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Determination of Antibiotic Sensitivity Pattern of *Helicobacter pylori* Isolates from South India Population by Epsilometer Test (E-Test)

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ABSTRACT

Introduction: The importance of *H. pylori* as an etiological agent in gastroduodenal disease had suggested antibiotic treatment as a main target for the elimination of infection. The successful eradication of *H. pylori* infection was shown to resolve the gastritis, dramatically accelerate ulcer healing, reduce ulcer recurrence and the prophylactic effect on the recurrence of ulcer bleeding. **Materials and Methods:** In the present study a total six antimicrobial agents such as Ciprofloxacin, Metronidazole, Norfloxacin, Tetracyclin, Amoxycillin and Clarithromycin were used against 100 clinical isolates from patients with peptic ulcer, and non-ulcer dyspepsia. *H. pylori* was isolated from the gastric biopsies obtained from the clinical subjects. **Results:** Results of our study showed 100% resistance to Metronidazole (MIC > 256 µg/ml). 97% of the isolates were observed sensitive to Ciprofloxacin (MIC 0.38 µg), whereas Tetracyclin showed 96% sensitivity (MIC 0.25 µg - 0.125 µg). Resistance to amoxycillin, Norfloxacin, and Clarithromycin were 80%, 38% and 76% respectively (MIC > 256 µg/ml). **Conclusion:** It is observed that 96% - 97% of the clinical isolates were sensitive to Tetracycline and ciprofloxacin, where all isolates were found to be resistant to Metronidazole. Resistance to Amoxycillin and Clarithromycin were 80% and 76% respectively. Studies attributed the high level of resistance to the frequent use of the drugs to treat various other infections and ineffective drug control policy.

Keywords: *Helicobacter pylori*; Culture; Metronidazole; Tetracycline; Clarithromycin

1. Introduction

The importance of *H. pylori* as an etiological agent in gastroduodenal disease had suggested antibiotic treatment as a main target for the elimination of infection. The successful eradication of *H. pylori* infection was shown to resolve the gastritis, dramatically accelerate ulcer healing and reduce ulcer recurrence and the prophylactic effect of the recurrence of ulcer bleeding [1]. Several studies were carried out to find an effective and well tolerated antibiotic regime for the treatment of *H. pylori* associated diseases. Despite the common availability of various treatment regimes for *H. pylori* infection, eradication regime of choice has not yet been accepted. Few studies suggested that combined antibiotic regime with two antibiotics and one acid suppressive agent to be effective but its efficacy was observed to vary significantly when the regime was tested in different geographical regions, suggesting a need for a universally effective antibiotic regime of choice. The most commonly used antibiotics in combination therapies worldwide are Metronidazole, Ciprofloxacin, Amoxycillin, Clarithromycin, and Tetracyclin. But the antibiotic sensitivity pattern against these antimicrobials from various geographical regions shows diversified results particularly from the developing countries. Studies ascribed several factors that could lead to the treatment failure [2]. Presence of resistant organism or acquiring resistance to the antibiotics are the most important variables for an observed diversified cure rate [3,4]. Bacterial heterogeneity [5], dietary habits could also be shown to contribute significantly in several Asian and European countries, where resistance to antibiotics particularly for the Metronidazole and Clarithromycin through out 1990s. It is reported to vary between 10% - 80% among the different geographic regions [6,7]. Mollison *et al.* 1998 [8] reported 36% and 11% resistance to Metronidazole and Clarithromycin. Alarcon *et al.* (1999) [6] determined the

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frequency of resistance to Metronidazole, tetracycline, Amoxyicilin and Clarithromycin in 282 *H. pylori* clinical isolates from spain and evaluated the evolution of resistance over five years of their study. Data infers a steady increase in the metronidazole resistance from 9% to 33%. Similarl increase of resistance to Clarithromycin and Tetracycline was observed. Mendonca *et al.* (2000) [9] reported 42% resistance to Metronidazole, 29% to Amoxycillin and 7% for Clarithromycin and tetracycline. Similarly Mijaji *et al.* 1997 [10] reported a very high acquired resistance to Metronidazole 66.7% and 70.6% for Clarithromycin after the dual and triple therapy. In India studies reported a relatively high resistance to Metronidazole and Clarithromycin *i.e*. between 90% - 100% and 30 - 50 respectively [11]. Resistance to Amoxycillin was reported to be 40%. Thus as a result of changing patterns of antibiotic sensitivity between different geographic regions and also within the same region it is suggested valuable to test the sensitivity regularly before deciding the therapeutic regime.

Additional limitations to the treatment failure could be the inaccessibility of the antibacterial agent to the organism residing deep at the inter cellular junction, or inside the gastric mucous cell hence it re-establishes the infection after the treatment, as suggested by the transmission electron microscopy and similar DNA patterns of the *H. pylori* strains before and after treatment [12,13]. The bioavailibity and stability of the drug at gastric pH is an important factor for determining the choice of eradication therapy. Metronidazole is very stable at pH ranging from 2.0 - 7.0 but increasing prevalence of pretreatment resistant strain hampered its use as a drug of choice [14]. Resistance to Amoxycillin though not reported commonly, the drug is less stable particularly at low pH. Clarithromycin is the most acid labile drug commonly used to eradicate the *H. pylori*. However significant increase in the efficacy of these drugs is observed by the co-administration of Proton Pump Inhibitors [15]. Thus *in vitro* antimicrobial susceptibility test helps in predicting the clinical response to treatment and prevalence of antibiotic resistant strains between the different geographic regions and also among the sub groups within the study population and guide the selection of antibiotics.

The Minimum Inhibitory Concentration (MIC) of an organism is the lowest concentration of an antibiotic that will inhibit its growth. Bacteria are classified as sensitive, intermediate or resistant based on the breakpoint MIC values that are arbitrarily defined and reflect the achievable levels of the antibiotic, the distribution of MIC's for the organism and their correlation with clinical outcome. Disk diffusion, agar dilution and Epsilometer test (E-Test), where the two fold serial dilution of antibiotics were in-corporated in to tubes of broth, agar plates or on a paper strip respectively, are different methods to measure the MIC of the organism.

The Epsilometer test (E-Test AB Biodisk, solna, Sweden) is a recently developed technique for the quantitative determination of susceptibility to antimicrobial agents in a variety of bacteria and fungi [16]. The test is based on the combination of concepts of both the agar dilution and disk diffusion methods but differs from the conventional disk diffusion method by the use of the predefined exponential gradient of antibiotics with the concentration minimum (least concentration) and the concentration maximum (highest concentration). The gradients cover a continuous concentration range depending on the antibiotic, which corresponds to 15 two old dilution in a conventional MIC method. The E test is also much less labor intensive and is easier to perform than agar dilution and broth dilution method, which also allows the test to be quickly and economically adapted into the laboratory work flow.

The MIC value is read from the scale in terms of μg/ml where the ellipse edge intersects the strips. Studies have assayed several antimicrobials commonly used to treat infection using E-Test and reported a good correlation with standard methods. The resistance breakpoint used for is Metronidazole > 8 μg, Clarithromycin > 1 μg, Tetracyclin > 2 μg, Amoxycillin > 8 μg, Norfloxacin > 8.0 μg and Ciprofloxacin > 1.0 μg [17].

The Present study was designed to determine the sensitivity pattern of various commonly used antimicrobial agents to treat the infection. In this study a total of six antimicrobial agents such as Ciprofloxacin, Metronidazole, Norfloxacin, Tetracyclin, Amoxycillin and Clarithromycin were tested by E-Test against 100 clinical isolates from patients with peptic ulcer, and non-ulcer dyspepsia. Having established the prevalence of *H. pylori* infection, patients are currently treated (with a view to eradication) with combination therapy (amoxicillin, ciprofloxacin and a proton pump inhibitor/bismuth). The relationship between antimicrobial resistance and the successful treatment of *H. pylori* infection has dictated that a study of the incidence of acquired antimicrobial resistance among *H. pylori* isolates obtained within our environment be determined hence the importance of this study.

2. Materials and Methods

2.1. Media Preparation

1.4 gm of Brucella Agar media was dissolved in 100 ml of distilled water and the media was autoclaved at 120 lbs for 15 minutes. The medium was supplemented with 7% sheep blood.

2.2. Procedure

In the present study a total of six antimicrobial agents

such as Ciprofloxacin, Metronidazole, Norfloxacin, Tetracyclin, Amoxycillin and Clarithromycin were used against 100 clinical isolates from patients with peptic ulcer, and non-ulcer dyspepsia. *H. pylori* was isolated from the gastric biopsies obtained from the clinical subjects. After the primary identification of the bacterium by the conventional biochemical method, bacteria were subcultured on the brucella agar plates supplemented with sheep blood. Colonies were suspended in 1 ml of Brucella broth to achieve turbidity equivalent to that of no 3 McFarland turbidity standard. 140 mm diameter agar plates were inoculated by swabbing of the surface. The plates were incubated at 37˚C under microaerophillic conditions. MICs were read after 48 hrs of incubation on the basis of the intersection of the elliptical zone of the growth inhibition with the MIC scale on the E-Test strip (**Figure 1**).

3. Results

The 100 *H. pylori* culture isolated from Peptic Ulcer and Non-Ulcer Dyspepsia patients were tested for their antimicrobial sensitivity by E-Test with six antimicrobials. Results of our study showed 100% resistance to Metronidazole (MIC > 256 μg/ml). 97% of the isolates were observed to be sensitive to Ciprofloxacin (MIC 0.38 μg), whereas Tetracycline showed 96% sensitivity (MIC 0.25 μg - 0.125 μg). Resistance to amoxycillin, Norfloxacin, and Clarithromycin were 80%, 38% and 76% respectively (MIC > 256 μg/ml) (**Table 1**).

4. Discussion

In vitro sensitivity testing of *H. pylori* is regarded as an important test as no regimen is proved to be universally successful. Sensitivity profile is recommended to be determined regionally and periodically before starting the treatment. In this study six commonly used antibiotics were tested by E-Test against 100 clinical *H. pylori* isolates obtained from different diseased group. Our data on 100 clinical isolates showed 95% - 97% sensitivity to Ciprofloxacin (MIC-0.38 μg) and Tetracycline (MIC-0.25 μg and 0.125 μg) and all the clinical isolates were found to be resistant to Metronidazole (MIC < 256 μg). Resistance to Clarithromycin, Amoxycillin, and Norfloxacin is 76%, 80% and 38% respectively (MIC < 256 μg).

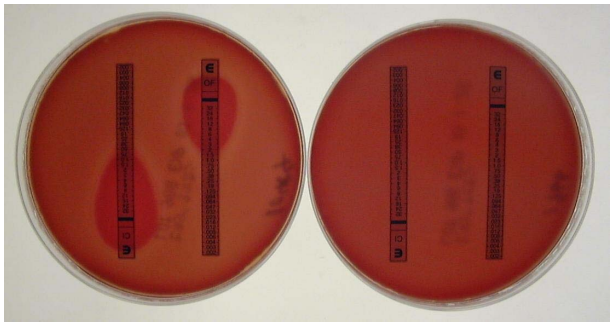

Figure 1. Photograph showing the sensitivity and resistance towards different antibiotics by *Helicobacter pylori*.

Table 1. Sensitivity of *Helicobacter pylori* isolates (n = 100) to different antimicrobial agents.

Antimicrobial Agent Tested	% of Isolates Inhibited	% of Isolates Inhibited	Resistance %
Amoxyicilin	16% = 0.25 mcg	4% = 32.0 mcg	80%
Ciprofloxacin	97% = 0.38 mcg	-	3%
Tetracycline	66% = 0.25 mcg	30% = 0.125 mcg	4%
Norfloxacin	40% = 1.5 mcg	12% = 4.0 mcg	38%
Clarithromycin	10% = 8.0mcg	16% = 48.0 mcg	76%
Metronidazole	-	-	100%

High resistance observed for Metronidazole could be due to the frequent use of the antibiotic in the empiric treatment of diarrhea [16]. The use of Metronidazole for dental infections may also add to selection pressure. In addition antibiotics self-medication is encouraged by free access and over the counter purchase and by ineffective drug control policy. This could be a contributing factor for the very high level of resistance of *H. pylori* to Amoxyicilin (80%), Clarithromycin (76%), Metronidazole (100%) [18]. Though these data sets are difficult to directly compare, it appears that Metronidazole and amoxicillin resistance have remained relatively stable while Clarithromycin resistance has increased. The increasing background rate of Clarithromycin resistance provides at least a partial explanation for the decreasing efficacy of traditional Clarithromycin-containing regimens. It is quite clear that Clarithromycin resistance, which has been attributed to several different point mutations in the peptidyltransferase region encoded in domain V of the 23S rRNA gene (142), is associated with a high rate of treatment failure when Clarithromycin containing regimens are employed (148 - 150) [19].

In one study, scientist determined the frequency of resistance to Amoxycillin, Tetracycliin, Metronidazole and Clarithromycin in 282 *H. pylori* isolates from Spain and evaluated the evolution of resistance over five years of the study. The overall percentage of resistance for Metronidazole was 19.9% and 3.5% for Clarithromycin. They observed a steady increase in resistance to metronidazole from 9% in 1991 to 21.6% in 1995. No Amoxycillin and Tetracyclin resistance was observed in the strains tested against Metronidazole, Tinidazole and Clarithromycin. Resistance to Amoxycillin is 73%. However a comparatively lower resistance was observed at lucknow, 66% for Metronidazole and 28% for Ampicillin

[11]. This difference in the sensitivity pattern observed in western as well as in Asian isolates could be due to the frequent use of the antibiotics to treat other infections. Moreover, ineffective drug control policy also has a contribution. Another Possibility could be due to infection with different strains of *H. pylori.*

Results of our study support that Tetracyclin and Ciprofloxacin could be the antibiotics of choice in the eradication of *H. pylori.* It would be necessary to investigate ciprofloxacin in any eradication treatment regime in our setting, since it appears to be the most active antibiotic in eradicating *H. pylori* in this environment.

5. Conclusion

It is observed that 96% - 97% of the clinical isolates were sensitive to Tetracycline and Ciprofloxacin, where all isolates were found to be resistant to Metronidazole. The same resistance to metronidazole has also been reported from other geographic regions. Resistance to Amoxycillin and Clarithromycin were 80% and 76% respectively. Studies attribute the high level of resistance to the frequent use of the drugs to treat various other infections, ineffective drug control policy and infection with different strains of *H. pylori.* The relationship between antimicrobial resistance and the successful treatment of *H. pylori* infection has dictated that a study of the incidence of acquired antimicrobial resistance among *H. pylori* isolates obtained within our environment be determined hence the importance of this study. The ministry of health should have an effective drug control policy. Furthermore, there is the need to continue the evaluation of new treatment agents such as NE-2001 [20], older agents such as nitrofurantoin [21] or introduction of herbal management in order to eradicate *Helicobacter pylori*. Future case-control studies employing larger sample size are needed to demonstrate the effectiveness or otherwise of therapy in the different patient groups. A long-term follow-up of our patients will also contribute to the development of guidelines on the issues of referral, diagnostic methods and treatment of *H. pylori*.

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