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In vitro Susceptibility Testing of *Helicobacter pylori* to Metronidazole, Amoxicillin, Tetracycline and Ciprofloxacin in Iran

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Abstract: A cross-sectional study was carried out at the endoscopy unit of Bagqiyatollah University of Tehran. From February 2004 to June 2006, 305 consecutive dyspeptic patients were evaluated. With regard to standard methods, biopsies were taken and cultured. For each isolates Minimum Inhibitory Concentrations (MIC) for Metronidazole (MTZ), Amoxicillin (AMX), Tetracycline (TC) and Ciprofloxacin (CIP) were determined by Epsilometer test. Considering standard resistance breakpoints, our data was analyzed and resistance rates were reported. One hundred forty nine patients, had a positive culture. The resistance rate in H.P. isolates were as follows: MTZ (60.4%), CIP (40.3%), AMX (15.4%) and TC (10.7%). 82.6% of isolates were resistance to at least one drug. In resistance group: 56, 39 and 5% of isolates displayed single, dual and triple drug resistant pattern, respectively. In the different age groups or sex groups there was no difference ($p>0.1$) in the antibiotics resistance rates except for TC ($p = 0.01$) which was more common in older groups (17.1% versus 4.1%). In present study we confronted with markedly high rate of resistance to metronidazole and ciprofloxacin, furthermore other antibiotics displayed significant resistance rate and there was some level of overlap between resistances to these drugs. It seems that in Iran HP eradication is more difficult and demand more concern.

Key words: *In vitro*, susceptibility, *Helicobacter pylori*, Iran

INTRODUCTION

Helicobacter pylori (HP) is one of the most common bacterial infections affecting nearly half of the world's population. It is very common in developing counties like Iran, in which HP infection prevalence reaches to almost 60% of general population (Amini, 2005; Go, 2002) has been implicated as a predisposing factor in chronic active gastritis, duodenal ulcer, gastric ulcer, gastric cancer and gastriclymphoma (Suerbaum and Michetti, 2002). Thus its eradication is an important issue, but the resistant strains has been emerged in most part of the world, especially in countries like Iran (Frenck and Clemens, 2003; Malekzadeh *et al*., 2004). There is a great diversity in rate and type of this resistance with regard to geographical location and time of the study (Amini, 2005). It's appearing that the optimal regimens vary for each geographic region; clarithromycin, metronidazole, amoxicillin, bismuth and omeprazole are the most effective drugs in western countries, while they are seemed less

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effective in Iran. At the present moment, there is an anarchism in introduction of practical HP eradication regimens in Iran (Amini *et al*., 2005; Malekzadeh *et al*., 2004). Therefore we must consider trial of other new drugs and revolutionize our knowledge about routine drugs in domain of HP eradication. Recent studies imply on effectiveness of fluoroqiunolons like ciprofloxacin in this domain (Amini *et al*., 2005; Turel *et al*., 1998; Boyanova *et al*., 2000; Falsafi *et al*., 2004), furthermore in two recent clinical trials in Iran, researchers found that eradication rate of ciprofloxacin is comparable to an introduced effective but not practical drug in Iran, i.e., furazolidone (high rate of side effects) (Amini *et al*., 2005; Malekzadeh *et al*., 2004). Moreover the body of our knowledge about fluoroqiunolons in Iran is still not adequate; therefore an *in vitro* study perform to evaluate the resistance pattern of HP to routine drugs such as metronidazole, amoxicillin and tetracycline and new drugs like ciprofloxacin to help to introduce new regimens in HP eradication field in countries with high metronidazole resistance rate (Fakheri *et al*., 2001).

MATERIALS AND METHODS

Study Design

We performed a cross sectional study, from February 2004 to June 2006, in which 305 consecutive dyspeptic patients (with regard to definition of dyspepsia in Rome II criteria) were evaluated at the endoscopy unit of Bagqiyatollah University of Tehran, one of the major referral centers in Iran.

Endoscopy

During each endoscopy, two biopsy specimens were obtained, the first from the antral mucosa of the gastric greater curvature within approximately 5 cm from the pylorus and the second from corporal mucosa near gastric angula. Then the specimens were placed into Cystein albimi broth with 20% glycerol as a transport media, they were rapidly labeled, then frozen at 70 centigrade and transported to the laboratory in dry ice. All specimens were processed within 2 h in the laboratory we let all the specimens melt in room temperature.

Culture

The biopsy specimens were minced and homogenized in physiological sterile saline (0.5 mL) and plated immediately on chocolate agar media (Columbia agar + 5% Sheep blood). The plates were then incubated for three days at 37°C in a microaerophilic atmosphere (9% CO_2, 11% O_2, 80% N_2 and 98% humidity). All the negative plates were kept for 14 days at the previous mentioned condition. All the transparent colonies with 1-2 mm diameters were identified by gram staining, catalase and urease tests. After identification by these conventional methods, all the positive colonies of one plate were touched by a cotton swab and were transported into 1 mL Brucella Broth media. The density of all suspensions were reached to Mc Farland Standard No. 4 Opacity (10^8 cfu mL^{-1}). A specimen was caught from each suspension to evaluate motility and morphology of bacteria by phase contrast microscopy. In the case of observing more than 25% immotile sticky bacteria or spherical forms we discarded the suspension and repeated the process of making the suspension. All the suspensions satisfied our previous mentioned criteria were inoculated by flooding and aspiration on to a chocolate agar media (Columbia agar + 5% Sheep blood). All the plates were let to dry at room temperature in 5-10 min.

MIC

For each isolates MICS for MET, AMX, TET and CIP were determined by a gradient diffusion method (Epsilometer test, E-test, AB Biodisk, Solna, Sweden). We put E-test strips on the surface of plates in sterile condition. Quality control was ensured by using organisms from the American Type

Table 1: MIC* values for definition of susceptibly status to antibiotics for Helicobacter pylori eradication

	Susceptibility status (mg L^{-1})		
Antibiotics	Susceptible	Intermediate	Resistant
Metronidazole	<4	4≤and<8	≥8
Amoxicillin	<8	-	≥8
Tetracycline	<4	-	≥4
Ciprofloxacin	<1	-	≥1

*: MIC = Minimal Inhibitory Concentration

Culture Collection, including *Pseudomonas aeruginosa* ATCC 85327, *Staphylococcus aurous* ATCC 29213, *Escherichia coli* ATCC 25922 and HP ATCC 26695, which were cultured under same pervious mentioned condition. MICs were read as the nearest concentration to intersect point of bacteria growth. Susceptibilities and resistances were reported with regard to reference values. (Boyanova *et al*., 2000; King *et al*., 2000; Ge *et al*., 2002; Shara *et al*., 2002) (Table 1).

Statistical Analysis

We used SPSS for Windows 11.5 (SPSS, Inc., Chicago, Illios) for reporting frequencies and means, Standard Error (SE) and analyzing our data by chi square and independent student t-test. Confidence Interval (CI) for every resistance frequency was calculated as: First, calculate the three quantities $A = 2r + z^2$; $B = z \sqrt{[z^2 + 4r (1 - r/n)]}$ and $C = 2 (n + z^2)$. Then, the confidence interval for the proportion is given by (A - B)/C to (A + B)/C and z is 196 for a 95% CI (Newcombe *et al*., 2002). A p-value <0.05 was considered statistically significant and all p-values were calculated two-sided.

We must mention that for preparing the suspension solution we used colonies which were 4 days old or younger and all the colonies which were harvested from the primary plate beyond 4 days, were re-cultured to produce younger isolates for susceptibility testing. The Institutional Review Board of Bagqiyatollah University of Tehran approved and financed this study.

RESULTS AND DISCUSSION

From three hundred and five consecutive dyspeptic patients, 149 patients who didn't mention any history of H.P eradication therapy, (48.8%) had a positive culture (61.1% male and 38.9% female, mean age (SE): 38.17 (1.07) years). The study population comprised all the isolates harvested from these 149 patients. These isolates evaluated under antibiogram test; overall results of antibiogram test are shown in Table 2.

By considering the intermediate group as susceptible group in the case of MET, we can say that 123 (82.6%, 95% CI 75.66-87.80%) strains from our study population were drug resistant. Of resistance group, 69 isolates (56%, 95% CI 47.27-64.55%) displayed single-drug resistant pattern from them, 40 isolates (58%, 95% CI 46.21-68.89%) were resistant to MET, 9 isolates (13%, 95% CI 7.02-22.97%) to AMOX, 18 isolates (26.1%, 95% CI 17.19-37.51%) to CIP and 2 isolates (2.9%, 95% CI 0.79-9.97%) to TET. Fifty four isolates (44%, 95% CI 35.45-52.73%) displayed more than one drug resistant pattern; In this group 48 isolates (88.9%, 95% CI 77.86-94.81%) displayed dual-drug resistant pattern, by considering this group we can say that, 29 isolates (60.4%, 95% CI 46.31-72.98%) were resistant to CIP-MET, 7 isolates (14.6%, 95% CI 7.25-27.17%) to CIP-TET, 4 isolates (8.3%, 95% CI 3.29-19.55%) to MET-AMOX, 4 isolates (8.3%, 95% CI 3.29-19.55%) to CIP-AMOX, 3 isolates (6.3%4, 95% CI 2.15-16.84%) to MET-TET and 1 isolates (2.1%, 95% CI 0.37-10.90%)to TET-AMOX).finally 6 isolates (11.1%, 95% CI 5.19-22.19%) displayed triple-drug resistant pattern, from this group, three isolates were resistant to MET-CIP-AMOX, two isolates to TET-CIP-AMOX, one isolates to MET-CIP-TET) and no isolates displayed quadruple-drug resistant pattern. We found out there was no statistically meaningful relationship between resistance to four studied antibiotics and patient's age and sex (Table 3).

Table 2: Percentage frequency of *Helicobacter pylori* susceptibly to the studied antibiotics

	Susceptibly status: No. (%)			
Antibiotics	Resistance	Intermediate	Susceptible	95% CI*
Metronidazole	80 (53.7)	10 (6.7)	59 (39.6)	45.69-61.50%
Amoxicillin	23 (15.4)	-	126 (84.6)	10.51-22.09%
Tetracycline	16 (10.7)	-	133 (89.3)	6.72-16.73%
Ciprofloxacin	64 (43)	-	85 (57)	35.28-50.98%

*: CI = Confidence Interval

Table 3: Background variables in a hospital-based cross-sectional *in vitro* study of Helicobacter pylori resistance

Antibiotics	Men in resistant group (%)	Men in susceptible group (%)	p-value*
Metronidazole	54.9	45.1	0.701(NS†)
Amoxicillin	56.5	61.9	0.626(NS)
Tetracycline	62.5	60.9	0.901(NS)
Ciprofloxacin	64.1	58.8	0.516(NS)
Antibiotic	Mean (SD†) age in resistant group	Mean (SD) age in resistant group	p-value
Metronidazole	37.35 (13.5)	37.13 (12.5)	0.41 (NS)
Amoxicillin	41.35 (15.65)	37.60 (12.55)	0.207 (NS
Tetracycline	44.06 (13.3)	37.47 (12.93)	0.057 (NS)
Ciprofloxacin	39.44 (12.7)	37.22 (13.3)	0.303 (NS)

*: p-values were calculated by unadjusted analysis, †: NS = not significant; SD = standard deviation

Metronidazole resistance have been emerging world wide and susceptibility to this agent is a major corner stone in therapeutic regimens efficacy and therefore in HP eradication failures around the world, the aim for initial eradication rates should be 85 to 95% in first line HP eradication therapy, with respect to this, Megraud has emphasis that in the case of resistance rate <30% to metronidazole, these agent could be used in therapeutic regimens with out *in vitro* susceptibly testing of HP (Megraud *et al*., 1998).

The present results and other similar studies in Iran demonstrate primary H.P. resistance to metronidazole is markedly upper than this limit and exceed 50% (Malekzadeh *et al*., 2004; Siavoshi *et al*., 2002) these evidence implies that without usage *in vitro* susceptibly testing of HP there is a necessity of moving this drug from first line to further lines of therapy.

Present study and similar studies display low number of resistant isolates to amoxicillin and tetracycline, although the rate of resistance to amoxicillin and tetracycline is higher than other developed countries but this deference is not enough to pose any changes on their efficacy in a country with a high Metronidazole resistance rate like Iran and this fact signifying the importance of consideration of them in H.P. eradication regimens (Siavoshi *et al*., 2000; Malekzadeh *et al*., 2004; Shara *et al*., 2002; King *et al*., 2000).

Furthermore we found zero resistance rates with quadruple pattern and this fact is parallel with studies which insist on usage of quadruple regimens in Iran (Malekzadeh *et al*., 2004). In present *in vitro* study we confront with a same significant level of resistance to ciprofloxacin as metronidazole but interestingly in our earlier clinical trial study we found that, a composition of ciprofloxacin with a bismuth salt and proton pump inhibitor has an efficacy like furazolidone, which in other recent studies presented as effective as claritromycin which is still effective and powerful drug in HP eradication in countries like Iran (Amini *et al*., 2005; Malekzadeh *et al*., 2004; Fakher *et al*., 2001; Falsafi *et al*., 2004; Asaka *et al*., 2001) by considering this obvious discrepancy with regard to recent evidences that support usage of fluoroqinulons in HP eradication (Amini *et al*., 2005; Boyanova *et al*., 2000; Fujimura *et al*., 2004; Di Caro *et al*., 2002) and knowledge that, there is a poor correlation between clinical outcome and *in vitro* susceptibility results in HP eradication domain, (Gisbert *et al*., 2002) we can postulated that ciprofloxacin (and may be other fluoroqiunolons) susceptibility judged by the E-test may not always predict the treatment outcome.

As for metronidazole, there may be a discrepancy between the E-test and the agar dilution method (Hachem *et al*., 1996; Osato *et al*., 2001). As for clarithromycin, the results of susceptibility obtained by the culture method may be different from those obtained by the Polymerase Chain Reaction method (Masaoka *et al*., 2004).

Furthermore we know that addition of Bismuth salt and omeprazole to antibiotics may be lead to achievement of higher eradication rate (Masuda *et al*., 2003; Saberi *et al*., 1995), may be this mechanism lead to display of higher eradication rate in our clinical trial than present study, Further more existence of discrepancy between *in vivo* and *in vitro* studies is in concordance with other evidences that questioned the true role of culture for HP management in clinical practice (Lee *et al*., 1999; Gisbert *et al*., 1999; Zullo *et al*., 2001) Indeed, *in vitro* antimicrobial sensitivity does not lead necessarily to eradication *in vivo* and vice versa (Zullo *et al*., 2003), with regard to these facts we suggest basing HP eradication regimens on *in vivo* clinical trials which were coupled with *in vitro* studies.

In conclusion we can say that in this study we confronted with markedly high rate of resistance to Metronidazole and Ciprofloxacin, furthermore other antibiotics displayed significant resistance rate and there was a some level of overlap between resistance to these drugs; these findings were in concordance with theory that HP in countries like Iran copying the same behavior of tuberculosis to eradication regimens, in addition to this we found that, like tuberculosis, for evaluation of HP resistance to at least fluoroqinulons like Ciprofloxacin we must rely on *in vivo* than *in vitro* studies, thus authors of this article suggest that maybe like tuberculosis in countries with high resistance rate to metronidazole such as Iran, we must more than other developed countries persist on strict usage of HP eradication drugs by considering the recommended regimens and stratification of our drugs to first, second and more lines of treatment and this must be presented according to parallel *in vivo* and *in vitro* studies.

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