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High prevalence of mixed infections by *Helicobacter pylori* in Hong Kong : Metronidazole sensitivity and overall genotype

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Summary

Background: Diversity in metronidazole susceptibility and genotypes of *Helicobacter pylori* have been reported with varying results in different areas.

Aims: To investigate the prevalence of multiple strain infection in a symptomatic Chinese population and to determine the metronidazole susceptibility pattern and genotypic characteristics of these infecting strains.

Methods: Gastric biopsies from antrum, body and cardia were taken during upper endoscopy in symptomatic patients referred to our Department. Pooled cultures and single colony isolates were obtained and tested for metronidazole susceptibility and RAPD fingerprint patterns.

Results: Four hundred and sixty-one isolates were successfully cultured from 46 patients. Fifty-seven percent of subjects had metronidazole resistant strains. Among them, 77% carried a mixture of sensitive and resistant strains, non-uniformly distributed in the gastric mucosa. Mixed genotypes were found by RAPD typing in 24% of subjects. These did not correlate with the metronidazole susceptibility / resistance pattern.

Conclusion: *H. pylori* infections with mixed metronidazole sensitive/resistant strains and mixed genotypes are common in Hong Kong. This makes it prudent to use bacterial strains from several biopsy sites when testing for traits such as drug resistance or virulence in relation to disease.

Introduction

Helicobacter pylori establishes long-term chronic infections in the human gastric mucosa that can lead to active chronic gastritis and peptic ulcer disease [1,2], and is an early risk factor for gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma [3, 4]. Metronidazole (Mtz) is frequently included as one component in multidrug treatment regimens. However, the prevalence of Mtz resistance (MtzR) in some populations is very high, and this decreases the overall success rate of Mtz-based eradication regimens [5,6, 7].

Two related chromosomal nitroreductase genes, *rdxA* (HP0954) and *frxA* (HP0642) in the genome sequence, have been implicated in susceptibility and resistance to Mtz of *H. pylori* strains from diverse parts of the world, including East Asia [8, 9, 10, 11]. The nitroreductase enzymes these genes encode can activate Mtz by converting it from a harmless prodrug to a mutagenic and bactericidal agent (probably hydroxylamine). Most MtzR clinical isolates contain loss of function mutations in *rdxA*, and inactivation of *rdxA* in MtzS strains is generally sufficient to make them resistant to moderate levels of Mtz (16 micrograms/ml, up from 1.5 micrograms per ml, when measured as in the present experiments). Resistance to higher levels of Mtz is common in clinical isolates (typically some half of those that are MtzR), and can be achieved by inactivation of *frxA* (an *rdxA* homolog, located elsewhere in the chromosome). In our hands, this depends on the strain already being mutant in *rdxA* [9]. Another group has suggested that *frxA* inactivation by itself might be sufficient to render a MtzS strain MtzR [10]. Studies demonstrating that Mtz activation is mutagenic [12] had suggested that yet other cellular nitroreductases might also activate Mtz when present in high concentration, but the full

hierarchy of nitroreductases that can contribute to Mtz toxicity to *H. pylori* has not been elucidated.

H. pylori is an extremely diverse species, and independent clinical isolates can usually be distinguished by any of several DNA fingerprinting methods, including the random amplified polymorphic DNA (RAPD) method used here [13]. Differences in DNA patterns of isolates from a particular patient indicate presence of more than one strain. However, studies of the frequency of mixed infections has given seemingly conflicting results, with some indicating that most infected persons harbor only one *H. pylori* strain [13,14,15], and others indicating frequent mixed infection by more than one strain [16,17,18].

Antimicrobial susceptibility testing has usually been applied to pools of colonies from a primary culture of gastric antrum alone. It has been noted however, that *H. pylori* isolates with identical RAPD patterns may show different patterns of Mtz susceptibility [17,19,20], although studies of the MtzR fractions from mixed but predominantly MtzS infections can sometimes identify strains of other genotype. Other anecdotal evidence has suggested that different strains can sometimes predominate in different sites. Most analyses of *H. pylori* in infected individuals have been carried out using US or European residents, but there is emerging evidence that different *H. pylori* genotypes predominate in different human populations [21], and that the spectrum of gastroduodenal diseases that *H. pylori* causes also differs regionally [22]. Therefore, the aims of this study were to investigate the prevalence of multiple strain infection in a symptomatic Chinese population and to determine the metronidazole susceptibility pattern and genotype variation of these infecting strains.

Materials and Methods

Subjects and Specimens

From November 1998 to June 1999, subjects presenting with upper abdominal symptoms referred for upper endoscopy to the Endoscopy Unit, Department of Medicine, Queen Mary Hospital and who fulfilled the inclusion and exclusion criteria were enrolled prospectively into the study. Subjects giving informed consent were included if they were between age 18 to 85 years old, and with no previous history of *H. pylori* eradication and no evidence of active bleeding on endoscopic examination. They were excluded if they have taken antibiotics, proton pump inhibitors, bismuth-containing compounds, or H₂-receptor antagonists within the recent four weeks; had a history of gastrectomy, or had severe concomitant medical illness.

Forty six *H. pylori* positive subjects were included in this study. There were 27 males and 19 females, with a mean age of 56 years, ranging from 22 to 85 years. All patients were ethnic Chinese residents of Hong Kong. No data on prior metronidazole exposure of these specific patients was available. However, typically such patients may have had about two to three courses of metronidazole therapy (for other ailments) during the preceding ten to twenty years.

Three gastric biopsies were taken for bacterial culture, one each from the antrum, body and cardia of the stomach of each patient. Each biopsy was taken from similar sites of antrum (2cm from the pylorus), body (greater curvature, midway between pylorus and oesophageo-gastric junction) and cardia (greater curvature, 4cm from the OG junction) of the stomach. Further biopsies were taken for rapid urease test and histology examination, after the biopsies for culture have been taken. All endoscopies were performed by a single endoscopist (BCYW) to ensure accurate collection of specimens from different sites of the stomach. Each biopsy specimen for culture was placed in a tube containing

0.5 ml of brain heart infusion (BHI, Oxoid, Basingstoke, UK) medium with 20% glycerol and immediately frozen at -70°C until further use.

Single and Multiple colony Strains Isolation

Each biopsy specimen was cultured on selective media (Columbia agar with 7% horse blood and *H. pylori* supplement; Oxoid, Basingstoke, UK) under microaerophilic atmosphere produced by a gas-generation system (CampyGenTM; Oxoid, Basingstoke UK) at 37°C for 3 to 6 days. *H. pylori* was identified by Gram stain morphology and biochemical testing. Two to four single colonies from each primary culture were picked randomly and sub-cultured to obtain single colony isolates, and the rest of the colonies on the same plate were then pooled to obtain multiple colony isolates. All single colonies had a low passage level (less than three passages) prior to susceptibility testing. A total of two to eleven single colony isolates and one to three multiple colony isolates were obtained from each patient.

Determination of Mtz Susceptibility and Selection of Mtz Resistant Isolates

All *H. pylori* cultures derived from single, and also from multiple colonies (pools of over five colonies) were tested for susceptibility to Mtz. Briefly, *H. pylori* cultures were suspended in BHI broth and adjusted to a McFarland turbidity of 1 (3×10^8 cfu/ml). Ten μl aliquots of each suspension were then spread and plated on medium containing 8 $\mu\text{g/ml}$ Mtz and on control medium that was free of Mtz. Growth on Mtz containing medium indicated resistance. Resistant colonies from pools were re-plated again on the same medium and re-grown to obtain pure MtzR cultures.

Preparation of Genomic DNA

For each patient and each biopsy site, *H. pylori* genomic DNA was prepared from unselected single colony isolates, and also from pools of more than ten colonies and from Mtz- selected isolates if available. Bacteria were grown to confluency, harvested with sterile cotton swab applicators, and washed once by centrifugation with TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0). Genomic DNA was prepared using the QIAamp Tissue Kit (Qiagen GmbH, Germany). The DNA concentration was determined spectrophotometrically at 260 nm.

RAPD Fingerprinting

RAPD (or arbitrarily primed PCR) DNA fingerprinting was used to detect DNA sequence diversity among *H. pylori* isolates[7]. Two arbitrary primers used in this study were 1281 (5'-AACGCGCAAC) and 1254 (5'-CCGCAGCCAA), and were synthesized by Genosys Biotechnologies (London, UK). PCR was carried out in a volume of 25 μ l, containing 10 ng of *H. pylori* genomic DNA; 4 mM MgCl₂; 20 pmol of primer; 1 U of Taq DNA polymerase (Boehringer Mannheim GmbH, Germany); 250 mM each of dCTP, dGTP, dATP, dTTP in 10 mM Tris-HCl (pH 8.3); 50 mM KCl; and 0.001% gelatin. PCR was performed with a thermal cycler (PTC-200, MJ Research, USA), with an initial step of denaturing target DNA at 94 °C for 5 min. This was followed by 40 cycles each of 94⁰ C for 1 min, 36⁰ C for 1 min , 72⁰ C for 2 min, and then a final cycle of 72⁰ C for 10 min. The PCR products were analyzed using 2% agarose gel electrophoresis. A positive (known sample of *H. pylori* DNA) and a negative control were included in each set of PCRs. When different RAPD profiles were obtained from isolates of a particular patient, the RAPD fingerprinting was repeated again, using fresh DNA preparations, to ensure that the differences were real.

Results

Endoscopic diagnosis showed gastritis in 16 patients (35%), duodenal ulcer in 15 patients (33%), gastric ulcer in 12 patients (26%), and gastric cancer in three patients (7%). All cancers were proven histologically.

Bacterial culture

H. pylori was successfully cultured from all three gastric sites (antrum, body and cardia) in 32 of 46 patients, from two of the three sites in ten subjects, and from one of the three sites in four subjects. All these 120 sets of cultures (41 from antrum, 42 from body, 37 from cardia) were also represented by two to five related single colony isolates per biopsy site, giving a total of 418 isolates. All isolates were tested for susceptibility to Mtz.

Metronidazole resistance

Subjects were classified into three groups based on metronidazole susceptibility patterns from both pools and single colony isolates. The first group, called the MtzS group, consisted of twenty out of 46 patients (43.5%) from whom only Mtz-susceptible (MtzS) *H. pylori* was cultured. The second group, called the MtzR group, consisted of six patients (13%) from whom all single colony isolates and pools were MtzR. The third group, called the mixed group, consisted of 20 patients from whom both MtzS and MtzR *H. pylori* were cultured. A simplified description of findings is given in Table 1. It was striking that with four of the 46 patients (Nos. 9, 39, 78 and 84) in the mixed group (8.7%) the pools seemed to be purely MtzS, but one or more of the single colony isolates were clearly MtzR. Around one third of the *H. pylori* infections in non-ulcer dyspepsia, duodenal ulcer and gastric cancer patients were MtzS, but two third of the infections in

gastric ulcer patients were MtzS (Table 2). Due to the small sample size, there was no statistical significant difference in the Mtz susceptibility and disease status.

The prevalence of MtzR correlated directly with the number of sites of biopsies obtained and the number of colonies (both pooled and single isolates) tested. When only the pooled cultures from either the antrum or the body were analyzed, the observed prevalence of MtzR were 32% and 36% respectively (Table 3). This increased to 46% when pooled cultures from both antrum and body were included, and further to 59% when all sites and single colony isolates were included. There was no obvious preference for colonization of the antrum vs. body and cardia with the MtzS or MtzR strains.

H. pylori was eradicated by triple or quadruple therapy containing metronidazole from each of the twelve (100%) patients that our tests had indicated were probably infected only with MtzS *H. pylori*. Conversely, of the 17 patients with MtzR strains who had metronidazole-based therapy, 12 (71%) of them had successful eradication and 5 of them did not. There was a significantly higher eradication rate in patients with MtzS *H. pylori* than those with MtzR strains ($p < 0.001$). However, there was no significant difference in the eradication rate of MtzR strains by using regimes with or without metronidazole, but the numbers in each subgroup were small.

RAPD Fingerprinting of H. pylori

The genetic diversity of *H. pylori* strains in a given patient was assessed by RAPD fingerprinting using 298 single colony isolates obtained from different gastric sites, 120 pooled cultures and 43 MtzR isolates, when present. Two different primers were used in separate reactions to give better sensitivity.

Uniform RAPD fingerprint patterns were found in comparisons of single colony

isolates, pooled cultures and the MtzR fraction of a MtzR/MtzS mixed infection (when present) in 35 of 46 (76%) patients (14 of 20 (70%) patients with MtzR/MtzS mixed infection) (Figure 1). Different RAPD fingerprinting patterns (overall genotype) were always found in different patients, as expected. More important, in 11 of the 46 (23.9%) patients, at least one isolate differed completely in RAPD profile from the other isolates from the same patient, and thus probably constituted different strains. Among them, nine patients had two different strains (Figure 2) and the other two patients had three different strains. In two patients (patients 8, 12) the different strains were detected among different single colony isolates. In four patients (patients 11, 28, 47, 52) the different strains were detected by using Mtz-selected as well as unselected isolates (Figure 3). In another four patients (patients 8, 12, 78, 91) the different strains were detected by using two instead of just one arbitrary primer (Figure 4). Results of RAPD patterns by using two ng of DNA were the same as using 10 ng DNA (Figure 5).

More than one RAPD pattern was observed in 31%, 27%, 17% of non-ulcer dyspepsia, duodenal ulcer and gastric ulcer patients, respectively. The isolates from the three gastric cancer patients were uniform in RAPD pattern in each case. Had we only compared the RAPD fingerprint patterns from pools from different biopsy sites (antrum, body and cardia) using a single RAPD primer, then the detected prevalence of mixed infections would have been reduced to 6.5% (3/46).

Correlation between Metronidazole susceptibility pattern and RAPD fingerprinting

More than one RAPD fingerprint pattern was found in three (patient 2, 8 and 85) of 20 (15%) patients with pure MtzS strains; one (patient 47) of six (17%) patients with pure MtzR strains and seven (patient 11, 12, 28, 52, 78, 86, 91) of 20 (35%) patients with mixed MtzS/R strains (overall $p=0.33$)(Table 1). On the other hand, mixed MtzS/R

strains were found in 13 out of 35 (37%) patients with a single RAPD fingerprint pattern and seven out of 11 (64%) patients with more than one RAPD pattern ($p=0.17$). There were trends that patients with mixed MtzS/R strains were more likely to have more than one RAPD pattern, and vice versa, although these did not reach statistical significance.

Discussion

We studied the susceptibility of *H. pylori* to metronidazole from infected adults in Hong Kong, in relation to overall bacterial genotype. This entailed phenotypic tests and arbitrarily primed PCR (RAPD) fingerprinting, carried out using both single colony isolates and pools of bacteria recovered from each of several biopsy sites per patient. More than half of our cohort of infected patients (26 of 46) carried at least some MtzR *H. pylori*. It was striking however that in most of these cases (at least 20 out of 26), the infections were mixed, containing both MtzS and MtzR organisms. The MtzS and MtzR isolates were often (13/20, 65%) of the same RAPD type. That is, they were closely matched in overall genotype. This is in accord with the suggestion that MtzR clinical isolates often arise during infections initiated by MtzS organisms by de novo Mtz-induced as well as selected mutation [8, 19]. It may also be explained by recent findings that Mtz is mutagenic for *H. pylori*, and indications that MtzR strains may be less vigorous than MtzS strains [10,12, J. Y. Jeong, and D. E. Berg, unpublished results].

Our definition of MtzR is the ability to grow in the presence of eight micrograms per ml of Mtz. This was chosen for the present large scale tests based on long established criteria [9], and indications from our own recent studies that most (but not all) clinical isolates resistant to 8 $\mu\text{g/ml}$ (using testing protocol presented here) are resistant to 16

µg/ml, and that about half of them are resistant to 32 µg/ml [9]. We found that resistance to only 8 µg/ml is attributable to leaky mutations in *rdxA*. Resistance to 16 µg/ml but not 32 µg/ml reflects null mutations in *rdxA*, and resistance to 32 µg/ml reflects null mutations in *rdxA* and *frxA* [9 and, J. Y. Jeong, and D. E. Berg, unpublished results]. Megraud et al had suggested that only resistance to at least 32 µg/ml predicted failure of Mtz-based eradication therapy [23]. The observation that 71% of patients in our study who were infected with MtzR strains had successful eradication of the infection may suggest that the threshold for metronidazole resistance in our study might be set too low.

The high prevalence of MtzR and MtzS/R mixed infection has been important clinically: all patients that seemed to be infected only by MtzS strains were successfully treated. The 30% treatment failures occurred only in patients that had been colonized with at least some MtzR *H. pylori*. In this context, it was also remarkable that some MtzR infections were nevertheless eradicated by Mtz-based therapy [present results, 24,25]. This might reflect sometimes achieving higher effective concentrations of Mtz in the gastric mucosa than even "resistant" strains can tolerate.

There was a discrepancy in results of some MtzR tests using single colonies and pools: four patients judged from tests using pools to have purely MtzS infections yielded at least one single colony isolate with a bona fide MtzR phenotype. Similarly discrepant results have also been reported by others [26]. Some of these cases might reflect chance, stemming from our having tested different colonies individually and in pools, and the relatively few colonies available for pooling from some of our biopsies. Perhaps also affecting this might be the apparently lower "fitness" of many MtzR *H. pylori* strains, suggested by inspection of colony size [10], and findings that MtzR *H. pylori* tend to be lost from mixed cultures when grown in direct competition with MtzS strains [J. Y. Jeong and D. E. Berg, unpublished results].

Several earlier studies have reported that individuals can be infected by strains of more than one genotype, with the fraction of persons found to carry multiple strains ranging from 0 to 85% (mean 20%) [14,15,17,18,19,27-33]. Some of the strains we recovered from mixed infections (24% of infections analyzed) were in a small minority, and were found only because numerous single colonies or Mtz-selected colonies were analyzed. The differences in apparent prevalence of such mixed infections in different reports can be attributed to a combination of factors, including sampling strategy used: from one vs. several gastric sites, and the sites of biopsy (all antrum, vs. antrum, body and cardia); tests of pools vs. single colonies from each biopsy; analysis of drug resistant fractions as well as unselected strains, to increase chance of detecting minority subpopulations; and the sensitivity of the strain typing method used. In any case, it is now clear that tests of Mtz susceptibility using only *H. pylori* from antral sites, as had been quite typical of studies in the past, may often not fully sample the entire *H. pylori* population throughout the stomach. We suggest that several different sites need to be analyzed, at least including both the antrum and the corpus.

We might expect the actual chance of finding mixed infections to also reflect the overall risk of infection in the society under study. However, a clinical trial in Guangzhou, China (where the overall risk of *H. pylori* infection should be more than in Hong Kong), had indicated that new adult infections are uncommon [34]. If this finding is applicable to the present Hong Kong Chinese population, it is attractive to imagine that the mixed infections by different genotypes found here may represent a long-term stable association, one that has persisted for years. Whether this indicates specialization of certain strains for just a subset of gastric mucosal niches remains to be determined.

In conclusion, our study has shown that mixed MtzS/R infections are common in Hong Kong, and that genotypes are not uniformly distributed throughout the gastric

mucosa. This makes it prudent to study strains from several biopsy sites when testing for traits such as drug resistance or virulence in relation to disease. Infections that were mixed in terms of overall genotype were found in 24% of patients, and this did not correlate with the 43.5 % of infections that were mixed with regard to Mtz susceptibility. Further studies are needed to address issues such as the effects of MtzR on virulence, the importance of Mtz-induced mutation in bacterial adaptation and evolution during chronic infection, competition among strains during mixed infection vs. the possibility of specialization of certain *H. pylori* strains for particular gastric sites.

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Table 1. Metronidazole susceptibility and RAPD patterns of *H. pylori* isolates from 24 out of 46 patients

Patient	Diagnosis	Mtz Susceptibility Pattern			RAPD Pattern	
		Multiple Colony	Single Colony	Isolates	Pattern	No.
<i>Mtz-sensitive (total = 20)*</i>						
2	NUD	As, Bs, Cs	AS1s,AS2s,AS3s,BS1s,BS2s,BS3s,CS1s,CS2s	uniform	2	mixed
8	NUD	As, Bs, Cs	AS1s,AS2s, BS1s ,BS2s,CS1s,CS2s	uniform	2	mixed
85	GU	As, Bs, Cs	AS1s,AS2s,AS3s,BS1s,BS2s,CS1s,CS2s,CS3s	uniform	2	mixed
<i>Mtz-resistant (total = 6)*</i>						
47	DU	Ar	AS1r,AS2r	uniform	2(A ^r)	mixed
<i>Mixed (total = 20)</i>						
9	GU	As, Bs, Cs	AS1s,AS2r,BS1s,BS2s,CS1s,CS2s,CS3s	mixed	1	single
11	DU	Ar, Bs	AS1r,AS2s,BS1s,BS2s	mixed	2(A ^r)	mixed
12	NUD	Ar, Bs	AS1s,AS2s, AS3r ,AS4s,BS1s,BS2r,CS1r,CS2r,CS3r	mixed	2	mixed
19	NUD	As, Br, Cs	AS1s,AS2s,AS3r,BS1s,BS2s,CS1s,CS2s	mixed	1	single
20	NUD	As, Br, Cr	AS1s,AS2s,AS3s,AS4s,AS5s,BS1r,BS2r,BS3r,CS1r,CS2r	mixed	1	single
28	NUD	As, Br, Cr	AS1s,AS2r,BS1s,BS2s,CS1s,CS2r	mixed	3(B ^r ,C ^r)	mixed
38	NUD	As, Br, Cr	AS1r,AS2r,BS1s,BS2s,CS1r,CS2s,CS3s	mixed	1	single
39	DU	As, Bs, Cs	AS1s,AS2s,BS1r,BS2s,CS1r,CS2s	mixed	1	single
51	GU	Ar, Bs, Cs	AS1s,AS2s,BS1s,BS2s,BS3s,CS1s,CS2s	mixed	1	single
52	DU	Ar, Bs, Cs	AS1s,AS2s,BS1r,BS2s,CS1s,CS2s	mixed	2(A ^r)	mixed
53	NUD	As, Br, Cr	AS1s,AS2s,BS1r,BS2r,CS1s,CS2s	mixed	1	single
56	CA	As, Br, Cr	AS1s,AS2s,AS3s,BS1s,BS2s,BS3s,CS1s,CS2s,CS3s	mixed	1	single
57	DU	Ar, Cs	AS1s,AS2s,CS1s,CS2s	mixed	1	single
60	NUD	Ar, Br, Cr	AS1s,AS2s,AS3s,BS1s,BS2s,BS3s,CS1s,CS2s,CS3s	mixed	1	single
63	DU	As, Bs, Cr	AS1s,AS2s,BS1s,BS2s,BS3s,CS1s,CS2s	mixed	1	single
78	NUD	As, Bs, Cs	AS1s,AS2s ,BS1s,BS2s,BS3r,CS1s,CS2s	mixed	2	mixed
84	NUD	Bs	BS1r,BS2s,BS3s,BS4s	mixed	1	single
86	GU	Br, Cr	BS1r,BS2r,BS3r ,CS1s,CS2s,CS3s	mixed	2	mixed
91	DU	Ar, Br, Cr	AS1r,AS2r,BS1s,BS2s,BS3s,CS1r,CS2s,CS3r	mixed	3(A ^r)	mixed
105	CA	Br,Cr	AS1r,AS2r,AS3s,AS4s,AS5s,BS1r,BS2r,BS3r,CS1r,CS2s,CS3s	mixed	1	single

*For pure Mtz-sensitive and pure Mtz-resistant group, only those with mixed RAPD patterns were shown here. In the pure Mtz-sensitive group, 3 out of 20 were shown, and for the pure Mtz-resistant group, 1 out of 6 was shown.

Notes: A: Antrum; B: Body; C: Cardia; S: Single colony isolate; s: Mtz sensitive; r: Mtz resistant; A^r,B^r,C^r : Mtz-selected isolates; NUD: non-ulcer dyspepsia; DU: duodenal ulcer; GU: gastric ulcer.

Isolates in Bold character: different RAPD pattern compared with others.

Table 2. Metronidazole Susceptibility Patterns in Different Diseases

Diseases	No. of patients	MtzS (%)	MtzR/Mixed (%)
Non ulcer dyspepsia	16	6 (37.5)	10 (62.5)
Duodenal ulcer	15	5 (33.3)	10 (66.7)
Gastric ulcer	12	8 (66.7)	4 (33.3)
Gastric cancer	3	1 (33.3)	2 (66.7)
Total	46	20 (43.5)	26 (56.5)

Table 3. Results of Metronidazole susceptibility by different biopsy sites

Sites	Available Patients	MtzS (%)	MtzR (%)
Antrum	41	28 (68)	13 (32)
Body	42	27 (64)	15 (36)
Cardia	37	22 (59)	15 (41)
Antrum + Body	37	20 (54)	17 (46)
Antrum + Cardia	35	19 (54)	16 (46)
Body + Cardia	34	18 (53)	16 (47)
Antrum + Body + Cardia	32	16 (50)	16 (50)
Antrum + Body + Cardia + Single isolates	32	13 (41)	19 (59)

Figure Legend

Fig 1. RAPD fingerprinting from a representative patient indicating identical RAPD patterns using two sets of primers in multiple colony isolates (MCI)(A: antrum; B: body; C: cardia), single colony isolates (SCI)(A1, B1, C1 from antrum, body, cardia, etc) and Mtz-selected colony isolates (MSI)(Ar, Br, Cr from antrum, body and cardia respectively). (M: marker)

Fig 2. Two different RAPD fingerprintings were found for strains isolated from body and fundus. (MCI: multiple colony isolates; SCI: single colony isolates; MSI: Mtz-selected colony isolates, similar to figure 1)

Fig 3. Mtz-selected isolate from the antrum (Ar) demonstrated a different RAPD pattern from strains isolated from multiple colony isolates and single colony isolates. (M: marker; MCI: multiple colony isolates; SCI: single colony isolates; MSI: Mtz-selected colony isolates, similar to figure 1)

Fig 4. Three different RAPD patterns were found by primer 1281, but not by primer 1254. (M: marker; MCI: multiple colony isolates; SCI: single colony isolates; MSI: Mtz-selected colony isolates, similar to figure 1)

Fig 5. Two pairs of single colony isolates from two representative patients (A, B) each demonstrated two different RAPD patterns, which were confirmed by using 2 ng and 10 ng template concentrations, and by using primers 1281 and 1254.