Antibiotic Resistance and Failure of Eradication of Helicobacter Pylori in Egypt

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ABSTRACT

Background: Helicobacter pylori is a Gram negative bacterium that colonizes human gastric mucosa and is one of the most common bacterial pathogens worldwide with a prevalence of up to 90% in developing countries. It is the primary cause of peptic ulcer disease and an etiologic agent in the development of gastric cancer. H. pylori infection is curable with regimens of multiple antimicrobial agents. However, antibiotic resistance is a leading cause of treatment failure. The aim of this study is to assess the prevalence of H. pylori in gastric biopsies taken from Egyptian patients by using invasive methods and study the role of antimicrobial agents in elimination of this bacterium.

Methodology: From 50 patients, 3 antral gastric biopsies were taken from the greater curvature about 2 cm from pylorus. The first biopsy was for direct Gram’s stain and culture (using Blood agar) to apply traditional biochemical tests and antimicrobial susceptibility test to different antibiotics. The second biopsy was used for rapid urease test (using modified Christensen’s urea broth) and the third biopsy was kept in deep freezer at -70°C in brain heart infusion broth for PCR assay using UreC gene.

Results: Among 50 patients, 22 (44%) were positive by culture, 17 (34%) were positive by direct Gram’s stain with 77.3% sensitivity, 100% specificity and 90% accuracy, while 19 (38%) were positive by rapid urease test with 63.6% sensitivity, 82.1% specificity and 74% accuracy, and 25 (50%) were positive by PCR with 95.5% sensitivity, 89.3% specificity and 92% accuracy. By antimicrobial susceptibility testing using disc diffusion method, it was shown that the highest susceptibility of the isolated H. pylori strains was to amoxicillin (90.9%) followed by tetracycline (81.8%), Gentamicin (54.5%), Erythromycin (18.2%) and Ciprofloxacin (9.1%). However, no one (zero%) was highly sensitive to Metronidazole.

Conclusion: Some of the antibiotics widely used in Egypt are no longer suitable for treatment of Helicobacter pylori and new antibiotics regimens are needed to eradicate this organism.

Key words: H. pylori and antimicrobial agents eradication.

INTRODUCTION

H. pylori is a Gram negative spiral shaped, measuring 2 to 4 µm in length and 0.5 to 1 µm in width, multi-flagellated bacteria found almost exclusively on human gastric mucosa. It contains a hydrogenase which can be used to obtain energy by oxidizing molecular hydrogen (H2) that is produced by intestinal bacteria. The bacterium is a microaerophilic and capnophilic organism, slowly growing with rigorous culture demands.

Helicobacter pylori plays a vital role in the pathogenesis of several gastro duodenal pathologies makes its diagnosis necessary in many different circumstances. Since, the diagnosis of this bacterium is an essential element in the management of many common gastrointestinal pathologies.

Several virulence factors of H. pylori have been identified. The most studied is CagA (Cytotoxin associated gene Antigen) which according to numerous studies, is associated with peptic ulcers, precancerous conditions and gastric cancer in the Western world. Another well-known virulence factor, VacA (vaculating cytotoxin A) is associated with peptic ulcer, adenocarcinoma and gastric cancer.

Antibiotic resistance is an ever increasing problem with the treatment of most microbial infeccon including Helicobacter pylori and has become a growing problem world wide with eradication of this organism.

Aim of the Work

So this study was conducted to evaluate recent changes of antibiotic resistant pattern of Helicobacter pylori isolated from Egyptian patients to the most common antibiotics used for treatment of this organism.

MATERIALS & METHODS

50 patients with age ranging from 18 to 70 years suffering from abdominal complaints underwent gastroendoscopic examination. From each patient, 3 antral gastric biopsies were taken...
from the greater curvature about 2 cm from pylorus. (Prior to endoscopy a consent were taken from each patient). The first biopsy was transported to Microbiol. Lab. Within 2 hrs for direct Gram stain and culture. The second biopsy was used for rapid urease test and the third biopsy was kept in deep freezer at -70°C in brain heart infusion broth (BHIB: Oxoid) containing 10 % glycerol for PCR assay. (19)

The culture was performed on Blood agar (Oxoid Columbia agar base) figure1. Plates were incubated at 37°C for 3-5 days in a microaerophilic atmosphere (Campy Pak; Becton Dickinson). Identification of  H. pylori was made by Gram staining of the colonies, lack of aerobic growth on blood agar plates, and testing for the presence of urease, oxidase and catalase. (10) and an inoculum was spread all over agar surface using cotton swab for application of antimicrobial Susceptibility testing using; Amoxicillin, Tetracycline, Gentamicin, Erythromycin, Ciprofloxacine and Metronidazole. The Second biopsy was used for Rapid urease test where inoculated in 10% Urea broth with phenol red as an indicator. The presence of urease was indicated by colour change from yellow to pink. (2).

The third biopsy was used for extraction of DNA using DNeasy Blood and Tissue Kit(Qiagen Gmbh, Hilden,Germany). The extracted DNA was used for PCR using primers for the amplification of a highly conserved region Ure c gene( glm M gene) in which the size of the amplified product obtained is 294 base pair in (photo1) (7). The PCR protocol used was described by Lu et al. (11). PCR reactions were performed in a total reaction volume of 50 ul containing 25 ul 2x PCR Master Mix (Ferments USA)which compose of (Tag DNA polymerase recombinant in reacton buffer 0.05 units/ul, Mg cl2,4 Mm, d NTP s (dATP, d GTP, dTTP and dCTP,0.4Mm of each).To 25 ul 2x Master Mix we added 5 pm of each of the forward primer (5 AAG CTT TTA GGG GTG TTA GGG GTT T3)and the reverse primer (5 AAG CTT ACT TTC TAA CAC TAA ACG C3). (Biron, Germany). Then 5ul of the template DNA were added and the volume was completed to 50 ul by nuclease free water. The samples were maintained at 94 °C for 5 minutes and then 34 cycles (94 °C for 1 minute, at 56 °C for 1 minute and at 72 °C for 2 minutes) using DNA thermal cycler(Perkin Elmer ,USA). The PCR products were run on a 2% agarose gel electrophoresis and the products were visualized by ethidium bromide using UV transillumination (Cole-Parmer instrument CO, Chicago USA).

Statistical Methods:
The statistical analysis was performed using SPSS version 16 .P values less than 0.05 were considered to indicate significance.

RESULT

Among 50 patients, 22 (44%) had positive Culture results, 17(34%) had positive direct Gram's stain findings, 19(38%) had positive Rapid urease test results, and 25 (50%) had positive PCR results.In accordance with the culture as a gold standard; the sensitivity, specificity, positive predictive value, Negative predictive value and accuracy for Direct Gram's stain were 77.3%, 100%, 100%, 84.8% and 92%,respectively, the sensitivity, specificity, positive predictive value, Negative predictive value and accuracy for rapid urease test were 63.6%, 82.1%, 73.7%, 74.2% and 74%,respectively and the sensitivity, specificity, positive predictive value, Negative predictive value and accuracy for PCR were 95.5%, 89.3%, 87.5%, 96.2% and 92%,respectively as shown in table (1) which indicates that, The PCR method is the most sensitive method (95.5%) followed by direct Gram stain (77.3%). Direct Gram stain method is the most specific method (100 %) followed by PCR method (89.3%). While the rapid urease test is the least specific (82.1%). As regarding to antimicrobial susceptibility testing by disk diffusion method, it was shown that, the highest susceptibility of the isolated  H.pylori strains was to amoxicillin (90.9%) followed by tetracycline (81.8%) and Gentamicin (54.5%). On the other hand, all the isolated  H.pylori strains were resistant to metronidazole (91.0%). As shown in table (2) from which it was concluded that the the antimicrobial agent Amoxicillin and Tetracycline are the most drugs of choice for  H.pylori treatment. The result is statistically significant (P<0.001).
Figure (1): Agarose gel showing *H. pylori* DNA band at 294bp. 
Lane(1): Molecular size standard (1-kb DNA lader, Gibco BRL). 
Lane(5, 6, 7): Positive gastric biopsy specimens for *H. pylori*. 
Lane(2): Negative control. 
Lane(3): Positive control. 
Lane(4, 8): Negative gastric biopsy specimens for *H. pylori*.

Table (1): Validity of different laboratory methods used as confirmed by Culture for identification of *H. pylori*

<table>
<thead>
<tr>
<th>Screening test</th>
<th>Culture method (reference test)</th>
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<tbody>
<tr>
<td></td>
<td>Positive n= 22 Negative n= 28</td>
</tr>
<tr>
<td>Direct Gram stain</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>17 (a) 0 (b) (77.3) (100.0) 100 84.8 90.0</td>
</tr>
<tr>
<td>Negative</td>
<td>5 (c) 28 (d)</td>
</tr>
<tr>
<td>Rapid urease Test</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>14 (a) 5 (b) (63.6) (82.1) 73.7 74.2 74.0</td>
</tr>
<tr>
<td>Negative</td>
<td>8 (c) 23 (d)</td>
</tr>
<tr>
<td>PCR</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>21 (a) 3 (b) (95.5) (89.3) 87.5 96.2 92.0</td>
</tr>
<tr>
<td>Negative</td>
<td>1 (c) 25 (d)</td>
</tr>
</tbody>
</table>

Table (2): Antimicrobial susceptibility of the isolated *H. pylori* strains.

<table>
<thead>
<tr>
<th>Antimicrobial agents</th>
<th>Sensitive strains No (%)</th>
<th>Intermediate strains No (%)</th>
<th>Resistant strains No (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td>20 90.9</td>
<td>2 9.1</td>
<td>0 0.0</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>18 81.8</td>
<td>4 18.2</td>
<td>0 0.0</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>12 54.5</td>
<td>9 40.9</td>
<td>1 4.5</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>4 18.2</td>
<td>10 45.4</td>
<td>8 36.3</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>2 9.1</td>
<td>11 50.0</td>
<td>9 40.9</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>0 0.0</td>
<td>2 9.1</td>
<td>20 90.9</td>
</tr>
</tbody>
</table>
DISCUSSION

In the routine clinical diagnostics, rapid urease test, histopathological examination, culture, direct Gram's staining and PCR assay are valuable methods for detection of H. pylori in gastric biopsies\(^{(29)}\). In our study, Culture technique was used as a gold standard for diagnosis of H. pylori infection where 22(44%) among 50 patients was positive using culture. Since, culture proved a very successful method for detecting H. pylori in gastric biopsy specimens and this coincide with that reported by Ozcakir et al.\(^{(18)}\) who reported that among 148 patients with dyspeptic complaints, 70(47.2%) were positive by using culture. The rapid urease test is a simple, inexpensive method for detecting H. pylori in gastric tissues, in our study, 19(38%) among 50 patients was positive using rapid urease test and this agreed with that reported by Oyedeji et al.\(^{(17)}\) who reported the positive rate of 435 stomach mucosal biopsies taken from 145 consecutive patients, was 61 (42.1%) using RUT. and disagreed with that reported by Sengupta et al.\(^{(20)}\), found that of 25 patients, 24 were positive(96%) by using RUT. this difference may be attributed to that false-positives can occur owing to the presence of other urease-positive bacteria in the gastric tissues or reflux of alkaline bile into the stomach\(^{(4)}\), the Direct Gram's stain showed 17(34%) was positive and this is in close agreement with that of Murata et al.\(^{(14)}\) who found that direct gram's stain was positive 609(44.6%) in specimens taken from tunica mucos vestibulum ventricul and tunica mcosa corpus ventriculi. The PCR technique is an accurate for diagnosis of H. pylori as among 50 patients, 24(48%) was positive with PCR assay. This is in close coinincence with that repored by Stella et al.\(^{(24)}\) who found that 3/7(42.8%) patients were positive by using PCR with glmM primers, they concluded that PCR test using glmM gene appears to be the most reliable test for H. pylori diagnosis, and Ah Ra Cho et al.\(^{(31)}\) who found that the positivity rates of infection from 90 patients was 42.2% using PCR assay. in relation to validity of these tests, it was shown that, the The Direct Gram’s staining has 77.3% sensitivity, 100% specificity, 100% PPV, 84.8% NPV and 90% accuracy. and this coincide with that of Sadeghfard et al.\(^{(30)}\) who reported that the sensitivity, specificity and accuracy of direct Gram stain when Culture was taken as a gold standard are 89.7%, 96.9% and 86.3% respectively, while Rapid urease test has 63.6% sensitivity, 82.1% specificity, 73.7% PPV, 74.0% NPV and 74% accuracy when Culture was taken as a gold standard and this is similar to that of Taj et al.\(^{(28)}\) who reported the sensitivity and specificity of urease test was 67% and 85% respectively with culture as a reference. but disagreed with that reported by Kalem et al.\(^{(8)}\) who found that the sensitivity and specificity of RUT with a reference culture were 97.5% and 20.7% respectively, PCR assay has, the sensitivity, specificity, PPV, NPV and accuracy when culture was taken as a gold standard were 95.5%, 89.3%, 97.5% and 92.0% respectively. and this is strongly agree to that reported by Stella et al.\(^{(24)}\) who found that the PCR assay using glmM primers has 100% sensitivity, 74.1%-85.1% specificity, 68.2%-75% PPV and 100% NPV with culture and RUT as a gold standards. in the present study, the isolated H. pylori strains were highly sensitive to amoxicillin (90.9%), Tetracycline (81.8%) and showed intermediate susceptibility to Gentamicin (54.5%) while it showed 10% resistance to Metronidazole and 40.9% resistance to Ciprofloxacin. This result is very important as metronidazole is acorner stone of many triple-therapy formulation for the eradication of Helicobacter pylori and this agreed with several studies which revealed that resistance to metronidazole approach 90% in many developing countries and even in Western Europe it ranges from 5 to 50%. In Egypt, a universal high level primary metronidazole resistance in children compared to lower resistance to other selected antibiotics was reported by Sherif et al.\(^{(12)}\). And our results also agreed with Gholam et al.\(^{(6)}\) who found that 24 patients were resistant to metronidazole (84.1%), 4.1% to erythromycin and they are highly sensitive to Tetracycline, Amoxicillin and furazolidene. and Treiber et al.\(^{(26)}\) who found that H.pylori resistance rate to Metronidazole is 29.1- 41%, but disagreed with that reported by Smith et al.\(^{(23)}\) who documented that H.pylori strains were 100% resistance to Amoxicillin.

CONCLUSION

Polymerase chain reaction is the most accurate for diagnosis of H.pylori in gastric biopsy specimens. Some of antibiotics widely used in Egypt are no longer suitable for treatment of Helicobacter pylori and new antibiotics regimen are needed to eradicate this organism, since, Amoxicillin and
Tetracycline were taken as good antibiotics for treatment.

REFERENCES


