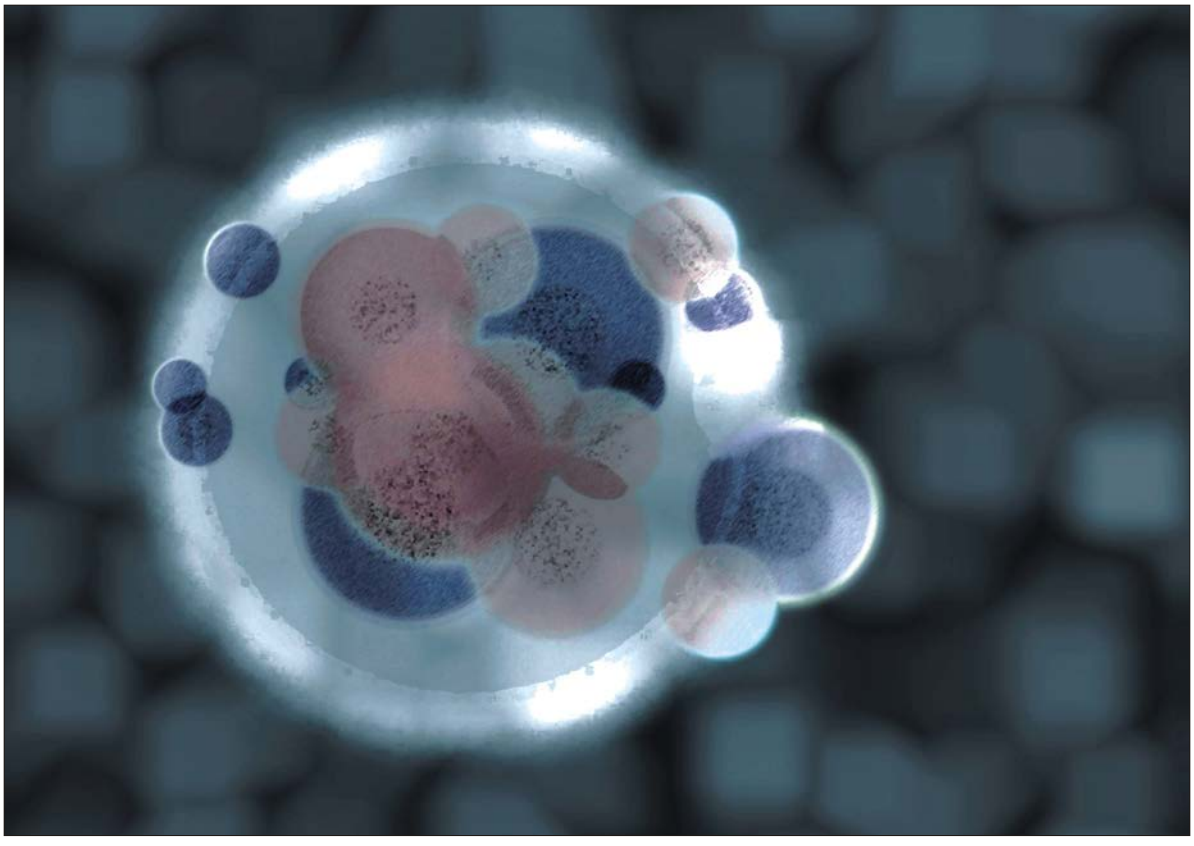

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Metronidazole Resistance of *Helicobacter pylori* Clinical Isolates in a Hospital in Iran and Protein Pattern of Two Strains Showing Differences in Susceptibility

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Abstract: Metronidazole resistant *H. pylori*'s clinical isolates from biopsy specimens at a university hospital in Northeast Iran were examined. Anti-*Helicobacter pylori* assay was performed by the filter paper Disc Diffusion Method (DDM) on modified egg yolk emulsion agar. Twenty two (56.41%) of 39 pure isolates of *H. pylori* were resistant to metronidazole. Metronidazole sensitivity determination of *H. pylori* isolates before treating patients is essential. Protein patterns of two strains showing differences in susceptibility toward metronidazole were different which indicates variation between two strains of *H. pylori*.

Key words: *Helicobacter pylori*, metronidazole, resistance, disk diffusion, protein pattern

INTRODUCTION

Helicobacter pylori is a spiral Gram negative, microaerophilic bacterium that is able to colonize the acidic environment of stomach (Murray *et al*., 2005; NachamKin and Skirrow, 1998). It infects more than half of the world's population (Dunn *et al*., 1997) and causes upper gastrointestinal tract disorders such as chronic gastritis, peptic ulcer disease and gastric carcinoma (Tytgat and Rauws, 1989). There is some genetic/phenotypic diversity between *H. pylori* populations (Ahmed and Sechi, 2005; Blaser and Berg, 2001) due to point mutations and larger substitutions, insertions, or deletions that may involve one or more genes or multigene segments (Blaser and Berg, 2001). Although there are several drug treatment regimens for these significant infections, including Colloidal Bismuth Subcitrate together with antibiotics such as amoxicillin and metronidazole (Edwards, 1993), sometimes eradication failure is seen. Increased antibiotic resistance of *H. pylori* strains may develop, leading to relapse (Goddard and Logan, 1996). The prevalence of antimicrobial resistance varies with geographical regions (Nahar *et al*., 2004). Metronidazole is one of the most common antibiotics that are used for treatment of *H. pylori* infection and bacterial resistance to this antibiotic is a serious and growing problem (Jenks and Edwards, 2002; Valdez *et al*., 1998). In the present study, after isolation and identification of clinical isolates of *H. pylori*, their sensitivity toward metronidazole were assessed and the protein pattern of two isolates that were different in sensitivity were compared.

MATERIALS AND METHODS

Isolation of bacteria: Between September 2004 and November 2005, 392 biopsy specimens were examined from 17 Shahrivar Hospital of Mashhad (Khorasan province, Iran). Biopsy specimens were transported in Stuart transport medium (Merck, Germany) less than 4 h to laboratory. To diagnose *H. pylori* infection, each biopsy specimen was examined by direct Gram staining, rapid urease test (Chem. Enzyme) and culture after homogenization. Primary isolation was performed on selective Brucella agar (Merck, Germany) supplemented with horse blood 5-7% (v/v) and starch (0.1% w/v, Merck, Germany), vancomycin (Lilly, USA) 5 mg L^{-1}, trimethoprim (Roche, Swiss) 5 mg L^{-1}, polymyxin B (Merck, Germany) 2500 μL^{-1}, amphotricine B (Bristol, England) 10 mg L^{-1}. Bacteria were incubated for 5-7 days under microaerophilic condition (10% CO_2 and 90-100% humidity) at 37°C.

Following primary isolation, *H. pylori* bacterial cells were identified according to colony morphology, Gram staining, rapid urease$^+$, catalase$^+$, oxidase$^+$, H_2S^- and nalidixic acid resistance (Boyanova *et al*., 2003; Goodwin and Armstrong, 1990).

Anti-*Helicobacter pylori* assay: Thirty nine clinical isolates from biopsy specimens were used. Growth inhibition was performed by the filter paper Disc Diffusion Method (DDM) on modified Egg Yolk Emulsion agar (EYE agar) at 37°C under microaerophilic conditions. Briefly each 100 mL of EYE agar contained Mueller-Hinton

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agar (Pronadisa-Madrid) 3.8 g, egg yolk emulsion 7-10%, triphenyl tetrazolium chloride (Merck, Germany) 4 mg (Tabak *et al*., 1999; Valdez *et al*., 1998).

The turbidity of bacterial suspension was equivalent to McFarland tube No. 4 (10^8 cfu mL^{-1}). Standard commercial discs of metronidazole (5 μg $disc^{-1}$) were used. The plates were incubated for 3-4 days under microaerophilic conditions at 37°C (McNulty *et al*., 2002). The zones of inhibition were measured and reported in millimeters (average diameter by two repetitions). Strains were considered resistant to metronidazole if the inhibitory zone was <16 mm (McNulty *et al*., 2002).

SDS-PAGE: Protein patterns of two strains showing differences in susceptibility toward metronidazole were determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by Laemmli method. Pure colonies of bacteria were collected from surface of Brucella agar and washed three times with PBS and then lysed in 100-200 μL of Laemmli sample buffer containing 5% (v/v) β-mercaptoethanol, 2% (w/v) SDS, 10% (v/v) glycerol and 125 mM Tris-HCl pH 6.8 (Mizoguchi *et al*., 1998). Samples were placed on shaker for 15 min and incubated at 37°C for 20 min. Later protein assay of two bacterial suspensions by Bradford method (Bradford, 1976), 50 μL of each sample was subjected to SDS-PAGE on a 12% acrylamide slab gel (Laemmli method) with a ladder standard (Sigma marker). Following electrophoresis, the gel was silver stained (Schevchenko *et al*., 1996).

RESULTS

H. pylori infection was detected in 231 (62.56%) of the total 392 patients. Samples were considered positive only if their culture or 2 out of 3 diagnostic methods were positive (Boyanova *et al*., 2003).

As shown in Table 1, 22 (56.41%) of 39 pure isolates of *H. pylori* were resistant, two (5.13%) of them were intermediate and 15 (38.46%) were sensitive to metronidazole.

As shown in Fig. 1, protein patterns of two strains showing differences in susceptibility toward metronidazole were different. This indicates variation among two strains of *H. pylori*. Many of bands are the same for two strains, but there is one band in strain 24 (resistant to metronidazole) in region of 55-66 kDa which is different from strain 36. In other words one band was different in protein pattern of two strains of *H. pylori*. In addition, the band with molecular weight less than 24 kDa in strain 36 is thicker than the same band in strain 24 which probably indicates its higher expression.

Table 1: Inhibitory effects of metronidazole (5 μg $disc^{-1}$) on 39 clinical isolates of *H. pylori*

Strain	Inhibition zone (mm)	Strain	Inhibition zone (mm)
1	12.0	21	46.0
2	9.0	22	52.0
3	9.0	23	10.5
4	28.0	24	6.0
5	10.0	25	28.0
6	6.0	26	19.0
7	41.5	27	47.5
8	11.0	28	10.0
9	50.0	29	9.5
10	51.0	30	9.0
11	6.0	31	9.0
12	16.5	32	50.5
13	6.0	33	13.0
14	6.0	34	11.0
15	9.5	35	51.0
16	55.0	36	48.0
17	9.5	37	10.5
18	10.0	38	9.0
19	29.5	39	46.0
20	50.5	-	-

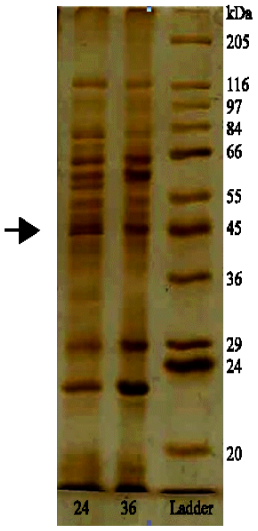

Fig. 1: Protein pattern of two strains (24 and 36) showing differences in susceptibility toward metronidazole by SDS-PAGE. Arrowhead indicates the additional band in strain 24

DISCUSSION

Prevalence of *H. pylori* is different among populations and it depends on used methods, social-economical conditions in childhood and geographical situation. In this study, its prevalence was 62.56%.

We also report the susceptibility to metronidazole of *H. pylori* clinical isolates from a university hospital in Iran. A high rate of resistance to metronidazole was found throughout the study; these results are similar to frequencies reported for other developing countries.

Resistance of isolated *H. pylori* strains toward metronidazole were reported 20% in a hospital in New Zealand (Ahmed *et al*., 2004). Resistance to metronidazole and tinidazolewere 72 to 79% and 71 to 81%, respectively by modified disc diffusion for *H. pylori* strains isolated from patients at the Pediatric Medical Center of Tehran, Iran (Falsafi *et al*., 2004). 77.8% of *H. pylori* strains were resistant to metronidazole in the Ninth People's Hospital of Shanghai (Wu *et al*., 2000).

Metranidazole resistance is a serious and increasing problem. Metronidazole resistance in Iran might be due to frequent use of metronidazole for parasite infections and gynecological problems. So, antibiotic sensitivity determination of bacteria before treating patients is essential, rather than giving empirical treatment.

In earlier research, strain variations of *H. pylori* have been studied not only from the viewpoint of genome (De Vries *et al*., 2003; Wang *et al*., 2003) but also at the protein level (Enroth *et al*., 2000; Govorun *et al*., 2003) and all of them showed diversity among clinical strains of *H. pylori*. In present study, two clinical isolates of *H. pylori* having different susceptibility toward metronidazole showed different protein patterns by SDS-PAGE confirming diversity among strains.

In this study, we found high rate of metronidazole resistance of *H. pylori* isolates in a hospital in Iran that may develop leading to relapse. Also, we showed protein variation among two *H. pylori* isolates from the viewpoint of whole protein pattern that it might be due to changes among strains. It is necessary more research to identify these different proteins and their coding genes.

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