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ANTIMICROBIAL SUSCEPTIBILITY

Antibiotic susceptibility of *Helicobacter pylori* in Germany: stable primary resistance from 1995 to 2000

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The issue of antibiotic resistance in *Helicobacter pylori* is of particular concern and has become an important factor leading to eradication failure. This paper reports the prevalence of primary resistance to clarithromycin, amoxicillin, metronidazole and tetracycline among *H. pylori* isolates in the north-eastern part of Germany. A total of 1644 clinical *H. pylori* isolates was investigated over a period of 6 years from 1995 to 2000. The MICs were determined by the Etest. The overall rate of primary resistance was 26.2% for metronidazole and 2.2% for clarithromycin. No significant changes in the resistance rates during the period of investigation were observed. No isolate was resistant to amoxicillin or tetracycline. PCR-RFLP analysis for the detection of point mutations associated with clarithromycin resistance was performed with 36 *H. pylori* isolates. The A → G transition mutation at position 2143 was detected in 19 *H. pylori* isolates (52.8%), whereas the mutation at position 2142 was found in 13 isolates (36.1%).

Introduction

Therapy of *Helicobacter pylori* infection has become the undisputed first option for all patients with peptic ulcers and is advised for several other associated clinical conditions [1]. Eradication treatment usually consists of a proton pump inhibitor in combination with several antimicrobial agents. Those commonly used for the treatment of *H. pylori* infections include clarithromycin, amoxicillin, metronidazole and tetracycline. An increase in resistance rates to these antimicrobial agents is to be expected because of the increasing numbers of patients treated, imperfect patient compliance and increasing consumption of antibiotics in recent years [2, 3]. The principal reason for failure to eradicate infection is believed to be the combination of resistance of *H. pylori* with poor patient compliance. Reported prevalences of resistance to clarithromycin, amoxicillin and metronidazole vary widely among geographic areas [2, 4]. Knowledge of antibiotic resistance rates is important because treatment of *H. pylori* infection is started with a standard therapeutic regimen. Primary resistance decreases the success of *H. pylori* eradication, hence the local prevalence of resistance should be monitored [5].

For this reason the aims of the present study were: (i) to evaluate the prevalence of primary resistance to clarithromycin, amoxicillin, metronidazole and tetracycline among *H. pylori* isolates in the north-eastern part of Germany and to monitor changes over a 6-year period, (ii) to determine the distribution of MICs of these antimicrobial agents against *H. pylori* strains and (iii) to analyse the point mutations associated with resistance to clarithromycin.

Materials and methods

H. pylori isolates and growth conditions

A total of 1644 clinical isolates of *H. pylori* (909 from female and 735 from male patients; average age 43.2 years, range 19–78 years) obtained from 1995 to 2000 was included in this study. *H. pylori* was cultured from gastric biopsies (either from the antrum or the corpus) of patients who underwent a diagnostic gastroduodenoscopy in the Department of Gastroenterology, Hepatology and Infectious Diseases. Isolates were from patients with chronic gastritis (1403 cases),

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peptic ulcer (221 patients) and gastric cancer (20 cases). None of the patients had been treated with metronidazole or clarithromycin for the eradication of *H. pylori* infection.

H. pylori was cultured from gastric biopsy specimens on selective medium (Pylori Agar; bioMérieux, France) under micro-aerobic conditions at 37°C. Plates were examined after 2 days and up to 10 days of incubation. *H. pylori* was identified by typical morphology on gram-stained smears and positive urease, oxidase and catalase tests. *H. pylori* ATCC 49503 was used as a positive control.

Determination of MIC

MICs were determined by the Etest (AB Biodisk, Sweden) on blood agar plates [6]. Suspensions of *H. pylori* were adjusted to a McFarland standard no. 2 as the inoculum. After an incubation period of 3 days under micro-aerobic conditions, the MICs of clarithromycin, amoxicillin, metronidazole and tetracycline were determined. Strains with an MIC of >8 mg/L were considered resistant to metronidazole. For clarithromycin, resistance was defined as an MIC of >1 mg/L and for amoxicillin and tetracycline as an MIC of >2 mg/L.

Detection of point mutations associated with clarithromycin resistance

The point mutations of *H. pylori* were detected by restriction fragment length polymorphism (RFLP) analysis in one PCR-amplified gene segment. Bacterial DNA was extracted from 36 *H. pylori* isolates with phenotypic clarithromycin resistance according to standard procedures. One fragment of the peptidyltransferase region of the 23S rRNA of *H. pylori* was amplified by PCR. A primer extending from positions 1820 to 1839 (5′-CCACAGCGATGTGGTCTCAG-3′) and a reverse primer from positions 2244 to 2225 (5′-CTCCATAAGAGCCAAAGCCC-3′) were used to amplify a 425-bp DNA fragment [7]. PCR amplification was performed in a final volume of 50 μl, with 1 μg of extracted DNA and 20 pmol of each primer. After 40 PCR cycles with denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min and a final elongation step at 72°C for 7 min, PCR products were analysed on agarose 2% gels.

The amplified DNA was precipitated with ethanol and digested with restriction endonuclease *Bsa*I or *Bbs*I (New England Biolabs, Beverly, MA, USA). The products were incubated for 24 h at 50°C for *Bsa*I and at 37°C for *Bbs*I to detect the restriction site occurring with mutations A → G at position 2143 and position 2142, respectively. The restriction fragments were electrophoresed in an agarose 3% gel.

Statistical analysis

Differences in the proportions of patients with susceptible and resistant *H. pylori* strains in each year were analysed by Fisher's exact test with a 95% confidence interval. The frequencies of resistant *H. pylori* isolates from female and male patients and from patients with different clinical manifestations were also compared.

Results

The overall rates of primary resistance to metronidazole and clarithromycin in 1644 *H. pylori* individual patient isolates were 26.2% (95% CI 24.1–28.3%) and 2.2% (95% CI 1.5–2.9%), respectively (Table 1); 21 (58.3%) of the 36 isolates resistant to clarithromycin were also resistant to metronidazole. No resistance to amoxicillin or tetracycline was observed. There were no significant changes in the prevalence of primary metronidazole and clarithromycin resistance during the period of investigation (Fig. 1). The MIC50 and MIC90 values and the MIC ranges for each antibiotic are summarised in Table 2.

Metronidazole resistance was more frequent in *H. pylori* isolates from female than male patients (32.2% *versus* 20.8%), but this did not reach statistical significance ($p > 0.05$). Primary clarithromycin resistance was similar in *H. pylori* isolates from female and male patients (2.4% *versus* 2.0%).

No significant differences were observed in the prevalence of metronidazole and clarithromycin resistance between *H. pylori* isolates from patients with chronic gastritis and peptic ulcer.

PCR-RFLP analysis for the study of point mutations associated with clarithromycin resistance was performed for 36 *H. pylori* isolates. In 19 *H. pylori* isolates (52.8%) the A → G transition mutation at position 2143 was detected, whereas the mutation at position 2142 was found in 13 *H. pylori* isolates (36.1%). In four cases it was not possible to detect the mutations by restriction with *Bsa*I and *Bbs*I. These strains possibly carry a transversion mutation of A → C, which the restriction analysis would not detect.

Table 1. Overall antibiotic resistance of *H. pylori* isolates from 1644 patients

Agents	Number (%) of resistant isolates	95% CI
Amoxicillin	0 (0)	0.0–2.0
Clarithromycin	36 (2.2)	1.5–2.9
Metronidazole	431 (26.2)	24.1–28.3
Tetracycline	0 (0)	0.0–2.0

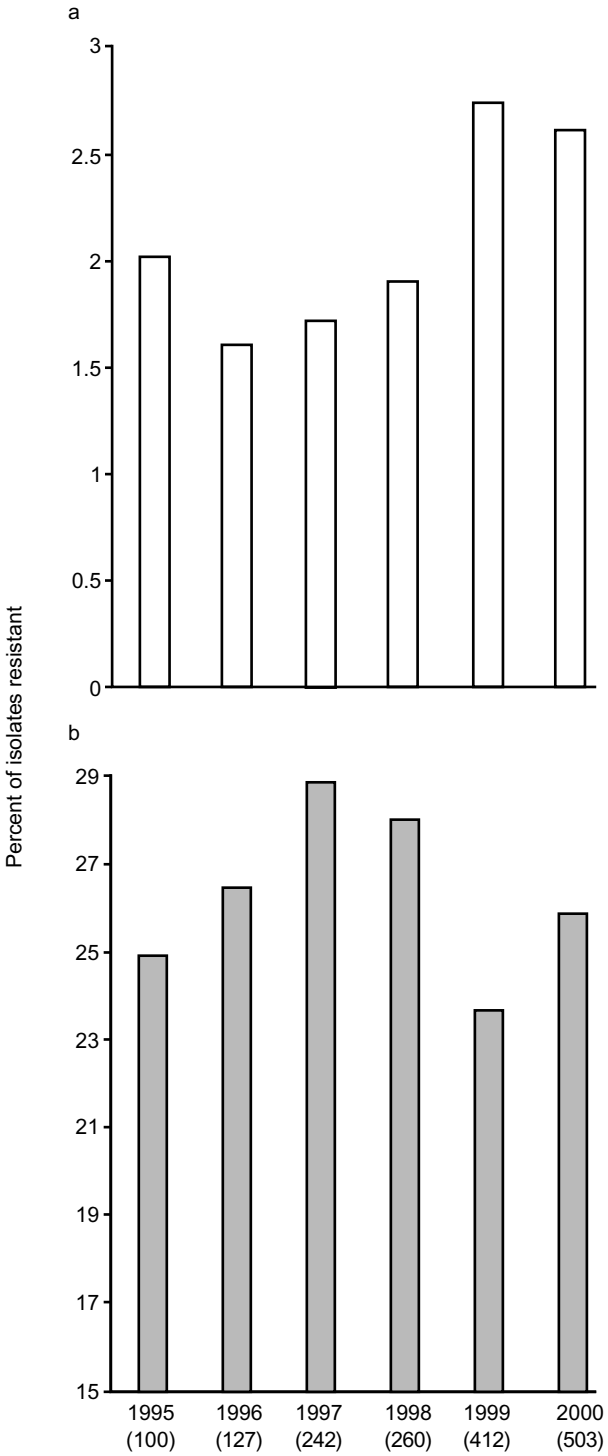

Fig. 1. Percentage of isolates resistant to (**a**) clarithromycin and (**b**) metronidazole during the investigating period (1995–2000). Numbers in parenthesis are the number of isolates examined each year.

Table 2. MICs (mg/L) of amoxicillin, clarithromycin, metronidazole and tetracycline for 1644 clinical isolates tested

Agent	MIC50	MIC90	MIC range
Amoxicillin	<0.016	0.023	<0.016–0.094
Clarithromycin	<0.016	0.064	<0.016–>256
Metronidazole	0.5	128	0.064–>256
Tetracycline	0.064	0.38	<0.016–0.5

Discussion

Resistance to antimicrobial agents in *H. pylori* is of particular concern because it is a major determinant in the failure of eradication regimens [8]. Antimicrobial drug resistance has been reported for nitro-imidazoles, macrolides, fluoroquinolones, amoxicillin, rifabutin and tetracyclines. Therefore, susceptibility testing of *H. pylori* is increasingly important. Two types of resistance must be considered: 'primary' resistance supposedly linked to previous exposure of the *H. pylori* strain to the antibiotic during treatment for another infection unrelated to *H. pylori*, and 'secondary' resistance resulting from treatment aimed at eradicating *H. pylori*. Resistance to metronidazole is the most common, ranging between 16% and 42% in Europe, while the overall prevalence of resistance to macrolides is lower, ranging between 1% and 12% in most countries [2, 4, 9, 10].

Nitro-imidazoles were among the first drugs identified as possessing a powerful anti-*H. pylori* effect. Metronidazole resistance occurs mainly by mutations in the *rdxA* gene of *H. pylori*, which encodes an enzyme that reduces metronidazole to active metabolites. Mutations in the *frxA* gene, encoding an NAD(P)H flavin reductase with high homology with the *rdxA* product can also affect metronidazole susceptibility [11].

In the current investigation the overall rate of primary resistance to metronidazole was 26.5%, which remained stable over the 6 years, and this confirms earlier results reported in 1998 [12]. Results from a European multicentre trial showed the level of overall metronidazole resistance to be 27%, with great variation observed among countries, from 16% in France to 42% in Norway [4]. In other studies, the prevalence of metronidazole resistance was reported to be 21% in the Netherlands, 20% in Spain and 29% in Bulgaria [3, 13, 14]. In the USA, metronidazole resistance was 37.4% in a large study conducted in a metropolitan hospital [15]. An increase in resistance to metronidazole from 30% to 48% over 5 years has been reported in Belgium [16], whereas no increase in metronidazole resistance rates occurred in this area of north-Eastern Germany over the last 6 years. The metronidazole MICs showed a normal distribution with an MIC50 of 0.5 mg/L and an MIC90 of 128 mg/L.

As reported by others, the results of the present study confirm that women are more likely to harbour resistant *H. pylori* strains than men (32.2% versus 20.8%, respectively) [2]. This supports the suggestion that resistance of *H. pylori* to metronidazole may partly be due to its frequent use in the treatment of gynaecological infections.

Several macrolides, alone or in combination with other antibiotics and a proton pump inhibitor, have been used in *H. pylori* eradication regimens, but among them

clarithromycin has been shown to be superior. This compound achieves high concentrations in the gastric mucosa and becomes highly concentrated intracellularly. Analysis of clarithromycin-resistant *H. pylori* strains revealed that point mutation in the peptidyl transferase domain of the 23S rRNA is the mechanism of resistance, and adenine to guanine transition mutations at position 2142 or 2143 were identified [17].

The rate of resistance to clarithromycin is consistently much lower than that to metronidazole. However, based on recent studies the prevalence of clarithromycin resistance varies considerably and ranges from 3.5% in Spain, to 5.7% in Italy and up to 7.7% in Korea [12, 18, 19]. The overall level of primary resistance (2.2%) observed in the study population is comparable to that observed in the Netherlands (1.7%) [3]. In the present study, the percentage of resistance was 2.6% in 2000, which is only marginally higher than the 1.6% found in 1996. The MIC distribution for clarithromycin in *H. pylori* was normal with an MIC90 of 0.064 mg/L. The situation is dramatically different in other countries such as Bulgaria, where clarithromycin resistance increased to 12.5% over 4 years [9].

Thirty-six clarithromycin-resistant *H. pylori* isolates were analysed by PCR-RFLP to detect point mutations. By restriction with endonuclease *Bsa*I and *Bbs*I it was possible to discriminate between the transition mutation A → G at positions 2142 and 2143. The mutation at position 2143 was detected in 52.8% of isolates and the mutation at position 2142 was found in 36.1%. In four cases it was not possible to detect mutations and these strains possibly had an A → C transversion mutation, without a restriction site. Conventional methods to determine antibiotic susceptibility of *H. pylori* are based on agar dilution testing or the Etest, which are time-consuming and require specific laboratory expertise. Therefore, DNA-based diagnostic methods may offer a rapid and alternative approach for macrolide susceptibility testing and could be useful when exploring the epidemiology of macrolide resistance in *H. pylori* [20].

In the present study all strains were susceptible to amoxicillin and tetracycline. These results confirm that amoxicillin-tolerant *H. pylori* strains identified by Dore and colleagues are still exceptional [21].

In conclusion, primary resistance rates for clarithromycin and metronidazole were stable over the last 6 years in the study population. The prevalence of metronidazole resistance is similar to the median rates in Europe, and the rate of clarithromycin resistance is still low compared with other areas. No resistance to amoxicillin or tetracycline was observed during the period of investigation. Therefore, clarithromycin-based triple therapy can still be recommended as first-line therapy for *H. pylori* eradication in the north-eastern part of Germany. Susceptibility testing of *H. pylori* isolates in different geographical areas is advisable because it is an aid to selection of optimal therapy regimens.

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