 34 (68%) of the 50 patients. Of these, reproduction took place in 21 (84%) of the 25 patients in the DU group and in 13 (52%) of the 25 patients in the duodenitis group. *Helicobacter pylori* positivity in culture is shown in Figures 1a and 1b.

*Helicobacter pylori* was positively identified in histopathological method from biopsy material in 35 of the 50 patients. Of these, reproduction took place in 20 (80%) of the 25 patients in the DU group and in 15 (60%) of the 25 patients in the duodenitis group and although there was a difference between the 2 groups it was not statistically significant ($p=0.2165$). *Helicobacter pylori* was produced in biopsy material culture in 21 of the DU cases and 13 duodenitis cases, and the difference between the 2 groups was statistically significant ($p=0.0322$).

Anti-CagA (IgG) was positively identified in serological methods in 37 (74%) of the 50 patients. Of these, serological positiveness was obtained in 22 (88%) of the 25 patients in the DU group and in 15 (60%) of the 25 patients in the duodenitis group. Using the serological method, serological positiveness was determined in 22 of the DU cases and 15 of the duodenitis cases, and this difference was significant ($p=0.05$). It was determined by E-test that 8.8% of the 34 *H. pylori* strains reproducing in culture were resistant to clarithromycin and 91.2% were sensitive to it. The figures were again 8.8% resistant and 91.2% sensitive using the agar disc diffusion method. It was determined using the E-test and agar disc diffusion methods that the 34 *H. pylori* strains were 100% sensitive to amoxicillin. Levels of resistance of 17.6% to metronidazole and 11.7% to azithromycin in *H. pylori* strains were determined using the agar disc diffusion method.

**Discussion.** In our study, the importance of the culture used in the diagnosis of *H. pylori* and of the histopathological and serological investigations used in diagnosis, the importance of the investigation of CagA (IgG) positivity in the identification of virulent strains, the correlation of the E-test and disc diffusion culture methods and the resistance of *H. pylori* to antimicrobials in our region were all investigated. Reproduction in culture was identified in all Gram positive cases. Furthermore, it was observed that Gram staining is significant since it is easily applied and a rapid and reliable method of pre-diagnosis. Bacteria of *H. pylori* morphology were identified in Gram stained preparates prepared from biopsy material in 34 of the 50 patients. This level was 84% in the duodenitis group and 52% in the DU group. *Helicobacter pylori* was positively identified in cultures in 34 (68%) of the 50 patients. Of these, reproduction took place in 21 (84%) of the 25 patients in the DU group and in 13 (52%) of the 25 patients in the duodenitis group. Mirghani et al. determined culture positivity levels of 56% in DU and 60% in duodenitis patients, and Cammarota et al. determined levels of culture positivity of 84.2% in DU cases and of 66.6% in duodenitis cases. These differences in the culture findings were explained in terms of the bacterium not being in a whole state in the stomach antrum submucosa and being maintained in patch style in specific regions. In another aspect, it may be said that the application of the culture method will not only be useful in determining the bacterium’s antibiotic sensitivity, but also from the point of view of diagnosis.

In addition to its place in pathological diagnosis, the way that histological examination permits the bacterium to be shown by staining has led some researchers to recommend this method as the gold standard. In our study, *H. pylori* was determined by the histopathological method in 35 (70%) of the 50 patients. Biopsy results were identified as positive in 20 (80%) of the 25 DU cases and 15 (60%) of the duodenitis cases. In addition, Anti-CagA (IgG) positivity was compared with histology and culture in order to determine the relationship between DU.
and duodenitis and Anti-CagA (IgG) positivity. The fact that serological methods in the diagnosis of \textit{H. pylori} do not require endoscopy, and are cheaper, faster and easier to apply than other methods. They are particularly recommended in initial screening examinations before endoscopy or therapy in dyspeptic patients below the age of 45. Different strains of \textit{H. pylori} exhibit different levels of virulence, and the CagA gene and high molecular weight products of this gene are important in determining this virulence.\textsuperscript{4} In this study, Anti-CagA (IgG) was investigated in 50 serum specimens using the ELISA method, and was determined as positive in 37 (74\%). Serological positivity was determined in 22 (88\%) of the 25 patients in the DU group and in 15 (60\%) of the 25 patients in the duodenitis group. This difference was statistically significant ($p$=0.005). \textit{Helicobacter pylori} infected patients may be seropositive while histology, culture and PCR are negative. This can be accounted for in 2 ways. The serology results may be erroneously positive, although this is unlikely with IgG ELISA; alternatively, the number of micro-organisms may be low for other methods in these patients. Serological tests are faster, easier and cheaper than urea breath test and endoscopic procedures. Serology is non-invasive and does not require the use of radioactive material. With these advantages serology is of considerable value in the diagnosis of \textit{H. pylori} and will be useful in the investigation of anti-Cag (IgG) positivity in the identification of virulent strains.

We did not used any other method in \textit{H. pylori} diagnosis, which is the PCR method, as various primers have been defined for cagA amplification, as particular primers need to be defined for every geographical region, otherwise false negative results can be obtained, and there are no defined primers for our region. One of the aims of our study was to examine the cagA difference between the DU and duodenitis patient groups, and this was found to be statistically significant. These results indicate that DU table had more virulent \textit{H. pylori} strains than duodenitis.

E-test and disc diffusion methods showed a rather good correlation, for which reason it was concluded that the disc diffusion method could be used in the determination of antimicrobial sensitivity in \textit{H. pylori} strains. It was determined by E-test that 8.8\% of the 34 \textit{H. pylori} strains reproducing in culture were resistant to clarithromycin, it was 8.8\% resistant using the agar disc diffusion method. It was determined using the E-test and agar disc diffusion methods that the 34 \textit{H. pylori} strains were 100\% sensitive to amoxicillin. Franzin et al\textsuperscript{12} also determined a sensitivity level of 100\% to amoxicillin using both methods, and despite the E-test being more expensive than the disc diffusion method they reported that it was an appropriate and easily applied method for the determination of antimicrobial sensitivity in clinical microbiology laboratories. In this study, levels of resistance of 17.6\% to metronidazole and 11.7\% to azithromycin in \textit{H. pylori} strains were determined using the agar disc diffusion method. Other study, metronidazole sensitivity in 100 \textit{H. pylori} strains using E-test, disc diffusion and agar dilution methods was investigated. They reported that the E-test and disc diffusion results were compatible and that disc diffusion was a good alternative for determining metronidazole sensitivity.\textsuperscript{13}

In conclusion, rising levels of antimicrobial resistance and the gravity of the diseases and complications to which it is linked, the diagnosis and standardization of treatment of \textit{H. pylori} is seen to be acquiring increasing importance. These resistance and multiple resistance levels are sounding a warning in terms of increasing surveillance on the subject of \textit{H. pylori}.

References