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ORIGINAL ARTICLE

Mandana Rafeey · Reza Ghotaslou · Solmaz Nikvash
Asghar Ashrafy Hafez

Primary resistance in *Helicobacter pylori* isolated in children from Iran

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Abstract *Helicobacter pylori*-associated infection is extremely common in Iran, as in other developing countries, but few data exist on the susceptibility of *H. pylori* to antimicrobials commonly used in the eradication schedules in this country. This study was performed to determine the resistance rate to six antimicrobial agents used in the treatment of *H. pylori* infection in dyspeptic Iranian children and to recommend an updated anti-*H. pylori* treatment regimen to use in children. All *H. pylori* isolated from children who were undergoing gastroscopy were prospectively collected and subcultured to yield their susceptibility to six antimicrobial agents, by E test and disk diffusion methods. Demographic data and presenting symptoms were also collected. A prospective study was carried out from January 2003 to January 2005 with 100 strains of *H. pylori* isolated from children (40 girls and 60 boys; age range, 1.5 to 16 years [mean, 9.22 ± 3.25 years]); the strains had been successfully subcultured to yield antimicrobial sensitivity. Overall the *H. pylori* resistance rate was 95% to metronidazole, 59% to amoxicillin, 16% to clarithromycin, 9% to furazolidone, 7% to ciprofloxacin, and 5% to tetracycline. The most common presenting symptom was abdominal pain. There were no statistically significant differences in antimicrobial resistance rates related to age, sex, or clinical presentation. In the Iranian children, the prevalence of *H. pylori* resistance was very high to metronidazole and amoxicillin, moderate to clarithromycin, and low to ciprofloxacin and tetracycline.

Key words Susceptibility · *Helicobacter pylori* · Pediatric population · Child

Introduction

The prevalence of *Helicobacter pylori* infection worldwide is approximately 50%, and it is as high as 80%–90% in developing countries.[1] *H. pylori* is an etiologic agent of peptic ulcer disease, primary gastritis, gastric mucosa-associated lymphoid-tissue lymphoma, and gastric adenocarcinoma.[2] The annual incidence of *H. pylori* infection is almost 4%–15% in developing countries, compared with approximately 0.5% in industrialized countries.[3] *H. pylori* infection is curable with multiple antimicrobial agents. Eradication therapy of symptomatic *H. pylori* infection substantially reduces the recurrence of associated gastroduodenal diseases. The most common causes of treatment failure are patient noncompliance, drug side effects, and antimicrobial resistance of the infecting *H. pylori* strain, with antimicrobial resistance being a leading cause of treatment failure.[4,5] Current practice dictates treatment of symptomatic individuals with a regimen containing two antimicrobial agents along with a proton pump inhibitor.[6] For successful eradication of bacteria, it is imperative that the clinician be aware of the current antimicrobial susceptibility profiles of isolates within the region. Therefore, this study was initiated to determine the antimicrobial susceptibility pattern among *H. pylori* isolates recovered from children in Northwest Iran.

The purpose of our study was to evaluate the prevalence of in vitro resistance to amoxicillin, metronidazole, clarithromycin, ciprofloxacin, furazolidone, and tetracycline before treatment in children and to recommend suitable modifications to the existing treatment regimen for *H. pylori* infection in Iran.

M. Rafeey[1] (✉)
Liver and Gastrointestinal Diseases Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

R. Ghotaslou · S. Nikvash
Department of Microbiology, School of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran

A. Ashrafy Hafez
Department of Family Medicine, School of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran

[1]*Present address:*
Childrens Hospital, Sheshglan Street, Tabriz, Iran, PO Box: 57367
Tel. +98-914-114-6982; Fax +98-411-526-2280
e-mail: mrafeey@yahoo.com

Subjects, materials, and methods

Subjects

Children referred for endoscopy for severe and/or recurrent upper gastrointestinal symptoms suggestive of organic disease (mostly recurrent abdominal pain or dyspepsia, nausea, vomiting for at least 3 months) were included. Informed consent from the parents was obtained, and demographic and clinical data were registered. All children had been living in Northwest Iran for at least 24 months and originated from Iran. The study protocol was approved by the ethics and research review committees of Tabriz Medical University.

Exclusion criteria

Treatment with proton pump inhibitor, antacid, antimicrobial, or nonsteroidal anti-inflammatory medication within the 3 months before endoscopy constituted exclusion criteria.

Inclusion criteria

Culture and histological examination of biopsy samples were carried out in a blinded manner. The presence of both a positive biopsy specimen culture and a positive histological examination was required for inclusion in the study.

Endoscopy

Upper endoscopy (XP20; Olympus, Tokyo, Japan) was performed by the same observer and under sedation in all of the patients. Biopsies were systematically taken from the duodenum (one or two pieces), gastric antrum (four pieces), and gastric body (one piece) and from the esophagus, only if endoscopic abnormalities were found. Two antral biopsies were used for culture and for urease test and the other biopsies were used for histopathology. Three antral biopsy specimens were taken and analyzed for histology and culture.

Histology

Biopsy specimens were processed in 4.5% buffered formalin and paraffin-embedded; sections were stained with hematoxylin and eosin. A modified Giemsa staining was used for *H. pylori* identification, and gastritis was evaluated according to the updated Sydney system.[7] The *H. pylori* density score, the chronic inflammation score (i.e., the mononuclear cell infiltration), and the active inflammation score (i.e., polymorphonuclear neutrophil infiltration) were determined separately and graded from 0 to 3 (for none, mild, moderate, and severe, respectively).

Culture

Antral biopsy specimens for culture were placed in a transport medium (Portagerm pylori; BioMerieux, Marcy L'Etoile, France) or sterile saline solution and processed within 3 h. *H. pylori* were isolated from gastric mucosal biopsies; briefly, biopsy specimens were ground between the frosted ends of two sterile microscope slides and plated on selective agar plates. The identification of the colonies was confirmed by morphology; Gram's staining; and oxidase, catalase, and urease production. Colonies were suspended in sterile saline and adjusted to a density equal to McFarland turbidity standard 3. The suspensions were spread onto the plates with sterile cotton swabs. The organisms were tested for their antimicrobial susceptibilities by growth under microaerophilic conditions for 3 days. The minimal inhibitory concentrations (MICs) of isolates were assessed by agar diffusion gradient test (E test; AB Biodisk, Solana, Sweden) on Mueller-Hinton agar supplemented with 10% horse blood for metronidazole, clarithromycin, and amoxicillin. After incubation, the concentration shown in the E test strip that was closest to the intersection point with growth on the plate (i.e., the lowest concentration of drug inhibiting visible bacterial growth) was defined as the MIC. The breakpoints used to define resistance were: metronidazole (MIC > 8 μg/ml), clarithromycin (MIC > 2 μg/ml), and amoxicillin (MIC > 0.5 μg/ml), according to the system of Glupczynski et al.[8] The strains were considered to be amoxicillin-, metronidazole-, and clarithromycin-resistant when the MICs were greater than 0.5, 8, and 2 μg/ml, respectively.[9] For tetracycline, furazolidone, and ciprofloxacin, a modified Kirby-Bauer disk diffusion method on the same solid medium was performed, with the use of an antibiotic disk (Oxoid, Basingstoke, UK), according to the Clinical Laboratory Standards Institute (CLSI).[10] After 2–3 days of microaerobic incubation, the growth inhibition zone diameters were recorded. *H. pylori* strains were considered as resistant when they exhibited growth inhibition zones of <20 mm for tetracycline (MIC 2 μg/ml), <20 mm for ciprofloxacin (MIC 0.5 μg/ml), and <13 mm for furazolidone (MIC 2 μg/ml).

Statistical analysis

Calculation of the mean, SD, and 95% confidence interval (95% CI) for all quantitative parameters was done with the SPSS system (SPSS, Chicago, IL, USA). For differences between groups and the prevalence of resistance, the χ^2 test and independent *t*-test were used. All tests performed were two-tailed, with *P* values of 0.05 or less considered significant.

Results

A prospective study was carried out from January 2003 to January 2005 with 100 *H. pylori*-positive children (40 girls and 60 boys; age range, 1.5 to 16 years [mean 9.22 ± 3.25

years]); 49.5% of the patients were less than 10 years old. The prevalence of *H. pylori* infection was 62%. Abdominal pain was the most frequent presenting symptom recorded in the patients in this study (31%); other frequent clinical symptoms were gastrointestinal bleeding (20%) and chronic vomiting (12%). Eighty-four percent of the patients were living in a city, but the rate of resistance was not significantly different between rural and urban areas. By endoscopy, gastric ulcer was found in 5 and duodenal ulcer in 1 of the 100 patients; erosive gastritis was present in 10 patients and 37% had antral nodularity. By histology, most patients showed moderate chronic gastritis (mean antral inflammation score, 1.7; range, 1–3) with a minimal degree of activity (mean antral activity score, 1.4; range, 0–2), and the mean *H. pylori* density score was 1.4 (range, 0–3). No relation was found between antibiotic susceptibility and peptic ulcer disease. The only significant risk factor linked to peptic ulcer disease was age; patients with peptic ulcer disease were significantly older than 10 years in comparison to patients with gastritis ($P < 0.001$). Among the 100 *H. pylori* isolates submitted to the survey, 95% were resistant to more than one antimicrobial agent. No significant differences were found between resistance rates in patients younger than 10 years and older children ($P > 0.05$) or between boys and girls. The primary resistance rates of the pediatric *H. pylori* isolates to the agents tested were: metronidazole 95%, amoxicillin 59%, clarithromycin 16%, furazolidone 9%, ciprofloxacin 7%, and tetracycline 5% (Table 1), while the resistance rates to combinations tested were: metronidazole +clarithromycin 42%, metronidazole+ciprofloxacin 6%, tetracycline+ciprofloxacin 3%, and clarithromycin+ciprofloxacin 4%.

Discussion

The regimens currently proposed as the first choice for *H. pylori* eradication, consist of a combination of a proton pump inhibitor and two antimicrobials, to be taken for 7–14 days. The occurrence of strains resistant to antibiotics is an emerging problem influencing eradication efficacy.[11–13] Rescue regimens must be selected on the basis of either previously administered antibiotics or the results of antimicrobial susceptibility tests, and multiple eradication failures are usually handled on a case-by-case basis by the specialist. Selection of the best first-line eradication therapy is important in avoiding primary failure and in a cost-effective approach to dyspepsia management and *H. pylori*-associated abdominal symptoms in children.

Table 1. Resistance to antimicrobial agents in 100 Iranian children

Antibiotic	Resistance	MIC 50 (μg/ml)	MIC 90 (μg/ml)	MIC range (μg/ml)
Amoxicillin	59%	0.015	0.125	0.015–1≤
Clarithromycin	16%	0.06	0.12	0.015–32≤
Metronidazole	95%	2	4	0.5–64
Tetracycline	5%	0.125	0.5	0.015–0.5
Ciprofloxacin	7%	0.06	0.25	0.03–0.25
Furazolidone	9%	0.125	0.5	0.008–4

We present the first pediatric Iranian study reporting clinical experience, *H. pylori* culture, and antibiotic resistance testing from a single center, during a 2-year period in Northwest Iran. Because the patients were enrolled from a population evaluated at a pediatric gastroenterology clinic, it is possible that the prevalence of *H. pylori* infection was higher than that in the general population in Iran. However, the prevalence was within the reported range from much of the developing world. Additionally, we have demonstrated that the antimicrobial resistance pattern of *H. pylori* in the present study is consistent with that in other studies from the region and studies from developing countries in general, compared with the results in the literature.[14–17]

In our study, the rates of resistance to the all the antimicrobials tested were not significantly higher for boys than for girls, and there was no significant difference in rates of resistant *H. pylori* strains according to age. A logistic regression model, done by Koletzko[18] in Europe, revealed a 2.25 times higher risk for primary clarithromycin resistance if the child grew up in Southern Europe ($P < 0.001$), but young age and male sex remained independent significant risk factors.

Siavoshi et al.[19] reported that resistance rates of *Helicobacter pylori* isolates from adults and children to metronidazole, clarithromycin, amoxicillin, tetracycline, and furazolidone were similar and were not significantly affected by age.

Clarithromycin resistance rates of over 20% have been reported in some areas of the world.[18–20] The resistance rate to clarithromycin in Iranian children was similar to those observed in most countries; Taiwan (18%), Greece (5.5%), and Italy (15.9%).[16,21–22] The use of clarithromycin, a drug for the management of respiratory tract infections, is recent in Iran, and the availability of expensive antibiotics in this country, such as second-generation macrolides may influence the antibiotic resistance pattern. Newer macrolides have been introduced in Iran since 2000, and their consumption is still low and relatively stable; the reason for the low level of resistance is not clear. Further surveillance will be needed to determine if resistance to clarithromycin increases.

Mohammadi et al.[23] examined the frequency of antibiotic resistance in Iranian *H. pylori* strains isolated from two major hospitals in Tehran. According to a modified disk diffusion test, 1.6% of the strains were resistant to amoxicillin, 16.7% to clarithromycin and 57.5% to metronidazole; there was no resistance to tetracycline. The prevalence of primary resistance to metronidazole in Iranian children was higher than that in most other countries studied except for Egypt (100%),[24] but our findings are consistent with previous reports, including studies in the Middle East, where metronidazole resistance was between 60% and 80%.[24,25] Safaalizadeh et al.[26] compared the in vitro efficacy of furazolidone with that of metronidazole, clarithromycin, amoxicillin, and tetracycline in 70 *H. pylori* isolates from dyspeptic patients. Of the isolates, 33% were

resistant to metronidazole, but all were susceptible to furazolidone.[26]

In Europe, multilogistic regression models revealed that patients born in Asia, Africa, or the Middle East had a 2.4 times higher risk (95% CI, 1.61–3.66) of being infected with a metronidazole-resistant strain than patients of equal age and the same sex born in Western, Northern, or Southern Europe ($P < 0.001$).[18] Metronidazole resistance shows significant geographic differences, but its rate is almost identical in children and adults.[24–25] Especially in developing countries, metronidazole is a widely used drug to treat patients with parasitic and gynecological diseases.[25–28] The metronidazole-resistant pediatric isolates in the present study exhibited a high level of resistance (95%). Resistance to metronidazole is mainly due to mutations in the *rdx*A gene encoding *rdx*A, an oxygen-insensitive nitroreductase.[29]

The detected resistance rate to amoxicillin was high in our study, reflecting the importance of its use in our country, especially in children. A high level of resistance to amoxicillin was also observed in *Shigella* spp., *Salmonella* spp., and *Campylobacter* spp. isolated in Iran.[30] Amoxicillin resistance was not considered important in the United States, Canada, and Italy until it was recently identified in these countries.[31] Resistance to amoxicillin was also observed in other studies.[32] No or rare resistance to amoxicillin (median MIC, 0.016μg/ml; range, 0.016–0.06μg/ml) was detected in Europe.[18,22,33,34] These studies[18,22,33,34] also suggest the possibility of resistance to antimicrobials, such as amoxicillin or metronidazole, in geographic areas that have a high prevalence of *H. pylori* infection, but have not yet been fully evaluated for their antimicrobial susceptibility.

Primary tetracycline resistance in pediatric *H. pylori* isolates is extremely rare and rates have been reported in some countries (Poland, 0.4%; Italy, 1.6%).[22,35] The higher tetracycline resistance in Iranian children (5%) could be associated with the high consumption of tetracycline in the past decades. In another study in Iran, antibiotic susceptibility testing of 70 pediatric *H.* pylori isolates was performed by using screening agar and disk diffusion methods. Susceptibilities to amoxicillin, clarithromycin, tetracycline, and ciprofloxacin were 58%, 68%, 68%, and 65%, respectively.[36]

Primary *H. pylori* resistance to ciprofloxacin in children has been detected only in Poland (1.2%);[35] in the present study, 7% of pediatric *H. pylori* isolates were ciprofloxacin-resistant, whereas the usage of fluoroquinolones in Iranian children was relatively low. The fluoroquinolones and tetracyclines are not ordinarily used in young children, because of their side effects. Fluoroquinolones affect the development of cartilage and tetracyclines are deposited in teeth and bony tissues during the first 6 years of life. This could explain the lack of *H. pylori* resistance to both these groups of antimicrobial agents in pediatric patients younger than 8 years of age. Currently, children with *H. pylori* infection have been reported to respond to a 14-day triple therapy regimen including amoxicillin and clarithromycin or amoxicillin and metronidazole, or metronidazole and clarithromycin.[35]

In conclusion, the evaluation of antibiotic resistance profiles in pediatric patients from different geographic areas can help in optimizing therapeutic regimens to prevent treatment failures. The use of culture and antibiograms might be useful in the selection of therapy regimens. Nonetheless, in vivo results will need to be carefully monitored to ensure that the treatment is effective. Continued surveillance to allow for the early detection of antimicrobial resistance development is critical and will need to be an ongoing endeavor. Metronidazole and amoxicillin resistance is much higher in our pediatric population than reported in other countries, and could be a major contributor to the breakdown of *H. pylori* eradication.

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