Evaluation of clinical and socio-demographic risk factors for antibacterial resistance of *Helicobacter pylori* in Bulgaria

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The aim of this study was to assess the clinical and socio-demographic risk factors for primary *Helicobacter pylori* antibacterial resistance. In total, 266 consecutive *H. pylori* strains, from untreated symptomatic adult patients who answered a questionnaire, were evaluated. Strain susceptibility to amoxicillin, metronidazole, clarithromycin and tetracycline was tested by a breakpoint susceptibility test. Metronidazole resistance was found in fewer (17.0 %) peptic ulcer patients than in non-ulcer subjects (28.3 %, $P=0.037$), as well as in fewer patients born in villages (12.7 %) than in those born in towns (27.6 %, $P=0.016$). Clarithromycin resistance varied from 8.8 to 23.4 % ($P=0.009$) within the hospital centres. The highest clarithromycin resistance rate was found in hospital centre A (23.4 %) compared to other centres (12.9 %, $P=0.041$). The factors sex, age, symptom duration, non-steroidal anti-inflammatory drug use, diabetes, type of profession and educational level were not associated with *H. pylori* resistance. Logistic regression revealed that the risk factors for metronidazole resistance were non-ulcer disease [odds ratio (OR) 1.95, 95 % confidence interval (95 % CI) 1.04–3.65] and a birthplace of a town (OR 2.64, 95 % CI 1.18–5.93). The hospital centre may be a risk factor (OR 2.07, 95 % CI 1.02–4.21) for clarithromycin resistance but further studies are required to verify this suggestion. In conclusion, the knowledge of the risk factors for *H. pylori* resistance to antibacterials could facilitate the treatment choice for *H. pylori* eradication.

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INTRODUCTION

Primary *Helicobacter pylori* resistance to antibacterial agents is an important factor for failure of eradication regimens; however, resistance rates may vary between patient groups according to the age, sex, disease, demographic and other factors (Megraud, 2004; Osato *et al.*, 2001; Koivisto *et al.*, 2004). It is important that the previous use of antibiotics can increase the primary *H. pylori* resistance to the agents (McMahon *et al.*, 2003).

Relatively few authors have evaluated birthplace and comorbidity, e.g. diabetes, as possible risk factors for primary *H. pylori* resistance (Katelaris *et al.*, 1998; Elviss *et al.*, 2005; Ojetti *et al.*, 2005). To the best of our knowledge, other patient characteristics, e.g. the duration of the symptoms or the patient's education level and type of profession, were not discussed in studies on *H. pylori* resistance status. The aim of the present study was to assess the influence of the hospital centre and patient characteristics such as age, sex, disease, place of residence, place of birth, presence of diabetes, non-steroidal anti-inflammatory drug (NSAID) use, and type of profession and education level, as possible risk factors for primary *H. pylori* resistance among untreated Bulgarian patients.

Abbreviations: 95 % CI, 95 % confidence interval; AUC, area under the curve; NSAID, non-steroidal anti-inflammatory drug; OR, odds ratio; ROC, receiver operating characteristic.

METHODS

Patients and specimens. In total, 266 consecutive *H. pylori* strains, isolated from untreated patients with upper gastrointestinal complaints who agreed to fill in a questionnaire (Table 1), were evaluated in 2004–2008. Participation was voluntary, and informed consent was obtained in each case. The patients were aged 18–40 years ($n=73$), 46–65 years ($n=138$) or 66–80 years ($n=55$). The classification of patients' diseases was based on endoscopic findings and clinical signs. The patients had duodenal ulcers ($n=80$), gastric ulcers ($n=20$), ulcer scars ($n=17$), chronic gastritis ($n=101$), gastro-oesophageal reflux disease ($n=47$) and hiatus hernia ($n=1$). In the questionnaire, the duration (in years) of the symptoms, use of aspirin or other NSAIDs, presence of diabetes, places of residence and birthplace, type of present or past profession, and education level were also recorded. Data about previous use of antibiotics by the patients were not available. The patients' specimens were taken in four hospital centres named as centre A (64 patients), centre B (102), centre C (46) and centre D (54). Two gastric biopsy specimens per patient were taken. The specimens were transported in Stuart transport medium (Becton Dickinson). The present study was approved by the Ethical Committee of the Medical University of Sofia.

Microbiology. The specimens were used for direct Gram staining, in-house rapid urease test and culture as described previously (Boyanova, 2007). Briefly, culture was performed on Columbia agar (Becton Dickinson) with 10% sheep blood (non-selective agar), and the same medium with 1% IsoVitaleX (Becton Dickinson) and the following agents: 10 µg vancomycin ml⁻¹, 5 µg trimethoprim ml⁻¹, 5 µg cefsulodin ml⁻¹ and 5 µg amphotericin B ml⁻¹ (selective medium). Plates were incubated at 35 °C for up to 11 days in a microaerophilic atmosphere (Campy Pak; Becton Dickinson). Identification of *H. pylori* was made by Gram staining of the colonies, lack of aerobic growth on blood agar plates, and testing for the presence of urease, oxidase and catalase.

Susceptibility testing. Susceptibility of the strains was evaluated by a breakpoint susceptibility test as previously described (Boyanova *et al.*, 2008). Briefly, about 30–60 µl *H. pylori* suspension, with a density corresponding to a McFarland turbidity standard of 2–3, were inoculated on Mueller–Hinton blood agar plates (National Center of Infectious and Parasitic Diseases, Bulgaria), containing one of the following drugs and concentrations: 8, 16 or 32 µg metronidazole ml⁻¹; 0.25, 0.5, 1 or 2 µg clarithromycin ml⁻¹; 0.5, 1 or 2 µg amoxicillin ml⁻¹; 4 µg tetracycline ml⁻¹. The plates were incubated microaerophilically at 35 °C for 2 to 3 days. Non-selective Mueller–Hinton blood agar plates were used for a control of strain viability. The breakpoints for resistance were: >8 µg metronidazole ml⁻¹, ≥1 µg clarithromycin ml⁻¹, >0.5 µg amoxicillin ml⁻¹ and >4 µg tetracycline ml⁻¹ (Megraud *et al.*, 1999; CLSI, 2000; Megraud & Lehours, 2007).

Statistical analysis. Statistical analysis was performed using SPSS 15.0 statistical software. The chi-square test and Fisher's exact test of independence were used to compare the following variables of interest: sex, disease, age, place of residence, birthplace, type of present or past profession, educational level, duration of symptoms, use of aspirin or other NSAIDs, presence of diabetes and hospital centre. Logistic regression was used for selection of significant predictor variables and to estimate odds ratios (ORs) of the same significant variables, as well as, if possible, to predict outcome. Receiver operating characteristic (ROC) curves were performed to test the ability to classify the cases as resistant or sensitive.

RESULTS AND DISCUSSION

Overall *H. pylori* primary resistance rates were: 1.1% for amoxicillin, 24% for metronidazole, 15.4% for clarithro-

mycin, 4.9% for tetracycline, and 4.9% for both metronidazole and clarithromycin (Table 1). No significant differences in the resistance rates were found between men and women ($P \geq 0.251$), and between patients aged >65 years and younger ones ($P \geq 0.207$). The results are similar to those reported by Elviss *et al.* (2005) and Kato *et al.* (2000), although other authors have found the female sex and age to be risk factors for metronidazole or clarithromycin resistance (Chisholm *et al.*, 2007; Osato *et al.*, 2001). Clarithromycin resistance has been associated with one or several of the following factors: age, female sex, geographical region, race, the patient's disease status and previous use of macrolides (Broutet *et al.*, 2003; Megraud, 2004; Toracchio & Marzio, 2003; Seow & Chew, 2004; McMahon *et al.*, 2003; Chisholm *et al.*, 2007; Duck *et al.*, 2004) (Table 2).

H. pylori virulence factors have been associated with both antibacterial resistance rates and success of eradication. Cure rates have been higher for patients with *cagA*⁺/*vacA* s1 strains compared to those with less virulent strains (Van Doorn *et al.*, 2000). Clarithromycin resistance has been more common in non-ulcer patients than in peptic-ulcer patients (Megraud, 2004). Possible explanations could be the higher prevalence of *cagA*-positive strains in the ulcer patients, the shorter generation time of the *cagA*-positive strains and their closer contact with gastric cells, which can render these strains more accessible to antibacterials, or a higher antibiotic consumption in non-ulcer compared to ulcer patients (Megraud, 2004). In addition to antibacterial resistance, other factors can also affect the eradication success of *H. pylori* infection. Following treatment by lansoprazole, amoxicillin and clarithromycin, the eradication of clarithromycin-susceptible strains has been influenced by differences in *S*-mephenytoin 4'-hydroxylase (CYP2C19) genotype (Kawabata *et al.*, 2003).

In the present study, the clarithromycin resistance was associated with hospital centre. The resistance rate was higher (23.4%, 15 of 64 patients) in the hospital centre A than in the other centres (12.9%, 26 of 202, $P=0.041$). Centre A is a large multi-profile hospital for active treatment and emergency medicine. For this reason, in centre A, there were more ulcer patients evaluated (70.3%, 45 of 64) than in the other centres (27.2%, 55 of 202, $P=0.0001$). However, unlike the patients from other hospital centres, those from centre A involved many (29.7%, 19 of 64) cases with complicated ulcer disease or a stomach operated on for peptic ulcer, including patients with bleeding ulcers (7), perforated ulcers (2), an operated stomach (9), and both perforated ulcer and operated stomach (1). Probably, the complex characteristics of the patients from the different hospital centres and/or the previous antibacterial use could be associated with the antibacterial resistance rates in *H. pylori*. According to several authors, the regional and inter-hospital differences in the rates of *H. pylori* resistance to antibacterials are not completely explained, but could be due to variations in either prescribing practice or previous use of antimicrobial agents for other infections (Elviss *et al.*, 2004; Kato *et al.*, 2000).

Table 1. Resistance of *H. pylori* to antibacterial agents according to the patient group

Parameter	Patient group	No. of patients	Amoxicillin resistance (%)	Metronidazole resistance (%)	Clarithromycin resistance (%)	Tetracycline resistance (%)	Metronidazole + clarithromycin resistance (%)
Sex	Female	103	0	21.4	15.5	6.8	2.9
	Male	163	1.8	25.8	15.3	3.7	6.1
Disease	Ulcer	100	1.0	17.0*	15.0	4.0	5.0
	Other	166	1.2	28.3*	15.7	5.4	4.8
Age (years)	18–65	211	0.5	24.6	15.2	4.7	5.7
	>65	55	3.6	21.8	16.4	5.4	1.8
Duration of symptoms (years)	≤10	195	1.5	24.1	13.8	5.6	4.1
	>10	71	0	23.9	19.7	2.8	7.0
Aspirin	User	18	0	16.7	5.6	0	0
	Non-user	248	1.2	24.6	16.1	5.2	5.2
Hospital centre	A	64	1.6	17.2	23.4*	3.1	7.8
	B	102	2.0	27.4	8.8*	2.9	2.0
	C	46	0	21.7	21.7*	8.7	4.3
	D	54	0	27.8	13.0	7.4	7.4
NSAID use	Users	87	2.3	24.1	14.9	5.7	4.6
	Non-user	179	0.6	24.0	15.6	4.5	5.0
Diabetes	Yes	24	0	37.5	12.5	8.3	0
	No	242	1.2	22.7	15.7	4.5	5.4
Place of residence	Town	246	1.2	25.2	15.8	4.5	5.3
	Village	20	0	10.0	10.0	10.0	0
Place of birth	Town	202	1.5	27.6*	15.3	5.4	5.9
	Village	64	0	12.7*	15.6	3.1	1.6
Present† or past‡ profession	Physical work	102	1.0	19.6	14.7	4.9	2.9
	Intellectual work	144	1.4	25.7	13.9	3.5	4.9
Present profession†	Physical work	78	1.3	20.5	14.1	6.4	3.8
	Intellectual work	118	0	25.4	13.6	2.5	5.1
Educational level	Higher	97	1.0	28.9	14.4	4.1	6.2
	Other	169	1.2	21.3	16.0	5.3	4.1
Total		266	1.1	24.0	15.4	4.9	4.9

*Statistically significant difference ($P<0.05$).

†Profession of the patients who actually work.

‡Profession of the patients before retirement for the retired people.

For clarithromycin resistance, the omnibus test of model coefficients was of borderline significance (chi-square=3.86, $P=0.05$). The Nagelkerke R-square value was 0.025. Logistic regression analysis showed that the patients from hospital centre A were two times more likely to have clarithromycin resistance than those from other hospital centres. Hospital centre may be a risk factor [OR 2.07, 95 % confidence interval (95 % CI) 1.02–4.21] for clarithromycin resistance, but because of the borderline significance, further studies are required to verify this suggestion (Table 3).

Metronidazole resistance has been associated by several authors with the previous use of nitroimidazoles and female sex (in women younger than 40–45 years), probably owing to the use of nitroimidazoles to treat gynaecological infections, as well as with ethnicity and place of residence (Megraud & Lehours, 2007; McMahon *et al.*, 2003; Meyer *et al.*, 2002; Chisholm *et al.*, 2007). The previous use of

nitroimidazoles for dental infections and, especially in the tropical areas, for parasitic diseases can also increase the primary *H. pylori* resistance to the agent (Megraud, 2004).

In the present study, metronidazole resistance was found in less (17.0 %, 17 of 100 patients) peptic-ulcer patients than in the rest of the patients (28.3 %, 47 of 166, $P=0.037$). Birthplace was also associated with *H. pylori* resistance to metronidazole. Metronidazole resistance was more common in patients born in towns (27.6 %, 56 of 203) than in those born in villages (12.7 %, 8 of 63, $P=0.016$). Another study has also detected non-ulcer patients to be associated with a higher metronidazole resistance rate (Lui *et al.*, 2003). An inverse association has been reported in Spain (Ferrero *et al.*, 2000).

For metronidazole resistance, the omnibus test of model coefficients indicated significance for the overall model (chi-square=11.0, $P=0.004$). The Nagelkerke R-square value was 0.061. Logistic regression revealed that the ulcer

Table 2. Some reported clinical and socio-demographic risk factors for antibacterial resistance in *H. pylori* in adult dyspeptic patients

Resistance to:	Risk factors for resistance	Country*	No. of patients	Patient group	Method of testing†	Reference
Metronidazole	Age (18–39 years), birthplace	Australia	237	Dyspeptic	Et	Katellaris <i>et al.</i> (1998)
	Birthplace, disease status (non-ulcer)	Bulgaria	266	Dyspeptic	BST	This study
	Previous use of antibiotics‡	Finland	342	Referred for gastroscopy	Et	Koivisto <i>et al.</i> (2004)
	Place of residence	India	259	Peptic ulcer	Et	Thyagarajan <i>et al.</i> (2003)
	Sex (female)	Italy	248	Dyspeptic	Et	Pilotto <i>et al.</i> (2000)
	Disease status (non-ulcer)	Singapore	120	Dyspeptic	AD	Lui <i>et al.</i> (2003)
	Peptic ulcer	Spain	300	Dyspeptic	Et	Ferrero <i>et al.</i> (2000)
	Birthplace	UK	402	Dyspeptic	MDDM and Et	Elviss <i>et al.</i> (2005)
	Sex (female)	UK	1064	Dyspeptic	MDDM	Parsons <i>et al.</i> (2001)
	Place of residence, sex (female), age (<45 years)	UK¶	1310	Dyspeptic	Et and MDDM	Chisholm <i>et al.</i> (2007)
	Sex (female), age (<70 years)	USA	1723	Dyspeptic	AD and Et	Osato <i>et al.</i> (2001)
	Sex (female), ethnicity	USA	3624	Peptic ulcer	AD and Et	Meyer <i>et al.</i> (2002)
	Hospital centre§	Bulgaria	266	Dyspeptic	BST	This study
	Previous use of antibiotics	Finland	342	Referred for gastroscopy	Et	Koivisto <i>et al.</i> (2004)
Clarithromycin	Disease status (non-ulcer)	France	2751	Dyspeptic	MDDM	Broutet <i>et al.</i> (2003)
	Age, sex (female)	Hungary	238	Dyspeptic	FISH	Buzás <i>et al.</i> (2007)
	Sex (female)	Italy	406	Dyspeptic	AD and BST	Toracchio & Marzio (2003)
	Age (older)	Spain	300	Dyspeptic	Et	Ferrero <i>et al.</i> (2000)
	Place of residence, sex (female), age (<45 years)	UK¶	1310	Dyspeptic	Et and MDDM	Chisholm <i>et al.</i> (2007)
	Sex (female), age (<70 years)	USA	1723	Dyspeptic	AD and Et	Osato <i>et al.</i> (2001)
	Place of residence, age (older), sex (female), disease status (inactive ulcer)	USA	3624	Peptic ulcer	AD and Et	Meyer <i>et al.</i> (2002)
	None	Bulgaria	266	Dyspeptic	BST	This study
	Sex (female), age (≥40 years), ethnicity	USA	3624	Peptic ulcer	AD and Et	Meyer <i>et al.</i> (2002)

*Data are arranged according to alphabetical order of the country of the study.

†Methods of testing used were: AD, agar dilution method; BST, breakpoint susceptibility method; Et, E test; FISH, fluorescent *in situ* hybridization MDDM, modified disc diffusion method.

‡For gynaecological infections.

§Borderline significance was found by logistic regression.

||For respiratory and dental infections.

¶England and Wales.

patients had a two times lower risk for metronidazole resistance (OR 0.51, 95 % CI 0.27–0.96) than the non-ulcer patients. The patients born in towns had OR value of 2.64 (95 % CI 1.18–5.93) of metronidazole resistance compared to those born in villages. Both the non-ulcer disease and birthplace in towns were important independent predictors of metronidazole resistance. Likewise, birthplace has been associated with metronidazole resistance, but not with clarithromycin resistance, in a study from the UK (Elviss *et al.*, 2005).

The influence of birthplace on antibacterial resistance rates of *H. pylori* could be associated with the local usage of antibacterial agents during childhood. It is well known that *H. pylori* infection is mainly acquired in childhood (Halitim *et al.*, 2006). In a study from India, metronidazole resistance has been also associated with geographical differences (Thyagarajan *et al.*, 2003). Another study from the USA, however, has not reported regional differences as a risk factor for *H. pylori* resistance to antibacterials (Osato *et al.*, 2001).

Table 3. Risk factors for *H. pylori* resistance to metronidazole and clarithromycin among Bulgarian symptomatic patients ($n=266$) confirmed by logistic regression analysis

Exposure (independent) variable	Outcome variable – resistance to:	B*	SE†	Wald‡	P	Exp(B)= OR§	95 % CI§
Disease status (peptic ulcer vs non-ulcer disease)	Metronidazole	0.77	0.32	4.33	0.037	0.51	0.27–0.96
Birthplace (town vs village)	Metronidazole	0.97	0.41	5.57	0.018	2.64	1.18–5.93
Hospital centre (centre A vs other)	Clarithromycin	0.73	0.36	4.05	0.044	2.07	1.02–4.21

*B, regression coefficient of the logistic regression.

†SE of the regression coefficients.

‡Wald statistics.

§95 % CI for Exp(B) (95 % CI for the OR).

The higher metronidazole resistance rate in the non-ulcer patients compared to those with ulcers may be due to the lower incidence of virulent (*cagA*⁺/*vacA* s1) strains, and the resulting slower strain proliferation and lower penetration of antibiotics in the less inflamed gastric mucosa (Van Doorn *et al.*, 2000; Megraud, 2004; Broutet *et al.*, 2003). Gisbert *et al.* (2001) have suggested that *H. pylori* eradication therapy could be more effective in peptic ulcer than in non-ulcer patients because of different infecting *H. pylori* strains, different severities of inflammation or patient compliance, age and gender, although another study did not confirm this difference (Huang *et al.*, 2005). Other factors that may be associated with the higher metronidazole resistance rate in the non-ulcer patients compared to the ulcer patients are poorer patient compliance of the non-ulcer patients, or higher antibiotic consumption in this patient group or both.

Combined resistance to clarithromycin and metronidazole has been associated with one or several of the following factors: female sex, older age and ethnicity (Seow & Chew, 2004; Meyer *et al.*, 2002). In the present study, the difference in rates of double resistance to clarithromycin and metronidazole between the patients born in towns (5.9%, 12 of 202 patients) and those born in villages (1.6%, 1 of 64, $P=0.312$) did not reach a significant level. Logistic regression analysis did not identify any independent predictor of combined resistance to clarithromycin and metronidazole.

From the ROC curves, the area under the curve (AUC) for clarithromycin was 0.57, SE=0.5, $P=1.13$ (95 % CI 0.47–0.67); the AUC for metronidazole was 0.62, SE=0.04, $P=0.003$ (95 % CI 0.55–0.70), and the AUC for both metronidazole and clarithromycin was 0.50, SE=0.04, $P=1.00$ (95 % CI 0.42–0.58). The results indicate that the ability to predict which patient will harbour a resistant *H. pylori* strain failed for clarithromycin, and for both metronidazole and clarithromycin, and was poor for metronidazole (Fig. 1).

Aspirin has been shown to enhance the *in vitro* susceptibility of *H. pylori* to amoxicillin, clarithromycin and metronidazole (Wang *et al.*, 2003). In the present study, the use of aspirin or

other NSAIDs, a duration of symptoms of >10 years and the level of education were not identified as risk factors for primary *H. pylori* resistance.

According to Pilotto *et al.* (2000), concomitant diseases and a histological activity of chronic gastritis have not been related to antibiotic resistance. However, diabetes has been associated with *H. pylori* infection in several studies (Ojetti *et al.*, 2005; Sargýn *et al.*, 2003). The lower eradication success in diabetic patients compared to controls has been explained by changed gastric microvasculature and a possible reduction of antibiotic absorption, possible gastroparesis and the common use of antibiotics for recurrent bacterial infections (Ojetti *et al.*, 2005). In the present study, diabetic patients had only a slightly higher (37.5%, 9 of 24 patients) metronidazole resistance rate than the rest of the patients (22.7%, 55 of 242, $P=0.106$); however, the difference was not significant possibly because of the small sample size of the diabetic patients.

In conclusion, the risk factors for metronidazole resistance identified by logistic regression were non-ulcer disease and

Fig. 1. ROC curve for metronidazole resistance in *H. pylori* strains from 266 symptomatic patients. AUC=0.62 (95 % CI 0.55–0.70). Solid line, ROC curve; broken line, random distribution.

a birthplace in a town. The hospital centre may be a risk factor for clarithromycin resistance but additional studies are necessary to confirm this association. Knowledge of the risk factors for *H. pylori* resistance to antibacterials could facilitate the treatment choice for *H. pylori* eradication and the recommendation of culture and susceptibility testing of strains for patients of the at-risk groups.

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