ANTIMICROBIAL SUSCEPTIBILITY OF *HELICOBACTER PYLORI* ISOLATED FROM GASTRIC BIOPSIES IN DYSPEPTIC PATIENTS

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Abstract. The objective of this study was to evaluate the prevalence of antimicrobial resistance in *Helicobacter pylori* isolated from the antrum and corpus of dyspeptic patients in Khon Kaen, Thailand, and to compare the antimicrobial susceptibility patterns of *H. pylori* isolated from the antrum and corpus in individual patients. Antimicrobial susceptibility was determined by disk diffusion, studying susceptibility to metronidazole, clarithromycin, amoxicillin, erythromycin, ciprofloxacin, and tetracycline. The *H. pylori* resistant rate to at least one of the six antimicrobial agents tested was 37%. The resistance rates were 30.2% for metronidazole, 9.2% for ciprofloxacin, 5% for clarithromycin, 2.4% for amoxicillin, and 1.7% for erythromycin and tetracycline. Single, double, and more than double antimicrobial resistances were found in 27.7, 6.7 and 2.5%, respectively. Antimicrobial susceptibility testing revealed 11 antibiotypes. The most common antimicrobial susceptibility pattern found was sensitivity to 6 antimicrobial agents (63%). *H. pylori* antimicrobial resistance in specimens isolated from the antrum and corpus were nearly equivalent, 37.3% (22/59) and 36.7% (22/60), respectively. Most of the *H. pylori* specimens isolated from the antrum and corpus in individual patients were identical (87.7%).

INTRODUCTION

The prevalence of *Helicobacter pylori* infection is approximately 50% worldwide, and is as high as 80-90% in developing countries (Lacy and Rosemore, 2001). *H. pylori* is strongly associated with some pathologies such as gastric and duodenal ulcers and related to gastric cancer (Nomura *et al*, 1991). Eradication of *H. pylori* infection is widely recommended as the most effective treatment for peptic ulcer disease and substantially reduces the recurrence of gastroduodenal diseases (Rafeey *et al*, 2007).

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Monotherapy with antimicrobials is unsuccessful in the eradication of *H. pylori* infection. Dual, triple or even quadruple therapy is necessary (Hoffman, 1997). Although *H. pylori* infection is curable with multiple antimicrobial agents, treatment failure does occur because of the increasing high prevalence of *H. pylori*-resistant strains (Megraud, 2007). Metronidazole, clarithromycin, amoxicillin and tetracycline are commonly used for the treatment of *H. pylori* infection (Megraud, 1997; Glupczynski, 1998). The prevalence of *H. pylori* resistance varies geographically, and therefore requires continuous monitoring.

Metronidazole resistance among *H. pylori* strains has been increased worldwide and resistance rates vary according to the population studied (Alarcon *et al*, 1999). It is higher in developing than in developed countries and

has reached 90% in India (Mukhopadhyay *et al*, 2000). The prevalence of clarithromycin resistance is usually low except in some countries (Osato *et al*, 1999; Samra *et al*, 2002; Sharara *et al*, 2002), whereas *H. pylori* resistance to tetracycline and amoxicillin is rarely encountered (Al-Qurashi *et al*, 2001; Sharara *et al*, 2002). Such antimicrobial resistance has been detected in several eastern European centers (Boyanova *et al*, 2002), however, there is little information regarding antimicrobial resistance in Thailand, especially in the Northeast.

The aim of this study, was to examine the prevalence of antimicrobial resistance in *H. pylori* isolates against six antimicrobial agents: metronidazole, clarithromycin, erythromycin, ciprofloxacin, amoxicillin, and tetracycline; to determine the susceptibility patterns of *H. pylori* isolates to antimicrobial agents used for *H. pylori* treatment, and to clarify the question whether antimicrobial resistance patterns vary between the antrum and corpus.

MATERIALS AND METHODS

Patients and bacterial strains

A total of 119 *H. pylori* isolates, 59 isolates from the antrum and 60 isolates from the corpus, were collected consecutively from 63 dyspeptic patients who had *H. pylori* culture positive results among 160 dyspeptic patients. The patients underwent upper gastrointestinal endoscopy in the Endoscopy Unit of Srinagarind Hospital, Faculty of Medicine, Khon Kaen University. The subjects were diagnosed as having non-ulcer dyspepsia (NUD), peptic ulcer dyspepsia (PUD), gastric carcinoma (GCA) or other gastrointestinal diseases (*eg,* GERD, duodenitis, etc). Patients who underwent antimicrobial therapy, such as bismuth treatment, proton pump inhibitors, or H_2-blockers within the previous month were excluded. Informed consent was obtained from each patient before being included in the study.

Detection and identification of *H. pylori* from biopsy specimens

Biopsy specimens were obtained from the antrum and corpus of each patient during endoscopy. Each antrum and corpus specimen was separately homogenized in 200 μl of normal saline and cultured on 7% human blood agar (Difco, Detroit, Michigan, USA) containing a supplement of 5 mg/l trimethoprim, 10 mg/l vancomycin, 5 mg/l amphotericin B and 5 mg/l cefsulodin [(SR147), Oxoid, Unipath Ltd, Basingstroke, Hamshire, England]. The plates were incubated at 37ºC under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2) and were examined at 4 and 7 days of incubation. Characteristic colonies of *H. pylori* were confirmed by Gram staining, oxidase, catalase and urease tests (Dunn *et al*, 1997). *H. pylori* isolates were stored at -70ºC until used.

Antimicrobial susceptibility by disk diffusion method

Frozen *H. pylori* isolates were grown on Columbia blood agar (Oxiod) plates supplemented with 5% human blood for 3 days under micro-aerophilic conditions at 37ºC. Colonies were suspended in 1.0 ml sterile saline solution and adjusted to the density equal to 3.0 McFarland standard ($1x10^9$ cfu/ml) (Xia *et al*, 1994). The suspensions were spread on Mueller-Hinton blood agar plates (Oxoid) with cotton swabs and then disks containing metronidazole (5 μg), clarithromycin (15 μg), amoxicillin (10 μg), tetracycline (30 μg), erythromycin (15 μg), and ciprofloxacin (5 μg) were placed on the agar surface. The plates were incubated under micro-aerophilic conditions for 3 days at 37ºC. After 3 days, the zone diameters were measured (Boyanova *et al*, 2000). The inhibition zone diameters were determined as resistant (R) or susceptible (S). A zone size ≤16 mm was considered resistant for metronidazole (Boyanova *et al*, 2000; Mishra *et al*, 2006), ≤25 mm for amoxicillin

resistance (Lang and Garcia, 2004) and ≤30 mm for clarithromycin, erythromycin, ciprofloxacin and tetracycline resistance. An inhibition zone greater than those measurements was determined to be susceptible (Boyanova *et al*, 2000). Quality control was ensured by using *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 85327 as controls.

Statistical analysis

The chi-square test was used for statistical analysis of antimicrobial resistance in *H. pylori* isolated from the antrum and corpus. $p<0.05$ was considered statistically significant.

RESULTS

Antimicrobial susceptibility by disk diffusion method

Of 119 *H. pylori* isolates, 75 (63%) were susceptible to all six antimicrobial disk agents. *H. pylori* antimicrobial resistances to metronidazole, ciprofloxacin, and amoxicillin as single antimicrobial agents were detected in 21.8, 5.0 and 0.8%, respectively (Table 1). Resistance to 2 or more than 2 antimicrobials was seen in 6.7 (8/119) and 2.5% (3/119), respectively. The antimicrobial susceptibility patterns were differentiated into 11 antibiotypes.

Antimicrobial susceptibility pattern and *H. pylori* isolated from the antrum and corpus in the individual patients.

Antimicrobial resistance was determined by site of infection (antrum or corpus), for the 6 antimicrobial agents. No significant differences in antimicrobial resistance of *H. pylori* were seen between the antrum and corpus isolates (37.3% and 36.7%) ($p = 0.962$) (Table 1). *H. pylori* antimicrobial susceptibility patterns were determined in 56 of 160 patients. *H. pylori* isolated from the antrum and corpus in individual patients had identical and non-identical susceptibility patterns in 87.7% and 12.5%; the difference was statistically significant ($p = 0.0$) (Table 2).

DISCUSSION

Antimicrobial resistance is a growing problem in *H. pylori* treatment. The problem concerning combined and multi-drug resistance in *H. pylori* is critical, as the efficacy of the current therapeutic regimens may be compromised (Boyanova *et al*, 2000; Mishra *et al*, 2006). Metronidazole, clarithromycin, amoxicillin and tetracycline are the most frequently used antimicrobials in the treatment of *H. pylori* infection, but antimicrobial resistance rates have been reported (Boyanova *et al*, 2002; Sharara *et al*, 2002; Debets-Ossenkopp *et al*, 2003; Eun *et al*, 2003). There is little information regarding antimicrobial resistance of *H. pylori* in Northeast Thailand, therefore we evaluated antimicrobial resistance in this region.

We evaluated 119 *H. pylori* isolates from the antrum and corpus of dyspeptic patients. Antimicrobial susceptibilities revealed 11 antibiotypes for the antimicrobial agents investigated. The most common antimicrobial susceptibility pattern found was all susceptible to 6 antimicrobial agents (63%). Of the 119 *H. pylori* isolates, 44 (37%) exhibited resistance to at least one of six antimicrobial agents. Antimicrobial resistances to metronidazole, clarithromycin, erythromycin, ciprofloxacin, amoxicillin, and tetacycline were 30.2, 5.0, 9.2, 2.4, and 1.7%, respectively. The antimicrobial resistance of *H. pylori* isolates to one, two, and multiple antimicrobial agents was found in 27.7, 6.7 and 2.5%, respectively. A previous study reported antimicrobial resistance rates to metronidazole, clarithromycin, amoxicillin, tetracycline, and multiple drugs in Bangkok was 30.4, 19.0, 13.9, 5.1 and 16.5%, respectively (Tangmankongworakoon *et al*, 2003). Our study showed that the prevalence of resistance to clarithromycin, erythromycin, amoxicillin, and tetracycline was lower than that reported in Bangkok except for the resistance to metronidazole.

The metronidazole resistance rate of

Table 1
Antimicrobial susceptibility (AS) patterns of 119 *H. pylori* isolates from the antrum and corpus in 160 dyspeptic patients.

AS type	Antimicrobial susceptibility pattern						No. of isolates		Total no. of isolates (%)
	MTZ	CLR	E	CIP	AMX	TE	Antrum (*n*=59)	Corpus (*n*=60)	
I	S	S	S	S	S	S	37	38	75 (63.0)
II	R	S	S	S	S	S	12	14	26 (21.8)
III	S	S	S	R	S	S	3	3	6 (5.0)
IV	S	S	S	S	R	S	1	0	1 (0.8)
V	R	R	S	S	S	S	1	1	2 (1.7)
VI	R	S	S	R	S	S	1	1	2 (1.7)
VII	R	S	S	S	R	S	0	1	1 (0.8)
VIII	R	S	S	S	S	R	1	1	2 (1.7)
IX	S	R	S	R	S	S	1	0	1 (0.8)
X	R	R	S	S	R	S	1	0	1 (0.8)
XI	R	R	R	R	S	S	1	1	2 (1.7)
Total No. of resistant isolates (%)							22/59 (37.3)	22/60 (36.7)	44/119 (37.0)

S = sensitive, R = resistant, CLR = clarithromycin, AMX = amoxicillin, E = erythromycin, CIP = ciprofloxacin

Table 2
Comparison of antimicrobial susceptibility patterns of *H. pylori* isolates from the antrum and corpus of 56 dyspeptic patients.

Antimicrobial susceptibility patterns in the antrum and corpus	No. (%) of patients
Identical	49 (87.5)
Non-identical	7 (12.5)
Total	56 (100)

H. pylori using the Epsilometer test (E-test) in Bangkok was high: 51.9% in one study (Wongkusoltham *et al*, 2001) and 30.4% in another study (Tangmankongworakoon *et al*, 2003), and is generally high in most Asian countries, such as 49.4% in Hong Kong (Wang *et al*, 2000), 41.9% in Korea (Eun *et al*, 2003), 50% in China (Yakoob *et al*, 2001), and 90% in India (Mukhopadhyay *et al*, 2000). Metronidazole is an antimicrobial agent widely used to treat patients with parasitic and gynecological diseases, especially in developing countries (Sack and Gyr, 1994; Wang *et al*, 2000; Samra *et al*, 2002). This may explain the high resistance rates to metronidazole. Our study demonstrated the prevalence of metronidazole resistance was lower than other Asian countries but was similar to that in Bangkok (30.4%) (Tangmankongworakoon *et al*, 2003). Our results suggest metronidazole combined with other antimicrobial agents may still be used for *H. pylori* treatment because there is relatively low resistance when it was tested in combination with other antimicrobial agents.

Clarithromycin resistance rates have been reported over 20% in some areas of the world (Taneike *et al*, 2002; Koletzko *et al*, 2006; Siavoshi *et al*, 2006), 19% in Bangkok (Tangmankongworakoon *et al*, 2003), 18% in Taiwan (Yang *et al*, 2001), and 15.9% in Italy

(Mentis *et al*, 1999). The resistance rate in Northeast Thailand is much lower (5%), similar to that of Greece (5.5%) (Street *et al*, 2001). The reason for the low clarithromycin resistance in our study is not clear. Clarithromycin, a second-generation macrolide that has been used for the management of respiratory tract infections in Thailand. However, it is still an expensive antimicrobial agent and perhaps this is the reason. Further surveillance should be considered because the use of clarithromycin combined with other antimicrobial agents for *H. pylori* treatment has been increasing (Hoshiya *et al*, 2000).

Several macrolides, alone or in combination with other antimicrobial agents and a proton pump inhibitor (PPI), have been used in *H. pylori* eradication regimens. In this study, we found the resistance rate of *H. pylori* isolates to erythromycin was low (1.7 %) and was lower than other studies, such as in Sofia, Bulgaria (3.9%) (Quintana-Guzman *et al*, 1998; Boyanova *et al*, 2000). These results suggest that erythromycin eradication regimens may be used in the treatment of *H. pylori* infection in this area.

The resistance rates to amoxicillin and tetracycline were low in this study (2.4 and 1.7%, respectively), which were lower than previously reported (Tangmankongworakoon *et al*, 2003). A few cases of tetracycline resistance in *H. pylori* were reported at the end of the last century (Kwon *et al*, 2000; Al-Qurashi *et al*, 2001; Samra *et al*, 2002; Sharara *et al*, 2002). In eastern Europe, resistance to amoxicillin and tetracycline have been detected in several centers (Boyanova *et al*, 2002). In Thailand, the resistant rates to amoxicillin and tetracycline were 13.9 and 5.1%, respectively (Tangmankongworakoon *et al*, 2003). The results suggest these antimicrobial agents may be used to eradicate *H. pylori* infection in our area.

Our results also showed resistance to ciprofloxacin by *H. pylori* was 9.2%, which is similar to previous reports from German (9%) and Portugal (9.6%) (Cabrita *et al*, 2000; Heep *et al*, 2000). Many studies have shown the prevalence of ciprofloxacin resistance as low (<4%) (Megraud, 1997; Piccolomini *et al*, 1997; Boyanova *et al*, 2000). The ciprofloxacin resistance exhibited was a combined antimicrobial resistance to metronidazole and/or other antimicrobial agents (Boyanova *et al*, 2000). Although ciprofloxacin is not a drug of choice in the treatment of *H. pylori* infection, its combination with amoxicillin may be considered as an alternative in cases of resistance to first-line antimicrobial agents (Megraud, 1998).

The major triple regimen therapy, recommended by the European *H. pylori* Study Group (1997), is the PPI-clarithromycin-amoxicillin regimen. Differences were observed between susceptible strains (87.8% eradication) and resistant strains (18.3% eradication). When the treatment regimen was PPI-clarithromycin-metronidazole, the eradication rate if the *H. pylori* was metronidazole susceptible was 97% and was 50% for resistant strains (Megraud and Lehours, 2007).

Our results indicate the PPI-clarithromycin-amoxicillin regimen may be used for the treatment of *H. pylori* infection in dyspeptic patients in this area, however other combined antimicrobial agents may also serve as alternate treatments.

We used the disk diffusion method instead of the E-test because it is cheap, easy to perform, and reliable (Chaves *et al*, 1999; Fukazawa *et al*, 1999; Boyanova *et al*, 2000; Eltahawy, 2002; Mishra *et al*, 2006).

The antimicrobial susceptibility patterns of *H. pylori* isolates obtained from the antrum and corpus in individual patients were determined and compared. *H. pylori* isolates obtained from the antrum and corpus were mostly identical in their antimicrobial susceptibility patterns (87.5%); in 12.5% the patterns were non-

identical. Our results indicate a single gastric biopsy from the antrum or the corpus alone is representative for the detection of antimicrobial susceptibilities of clinical *H. pylori* isolates. A previous study reported *H. pylori* isolates from the antrum were more likely to be antimicrobial sensitive, and the strains from the body and fundus were more likely to be resistant (Yakoob *et al*, 2001). The different antimicrobial susceptibilities may involve the interaction between two different *H. pylori* strains and the resulting predominance of a single strain (Hua *et al*, 1999). A single genotypic strain may exhibit different metronidazole susceptibilities (Hua *et al*, 2000).

Susceptible and resistant *H. pylori* strains may be present simultaneously in the same stomach (Arents *et al*, 2001; Kim *et al*, 2003). Therefore, gastric biopsies of patients who failed antimicrobial therapy should be collected from both the antrum and corpus to detect *H. pylori* resistant-strains.

In conclusion, the determination of *H. pylori* antimicrobial resistance in dyspeptic patients at Srinagarind Hospital, Khon Kaen can help clinicians to select an effective empiric treatment regimens. Surveillance of antimicrobial resistance needs to be performed constantly to detect antimicrobial resistance. The same antimicrobial regimen can be used to treat *H. pylori* infection in both the antrum and corpus of the stomach.

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