Efficacy of a Nitroimidazole Containing Triple Therapy Regime in Singapore

K L Ling, W Luman, B Ho, H S Ng

ABSTRACT

Background: There has been a gradual increase in the proportion of Singapore patients with metronidazole resistant strains of *Helicobacter pylori*. We studied the efficacy of a nitroimidazole containing regime in eradicating *H. pylori*.

Methods: Consecutive treatment naive patients with peptic ulcer disease and culture proven *H. pylori* were recruited. From each patient, two antral biopsies were taken for rapid urease test and two for histology. Two biopsies each from the gastric antrum and corpus were taken for *H. pylori* culture. Antibiotic sensitivity to amoxycillin, metronidazole, clarithromycin and tetracycline were tested using the disc diffusion method. Patients were treated with lansoprazole 30 mg bd, tinidazole 500 mg bd and clarithromycin 500 mg bd for seven days. Successful eradication was defined as either negative urea breath tests 4 and 12 weeks after treatment, or negative histology and culture at least four weeks after the end of treatment.

Results: A total of 64 patients were culture positive (51 males, 13 females). Forty-two patients had duodenal ulcers (DU), 17 gastric ulcers (GU), and 5 DU and GU. Metronidazole resistance was detected in 16 patients (25%). Three of the 16 patients (19%) had a mixed population of resistant and sensitive strains of *H. pylori*. None of the *H. pylori* isolates were resistant to amoxycillin, tetracycline or clarithromycin. Overall, eradication was achieved in 51/64 patients (80%). Eradication rate was 88% (42/48) among those with metronidazole sensitive strains, and 56% (9/16) among those with metronidazole resistant strains ($p < 0.02$).

Conclusion: A high proportion of our patients with metronidazole resistant strains of *H. pylori* failed eradication therapy when a nitroimidazole containing regime was used. It may not be appropriate to use a nitroimidazole containing regime empirically as the first line treatment without prior knowledge of the antibiotic sensitivity pattern of the *H. pylori* isolate.

Keywords: *Helicobacter pylori*, antimicrobial susceptibility testing, treatment failure

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INTRODUCTION

Helicobacter pylori colonises the human stomach and causes gastroduodenal diseases[1-3]. Upon establishment of colonisation in the stomach, the bacterium generally persists for life despite the immune and inflammatory responses of the host, and the hostile environment in the stomach[4]. Eradication of *H. pylori* is effective in the treatment of peptic ulcer disease and in preventing relapse.

In our previous retrospective study, a one week regime of omeprazole, clarithromycin and tinidazole was shown to be successful in 89.6% of patients[5]. However, no cultures were done for these patients. Since then, a continuing audit within the department has shown an increasing number of treatment failures with this regime. It was also reported that there was an increase in the proportion of patients in Singapore with metronidazole resistant strains[6,7]. In addition, there have been reports of *H. pylori* isolates with different antibiotic sensitivity in the stomach of the same patient, despite being genotypically similar by DNA fingerprinting[8-10].

The aim of this study is to investigate the efficacy of a one week regime containing lansoprazole, tinidazole and clarithromycin in our local population, and to correlate this with the metronidazole sensitivity of *H. pylori* isolated from both the gastric antrum and corpus.

MATERIALS AND METHODS

Patients

All patients seen in 1999 by one of the authors (LKL), with peptic ulcer disease and culture proven *H. pylori* infection were recruited. Patients must also be tested positive for *H. pylori* by either histology or a rapid urease test. Patients were excluded if there was a

previous history of gastric surgery, malignant gastric ulcer, in cases of pregnancy and if there was known allergy to one of the medications. Informed consent was taken from all patients. The trial protocol was reviewed and approved by the Ethics Committee of the Singapore General Hospital.

Endoscopy

Endoscopy was performed after six hours of fasting under a local anaesthetic agent (Xylocaine throat spray). Two antral biopsies were taken for histology, and two for the CLO test (Delta West, Bentley, Western Australia). In addition, two biopsies each were taken from the gastric antrum and corpus for culture. The CLO test was considered positive if the colour changed from yellow to pink within twenty-four hours. Histological slides were examined using standard haematoxylin-eosin stain.

Culture and Sensitivity Testing

Methods for isolation and culture of *H. pylori* were as described previously. The biopsies were transported in 0.85% sterile saline to the laboratory for processing within four hours. The two biopsies from each site were smeared onto a chocolate blood agar plate without antibiotics, before being smeared onto a chocolate blood agar plate supplemented with antibiotics[11]. The biopsy was then smeared onto a clean glass for Gram staining before being inoculated into urease reagent for rapid urease test. Identification of *H. pylori* isolates was based on Gram staining, colony morphology and positive reaction for catalase, oxidase and urease activity[7].

The *H. pylori* isolates were tested for their sensitivity to four antibiotics: tetracycline (10 µg/disc), amoxycillin (10 µg/disc), metronidazole (5 µg/disc) and clarithromycin (15 µg/disc) (Oxoid, England) by the disk diffusion method[12]. Three-day-old fresh bacterial suspension of *H. pylori* was diluted in sterile saline to about 3×10^8 CFU/ml. A 0.2 ml bacterial suspension was inoculated on horse blood agar plate. The plate was left to dry on the bench for 10 minutes. One antibiotic disc was placed at the centre of each plate which was then incubated in a 5% CO_2 incubator (Forma Scientific, USA) at 37ºC for three days. The inhibition zone was recorded accordingly. Resistance was defined as the absence of inhibition zone for tetracycline and amoxycillin, less than 15 mm for metronidazole[12,13], and less than 30 mm for clarithromycin[14].

DNA extraction

Plate culture of *H. pylori* was transferred into an Eppendorf tube and 1.5 mls of TE buffer (100 mM Tris-HCl and 1 mM EDTA) was added. The suspension was centrifuged at 8,000 X *g* and washed once with TE buffer. The pellet was suspended in 800 µl TE buffer. The bacterial suspension was incubated in 100 µl of 10 mg/ml lysozyme (Sigma) at 37ºC for 30 minutes, and then lysed with 100µl of 10% sodium deodecyl sulfate for a further 30 minutes at 37ºC. Following the addition of 5 µl of 10 mg/ml proteinase K (Boehringer), the mixture was incubated for one hour at 56ºC. DNA was purified by extracting twice with an equal volume of phenol, and once with an equal volume of chloroform. DNA was then precipitated overnight at - 20ºC with one volume of absolute ethanol and 20 µl of 3M sodium acetate. The DNA precipitate was washed once with 70% ethanol. The pellet was vacuum-dried using speed-vac (Savant) and resuspended in 200 µl sterile distilled water. This served as target DNA whose concentration was measured spectrophotometrically at λ_{260} nm. The target DNA was stored at 4ºC until use.

PCR-RAPD

The universal primer for PCR-based RAPD was randomly chosen according to Akopyanz et al[17] which allows for the fingerprinting of the whole DNA content of cells. The primer used in this study was 5'-AAGAGCCCGT-3'. PCR reaction was carried out in 25 µl volume. Fifty nanograms of *H. pylori* target DNA, 2 mM $MgCl_2$, 20 pmol primer, 1 unit of Taq DNA polymerase and 250 mM each of dGTP, dCTP, dATP and dTTP were placed in standard PCR incubation buffer containing 10 mM Tris-HCl, 50 mM KCl, 2 mM $MgCl_2$ and 0.01% gelatin (Promega, USA). The reaction mixture was overlaid with a drop of mineral oil to prevent evaporation. PCR was performed with a thermal cycler (Perkin Elmer, USA) consisted of an initial step of denaturation of target DNA at 94ºC for 5 minutes. This was followed by 39 cycles of denaturation at 94ºC for 1 minute, annealing at 36ºC for 1 minute and extension at 72ºC for 2 minutes. Ten microliters of the PCR products were electrophoresed in 1% horizontal agarose gels for 2 h at 80 V in TBE buffer. The gels were stained with ethidium bromide (1 µg/ml) and photographed with filtered UV illumination on Polaroid type 667 film.

Treatment

Eradication therapy consisted of lansoprazole 30 mg, clarithromycin 500 mg and tinidazole 500 mg all given twice a day for one week. This was prescribed to all patients tested positive for *H. pylori.* If the CLO test turned positive in an hour, eradication therapy was prescribed in the endoscopy suite. In these cases,

H. pylori infection was confirmed by culture afterwards. In patients with a positive culture, and either a positive histology or CLO test between 2-24 hours, therapy was started 2-3 weeks after endoscopy. In these cases, patients were treated with famotidine 20 mg bd while awaiting the culture and histology results. A pill count was done within seven days of completion of therapy.

Follow up

Patients with gastric ulcers (GU) had repeat gastroscopy at 4-6 weeks after therapy. Paired biopsies were taken from the gastric antrum and corpus for histology and culture. Eradication was defined as the inability to detect *H. pylori* in both histology and culture. For patients with duodenal ulcer (DU), eradication was defined as two negative urea breath tests done 4 and 12 weeks after the cessation of therapy. All acid suppression therapy was terminated at least one week before repeat endoscopy or urea breath test.

The ^{14}C urea breath test was carried out according to the method as described by Marshall[18]. Patients were administered with the ^{14}C urea solution (Sigma, St Louis), and a breath sample was obtained at 20 minutes into a liquid trap containing hyamine solution. The solution was then counted in a liquid scintillation counter (Wallac 1410, Pharmacia). This method has been previously validated[19].

Statistical Analysis

Statistical analysis was performed using the chi-squared test where appropriate. The level of significance was predetermined at $p = 0.05$.

RESULTS

A total of 84 patients who gave informed consent were gastroscoped. Nine were found not to have *H. pylori* by CLO test, histology and culture. 11 patients were positive for *H. pylori* by histology and CLO test, but were negative by culture. These patients were given eradication treatment but were not included in the outcome analysis.

H. pylori was isolated in at least one biopsy specimen from 64 patients. There were 51 men and 13 women. The median age was 50 years (range 19 to 80 years). Forty-two patients had DU, 17 had GU and 5 had DU and GU. Of the 64 patients, 46 showed the presence of *H. pylori* in both the gastric antrum and corpus specimens. In 18 patients, *H. pylori* was isolated only from the antrum.

Primary metronidazole resistance was found in 16 patients (25%). There was no primary resistance to clarithromycin, tetracycline or amoxycillin noted.

Table I. Treatment outcome.

Metronidazole Sensitivity \ Treatment Outcome	Total	Eradicated (%)	Failed (%)
Sensitive	48	42 (88)	6 (12)
Resistant*	16	9 (56)	7 (44)
Total	64	51	13

* Includes three patients with mixed metronidazole sensitivities

Table II. Characteristics of *H. pylori* isolates obtained from antrum and body.

No. of strains	Source of strain	Antibiotic susceptibility testing	DNA fingerprints
31	Antrum Body	TAMC-S TAMC-S	Identical
10	Antrum Body	TAC-S, M-R TAC-S, M-R	Identical
1	Antrum Body	TAC-S, M-R TAC-S, M-R	Similar
1	Antrum Body	TAMC-S TAMC-S	Similar
2	Antrum Body	TAC-S, M-R TAMC-S	Identical
1	Antrum Body	TAMC-S TAC-S, M-R	Identical

T: tetracycline; A: amoxycillin; M: metronidazole; C: clarithromycin; S: sensitive; R: resistant

The results of eradication treatment are shown in Table I. Overall, *H. pylori* eradication succeeded in 51/64 patients (80%). Among patients with a metronidazole sensitive strain, the eradication rate was 42/48 (88%), compared with a rate of 9/16 (56%) among patients with metronidazole resistant strains ($p < 0.02$).

Gastroscopy was repeated in the 22 patients with GU. Ulcer healing had taken place in all patients regardless of the success of eradication treatment. Among the six patients with GU who had failed treatment, *H. pylori* was successfully isolated in only two patients. Both showed isolates with secondarily acquired metronidazole resistance, but which had PCR-RAPD patterns identical to the pre-treatment patterns.

Table II shows the results of the genetic fingerprinting studies in relation to antibiotic sensitivity. Three of the 46 paired strains showed differing sensitivity to metronidazole. In one patient, the strain isolated from the antrum was sensitive to metronidazole while the strain isolated from the body was resistant. In another two cases, strains obtained from the antrum were resistant to metronidazole while strains obtained from the body were sensitive. All three pairs of strains demonstrated identical PCR-RAPD patterns.

Among these three patients with different metronidazole sensitivities, one failed therapy. This

patient presented initially with a GU. Cultures obtained from this patient post-treatment showed that the previously metronidazole sensitive isolate in the corpus had been replaced by a metronidazole resistant strain. The PCR-RAPD pattern was identical pre- and post-treatment.

It was found that all patients harboured a single strain of *H. pylori* based on PCR-RAPD fingerprinting. Of the 46 patients with positive cultures from the antrum and body, two pairs of DNA patterns showed subtle differences where up to one band was absent. This was interpreted as a minor variation due to the existence of a subclone of *H. pylori*[20,21]. Antral and corpus isolates of these two patients had identical antibiotic susceptibility patterns. One patient had isolates which were sensitive to metronidazole, while the other had isolates which were resistant.

Of the two patients with subclones of *H. pylori* in the stomach, the one with metronidazole resistant isolates failed eradication therapy. Eradication therapy was successful for the other patient.

DISCUSSION

Helicobacter pylori is a difficult organism to eradicate. Most regimes that are in use today contain at least three drugs: two antibiotics, a proton pump inhibitor with or without bismuth. In spite of this, none of these regimes achieve 100% bacterial eradication.

The success of *H. pylori* eradication regimes depends on patient's compliance and bacterial resistance to the antibiotics used. Resistance to amoxycillin and tetracycline is rare. Clarithromycin resistance is still low among treatment naive patients, occurring in 2-3%[7,22-24]. Resistance to clarithromycin decreases the eradication rate of clarithromycin containing regimes[25]. None of the patients in this study harboured *H. pylori* that had primary resistance to clarithromycin.

In our study, 25% of patients were resistant to metronidazole. This is lower than the 60-62% reported previously by our colleagues from Changi General Hospital and the National University Hospital[6,7]. These reports, however, included patients who had been given eradication therapy before, and therefore had secondary antibiotic resistance. The present study included only treatment naive patients. Another possibility is that the current sample size is small, and the metronidazole resistance rate obtained here is not a true reflection of that in the general population.

Differences in the rates of *H. pylori* resistance to nitroimidazoles may partly be due to different technical approaches in the laboratory. Testing for nitroimidazole resistance in the laboratory has not been standardised worldwide. The redox potential of the environment is not controlled. A number of authors have shown that it is possible to influence the result by pre-incubating the plates in an anaerobic atmosphere[26]. This may have led to an apparent instability of nitroimidazole resistance. The exact redox potential at which the test should be carried out has not been determined yet. In our study, we controlled this variable by standardising the procedures for all isolates tested.

Differences may also arise when different methods are used to test for metronidazole sensitivity. The E test and the disk diffusion methods are most commonly used. A minority of reports have used agar dilution. While some have suggested that the disk diffusion method is unreliable and that the E test should be used instead[27,28], others have shown that it gives comparable results to the E test and even agar dilution[29,30].

Another problem that may contribute to these differences is the existence of bacteria with different metronidazole sensitivity in the same patient. It is interesting that co-infections with both resistant and sensitive bacteria have not been described for other antibiotics. Weel JF et al reported that among 156 patients, 33% had populations of *H. pylori* which were heterogeneous in their sensitivity to metronidazole[10]. In our study, 3 out of 16 patients (19%) with metronidazole resistance showed a mixed population of metronidazole resistant and sensitive strains in different locations in the stomach. Sampling from only a single location for culture and antibiotic sensitivity testing, may therefore result in an under estimation of the rate of resistance.

The eradication rate among our patients with metronidazole resistant strains is 56% which is very close to the 57% as reported by Buckley et al[31]. Most investigators have reported eradication rates of between 60-83%[23,24,32]. A possible reason for this difference is our small sample size. Most studies, especially multicentre ones, have a larger sample size and therefore greater statistical power. However, the present study and that by Buckley et al had 64 and 87 patients, respectively. Another factor that may have contributed to this difference is the method by which metronidazole sensitivity was determined. Both this study and the one by Buckley et al used the disc diffusion test. The larger multicentre studies have used the E test. The advantages of the E test over disc diffusion has been discussed above.

Nevertheless, the significant difference in the eradication rate between metronidazole sensitive and resistant strains is in agreement with most reports published so far. The MACH 2 study showed that when a regime containing omeprazole, metronidazole and clarithromycin was used, the eradication rate for metronidazole sensitive strains was 95% versus 76% for resistant strains[23]. A meta-analysis by van der Wouden

et al showed that the efficacy of PPI based triple therapy dropped from 93% (86-99%) in metronidazole sensitive strains, to 69% (60-79%) in metronidazole resistant strains[32]. However, some authors have reported contrary results. Despite metronidazole resistance rates which range from 11-38%, they have reported no difference in the eradication rates between metronidazole resistant and sensitive strains[22,24,33].

The eradication rate among patients harbouring *H. pylori* with different metronidazole sensitivities appears to be lower than among those with sensitive strains only. In our study population, the eradication rate is 67% (2/3) in the former versus 88% (42/48) in the latter. These are however small groups of patients, and work with larger groups will be needed.

In conclusion, metronidazole resistance causes a significant decrease in the eradication rate among patients given a nitroimidazole containing regime. An alternative regime should therefore be used whenever possible. However, if it becomes imperative that a nitroimidazole regime be used, for example, when the patient is allergic to alternative antibiotics, culture and sensitivity testing for *H. pylori* from multiple sites in the stomach would be useful. A longer course of treatment with a quadruple regime may then give better results, than the one week nitroimidazole containing triple therapy[32].

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