High Prevalence of *Helicobacter pylori* infection with Dual Resistance to Metronidazole and Clarithromycin in Hong Kong


# Contributed equally to this work

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Abbreviation used in this paper: MIC, minimal inhibitory concentration; ClaS, clarithromycin susceptible; ClaR, clarithromycin resistant; PCR, polymerase chain reaction; RAPD, random amplified polymorphic DNA.

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Summary

Background: Metronidazole resistance is a common problem in most Asian countries, and clarithromycin has been widely used in Hong Kong. We determined the prevalence of *H. pylori* strains resistant to metronidazole and clarithromycin in Hong Kong and assessed the effect on eradication rates. We also determined the genetic mutation in relation to phenotypic divergence in clarithromycin resistant strains.

Methods: *H. pylori* were cultured from gastric biopsies obtained from 87 patients during upper endoscopy and minimal inhibitory concentrations (MIC) of metronidazole and clarithromycin were determined by Etest and agar dilution methods. Mutations in clarithromycin resistant strains were identified by PCR and restriction analysis. RAPD fingerprinting were performed on clarithromycin resistant and susceptible isolates.

Results: The prevalences of *H. pylori* strains resistant to metronidazole and clarithromycin were 49.4% and 10.8% respectively in Hong Kong. Dual resistance to metronidazole and clarithromycin were found in 7.2% of patients. The agreement between Etest and agar dilution methods was determined by error-rate bounded analysis as 95.4% for metronidazole and 100% for clarithromycin, respectively. Dual resistant strains reduced the eradication rate to 66.7%. Among clarithromycin resistant strains tested, all were due to A2144G point mutation in 23S rRNA gene. RAPD fingerprinting suggested various phenotypically mixed populations.

Conclusions: The prevalence of metronidazole resistant *H. pylori* strains remained static while the prevalence of clarithromycin resistant strains was not rare in Hong Kong. An alarming 7.2% of patients were dual resistant to the antimicrobials and had a definite impact on treatment success. All cases of resistance to clarithromycin were due to A2144G mutation in 23S rRNA of *H. pylori*. Mixed population is another factor affecting the eradication rate.
INTRODUCTION

*Helicobacter pylori* infection is a major cause of chronic active gastritis and peptic ulcer disease and has been implicated in the development of gastric carcinoma \(^1\)\(^-\)\(^3\). Eradication of *H. pylori* provides the most effective treatment for peptic ulcer disease. This generally requires use of a combination of antimicrobial agents, typically two antimicrobial agents and a proton pump inhibitor \(^4\)\(^,\)\(^5\). Primary resistance to some of the most effective antimicrobials, metronidazole and clarithromycin, is commonplace in many societies and is of particular concern as the major reason for treatment failure \(^6\)\(^-\)\(^8\). The prevalence of *H. pylori* resistance to metronidazole, for example, ranges from 10-45% in the United States and Europe to more than 50% in many developing countries. Increasing prevalence of resistance to clarithromycin have also been reported \(^8\)\(^-\)\(^10\), and it has been suggested that the prevalence of resistance to metronidazole and clarithromycin has increased significantly in many countries in recent years \(^10\)\(^-\)\(^13\). Differences among studies in reported prevalence of metronidazole resistance are attributable, variously, to different and changing local patterns of metronidazole usage (typically against parasitic, anaerobic and other infections) in various societies, and thus inadvertent selection for metronidazole resistance, and also to methodological variations and difficulties in interpretation of susceptibility test results.

*H. pylori* infection and associated gastroduodenal diseases is a major problem in Hong Kong, and indeed throughout all of China. The prevalence of *H. pylori* infection in Hong Kong is around 60%; and the prevalence was much higher in certain areas of China, reaching around 80%\(^12\). Here we have: (i) determined the prevalence of *H. pylori* infections that are resistant to metronidazole, clarithromycin, or both, in patients with upper gastrointestinal diseases; (ii) compared the accuracy of the Etest and agar dilution methods for routine susceptibility testing of metronidazole and clarithromycin; (iii) determined the effect of antimicrobial resistance on eradication rates; (iv) determined the genetic mutation of clarithromycin resistant (ClaR) strains in Hong Kong; and (v) identify differences in genotype in relation to clarithromycin resistance.
METHODS

Patients and strains

Patients who satisfied the following inclusion and exclusion criteria were prospectively recruited into this study. Inclusion criteria included age between 18 and 80 years with symptoms of dyspepsia, and no previous antimicrobial therapy to eradicate H. pylori infection. Exclusion criteria included previous gastric surgery, any use of bismuth, antimicrobial agents, H2-receptor antagonists, proton pump inhibitors or sucralfate within four weeks prior to endoscopic examination, or any of several concomitant medical illnesses including cardiac, respiratory, renal and liver diseases. The study population was randomly selected from three clinical studies on non-ulcer dyspepsia, duodenal ulcer and gastric ulcer. The studies were approved by local institutional human research review committee. One biopsy from the gastric antrum was collected during upper endoscopy, and transported to the laboratory immediately. H. pylori was cultured from gastric biopsy specimens on selective media (Columbia agar with 7% horse blood and H. pylori selective supplement; Oxoid, Basingstoke, UK) under microaerophilic conditions produced by a gas-generating system (CampyGen™; Oxoid, Basingstoke, UK) for 3 to 6 days. H. pylori was identified by Gram staining and by positive urease, oxidase, and catalase tests. Suspensions of H. pylori were stored at -80°C in brain heart infusion broth (BHI; Oxoid, Basingstoke, UK) with 20% glycerol.

Preparation for antimicrobial sensitivity testing

H. pylori was subcultured on Columbia agar with 7% horse blood to ensure good growth. Suspensions in BHI, yielding a viable count of 3x10^8 cfu/ml (equivalent to 1 McFarland turbidity standard unit), were used as the inoculum for agar dilution and Etest susceptibility tests. H. pylori strain 26695 with six derivatives that were resistant to different concentrations of metronidazole, from the Berg laboratory, were included in each run to serve as controls.
**Agar dilution**

Metronidazole powder (Fluka, Switzerland) and clarithromycin powder (kindly supplied by Abbott Laboratories, Chicago, USA) were dissolved in distilled water and ethanol separately, and were subsequently diluted in distilled water according to NCCLS document M11-A2. Columbia blood agar plates containing metronidazole in concentrations ranging from 0.5 to 128 μg/ml or clarithromycin in concentrations ranging from 0.125 to 32 μg/ml were prepared. One microlitre of each isolate suspension was inoculated onto antimicrobial containing plates, to give a bacterial density of 3x10^5 CFU/spot. Plates with no antimicrobials were inoculated at the beginning and end of each run as growth controls. Plates were allowed to dry, and then incubated for 72 hrs at 37°C under microaerophilic conditions.

**Etest**

From each suspension, 100 μl were spread on surfaces of Columbia blood agar plates with a cotton swab. A single metronidazole or clarithromycin Etest strip (AB Biodisk, Sweden) was applied to the surface of each dried plate. After incubation in microaerophilic conditions at 37°C for 72 hours, results were read according to the manufacturer’s guidelines.

**Definitions of susceptibility**

After 72 hours incubation under microaerophilic conditions, the MICs were determined. The MIC was recorded as the lowest concentration that inhibited visible growth of organisms. For metronidazole, resistance was defined as an MIC of >8 mg/ml. For clarithromycin, resistance was defined as an MIC of >2 mg/ml.

**Detection of mutations to clarithromycin resistance in 23S rRNA genes**
**Strains isolation and DNA preparation**

Strains initially identified as clarithromycin resistant (ClaR) were grown on plates without clarithromycin for three days and subcultured on clarithromycin-containing plates (0.5 μg/ml) to obtain ClaR isolates. *Five single colonies were picked from the drug-free plate and susceptibility testings of clarithromycin were done.* Genomic DNA was prepared from confluent plate cultures of ClaR and ClaS isolates by the CTAB-phenol extraction method.  

**PCR amplification:**

A 613 bp segment containing the peptidyltransferase region of the 23S rRNA gene, which contains the known sites of mutation to clarithromycin resistance, was amplified with the primers "forward" (5'-GGCTCTTTTGAGTCCTTTAGGACAA-3') and "reverse" (5'-CTCCATAAGAGCCCAGGAGCTTTACTACT-3'). PCR was performed using 10 ng of genomic DNA, 10 pmoles of primer, 1 unit of Taq DNA polymerase, 2.5 mM each of dATP, dTTP, dCTP, dGTP with standard PCR buffer in a volume of 20 μl for 35 cycles, with the following cycling conditions: 94°C for 1 min; 65°C for 1 min and 72°C for 1 min. PCR amplified product was then purified by Qiagen PCR purification kit (Chatsworth, CA).  

**Restriction digestion of PCR products**

The two most common mutations to clarithromycin resistance (changes 2143 A to G and 2144 A to G) result in new sites for restriction endonucleases BbsI and BsaI, respectively. To test for these changes in ClaR mutants, eight microliters of purified PCR product were incubated with BsaI for 24 hours at 50°C, and separately with BbsI for 24 hours at 37°C. Products were then electrophoresed in 1% agarose gels.  

**RAPD fingerprinting**

Random amplified polymorphic DNA (RAPD) PCR was carried out in 25 μl containing 10
mM Tris-HCl (pH 8.3), 50 mM KCl, 4 mM MgCl₂, 0.001% gelatin, 20 ng of *H. pylori* genomic DNA, 20 pmoles of primer 1254 (5’-CCGCAGCCAA), 1 unit Amplitaq DNA polymerase and 250 mM each of dCTP, dATP, dGTP, dTTP for 45 cycles of: 94°C, 1 min; 36°C, 1 min; and 72°C, 2 min, in a Perkin-Elmer TC480 thermal cycler. After PCR, 8 µl aliquots of products were electrophoresed in 1% agarose gels containing 0.5 mg/ml ethidium bromide and photographed under UV light.

**Data Analysis**

The error rate bounded method of Metzler and DeHaan 18 was used to compare MIC of agar dilution with MIC of Etest. The chi-square test and Fisher’s exact test were used to compare the difference in prevalence of drug-resistant *H. pylori* strains between different gender, age group, and endoscopic diagnosis of patients, and differences in eradication rates. A *P* value of <0.05 was considered significant.

**RESULTS**

**Prevalence of Metronidazole and Clarithromycin Resistance**

Eighty-seven patients (42 males, mean age 47.5 years; 45 females, mean age 51.6 years) who satisfied the inclusion and exclusion criteria were included in this study. *H. pylori* isolates were obtained from antral biopsy specimens of each of them. Endoscopic evaluation of the patients revealed 50 subjects with gastritis, 29 with duodenal ulcer, 7 with gastric ulcer, and 1 with gastric and duodenal ulcers. Of the 87 *H. pylori* isolates, 83 gave the same results by agar dilution and Etest methods. Resistance to metronidazole was found in 49.4% (41/83) isolates and resistance to clarithromycin in 10.8% (9/83). Six of the nine (7.2%) strains resistant to clarithromycin were also resistant to metronidazole (Table 1). Among these six patients infected with strains resistant to both metronidazole and clarithromycin, three were males and three were females. Endoscopic exam showed that three patients suffered from duodenal ulcer and three from chronic gastritis.
No significant differences in resistance rates to metronidazole and clarithromycin were seen between men and women (p>0.05). However, prevalence of metronidazole resistance was higher in strains from 18-40 year old patients (70.8%, 17/24) than from 41-60 or 61-80 year old patients (44.7%, 17/38; and 33.3%, 7/21, respectively) (p<0.05). Resistance to metronidazole was slightly higher in patients with duodenal ulcer than with chronic gastritis, but this difference was not significant (p>0.05). No differences were found in the prevalences of clarithromycin resistance among patients of different age groups or among patients with different diseases (p>0.05; Table 2).

**Comparison of Etest and agar dilution results for metronidazole and clarithromycin**

For many organisms, tests for colony formation by bacterial suspensions on agar containing defined levels of an antimicrobial (agar dilution) can be used as “gold standard” against which other methods of drug susceptibility testing can be judged. When resistance and susceptibility of *H. pylori* to metronidazole determined by agar dilution and Etest were compared, there was 95.4% (83/87) category agreement using the breakpoint of <=8 μg/ml as susceptible (Figure 1). Etest gave 2.3% (2/87) major error (resistant by the Etest and susceptible by the agar dilution method) and 2.3% (2/87) very major error (susceptible by the Etest and resistant by the agar dilution method).

The clarithromycin Etest, in contrast, correlated perfectly with the clarithromycin agar dilution, when using the breakpoint of <=2 ug/ml as susceptible (Figure 2); 100% (87/87) agreement, with no major error and very major error.

**Mutation in 23S rRNA gene in clarithromycin resistant strains**

Only five strains (strains 1, 2, 3, 4, 5) that were initially classified as clarithromycin resistance were able to grow on medium with clarithromycin (0.5 μg/ml), the other four strains (strains 6, 7, 8, 9) were likely to have mixed resistant and sensitive strains, with the resistant
colonies not surviving. Strain 4 was about 50% ClaR, whereas for strains 2 and 5, only about 5% isolates showed ClaR. All these five ClaR strains had the new BsaI site which is characteristic of the A2144G point mutation in 23S rRNA gene (Figure 3). None had a new BbsI cleavage site which is characteristic of the other relatively common mutation (A2143G).

For the mixed infection, defined as identification of both ClaR and ClaS single colonies from a single patient (strains 2, 4, 5), ClaR and ClaS colonies were isolated and RAPD fingerprinting were performed. Different RAPD patterns were found between ClaR and ClaS colonies for strain 4, whereas the identical RAPD patterns were observed between ClaR and ClaS colonies for strains 2 and 5 (Figure 4).

**Eradication rate and resistance**

Eighty of the 83 patients included in this study received triple therapy containing both metronidazole and clarithromycin, together with either omeprazole or ranitidine bismuth citrate. At 4 wks after the completion of treatment, 6 patients defaulted follow-up endoscopy and hence evaluation of compliance and treatment success was not possible. 74 patients fulfilled the follow-up endoscopy with biopsies and C\textsuperscript{13} urea breath test. Successful eradication was confirmed by negative results in all three diagnostic tests: rapid urease test, histology, and C\textsuperscript{13} urea breath test. Table 3 illustrated the effect of metronidazole and clarithromycin resistance on treatment efficacy. There was a higher eradication rate in those with dual susceptible strains (33/35, 94.3%) than those with dual resistant strains (4/6, 66.7%) (p=0.095). Resistance to metronidazole or clarithromycin alone did not significantly affect the eradication rates (90.3% and 100% respectively) compared with dual susceptible strains (94.3%).

Strains 2 and 5 were both dual resistant strains. The resistant strains contributed about 5% of the strain population (see above). Eradication was successful for strain 2 but not strain 5. Strain 4 was sensitive to metronidazole and the ClaR strains contributed about 50% of the strain population. Eradication was successful for strain 4.
DISCUSSION

The increasing prevalence of strains resistant to some of the most commonly used antimicrobial agents is an important cause for failures to eradicate *H. pylori* infections. Therefore, routine antimicrobial susceptibility testing of *H. pylori* by simple, inexpensive and standardized methods is recommended especially for patients who repeatedly fail treatment. In this study, the susceptibility of 87 *H. pylori* isolates to metronidazole and clarithromycin in vitro was determined by agar dilution and Etest methods, with generally excellent agreement (95% for metronidazole; 100% for clarithromycin). Therefore Etest is suitable for testing *H. pylori* resistance to antimicrobials, being simple to perform, less affected by bacterial inoculum size than agar dilution\textsuperscript{19} and ready to test single isolates as needed.

The present study showed that primary resistance of *H. pylori* to metronidazole and clarithromycin were found in 49.4% and 10.8% of patients respectively in Hong Kong. This primary resistance rate for metronidazole was similar to that observed over the past three years in this area\textsuperscript{20}, and further supported that the prevalence of metronidazole resistant *H. pylori* strains in Hong Kong was somewhere between that of the developed and the developing countries\textsuperscript{9,21,22}. Increased prevalence of resistance to metronidazole in patients in Hong Kong from 22% to 73.2% had been noted by a group from another center in Hong Kong from 1991 to 1996\textsuperscript{23}, whereas we did not observe such trend over the past three years (1996-1998). Traditionally metronidazole has been prescribed in Hong Kong, as in many other societies, for the treatment of gynecological infection. It is also widely used for treatment of anaerobic infections and amoebiasis in men and women, and no significant differences in the rate of metronidazole resistance were found between men and women in our study.

The prevalence of resistance to clarithromycin is generally much lower than that to metronidazole, although still very significant from a public health perspective: for example, less than or equal to 15% in Europe\textsuperscript{9}, 9.1% in Japan\textsuperscript{8}, and 6.1-12.6% in the United States\textsuperscript{10,24}. The resistance to clarithromycin observed in our study was 10.8% and was consistent with the data
reported worldwide. Although the overall prevalence of primary resistance to clarithromycin was still low, the increase in secondary resistance over a short period of time was worrisome. The emergence of ClaR strains after ineffective treatment against *H. pylori* is easily observed and we also found that more than 90% isolates cultured after clarithromycin-based treatment were resistant to the drug (data not shown). However, most of the resistance found in patients may reflect a history of usage of macrolides by them or their close contacts for other illnesses, generally at doses sufficient to select for new ClaR mutants (i.e. at doses that impair growth of normal wild type *H. pylori*, but that do not fully eradicate infection). Especially in areas of high prevalence of primary metronidazole resistance, metronidazole resistance could reduce efficacy of triple therapy and leads to secondary clarithromycin resistance, and these dual resistant strains are difficult to eradicate.

Most or all clarithromycin resistance is though to involve base substitution mutations in 23S rRNA at sites critical for macrolide interaction. We also confirmed that all the strains identified as ClaR were due to A2144G point mutation in 23S rRNA gene in our study. However, a study reported that clarithromycin resistance was not stable in 45% of normally ClaR strains of *H. pylori* in vitro and this phenomenon was also observed in vivo. In the present study, we found that for the nine strains that were initially confirmed as ClaR, three strains were mixed populations with ClaR and ClaS colonies and four strains lost the resistant population after storage and transportation. The possible reason might be that these four strains were also mixed populations, with a large quantity of ClaS colonies and a small quantity of ClaR colonies. The ClaS colonies showed a competitive growth advantage over the ClaR colonies, and the ClaR colonies did not survive after initial storage. Using a multiplex sequence analysis, Wang et al confirmed the decreased vigor of growth resulting from some other types of mutations (not A to G mutation) to clarithromycin resistance. Furthermore, the instability of clarithromycin resistance might also be attributed to the two copies of 23S rRNA per *H. pylori* genome. Mutation in one copy seems sufficient to confer clarithromycin resistance in certain strains.
For the three strains confirmed as mixed populations, RAPD fingerprinting of isolates from ClaR and ClaS colonies were compared. Different RAPD fingerprints were found in one patient (strain 4), meaning that the patient was infected with two different strains, one ClaR and one ClaS. An identical RAPD fingerprint was found for the other two patients (strains 2 and 5), suggesting that these patients were infected with a single strain, with part of the colonies acquiring resistance to clarithromycin via point mutation in 23S rRNA gene. Successful treatment was found in strains 2 and 4 but not strain 5. Several important points related to clarithromycin resistance were illustrated here. Mixed infections in relation to clarithromycin susceptibility were common in Hong Kong, as opposed to the study by Dore et al who showed that all isolates (multiple and single colonies) from ClaR patients were purely resistant \(^{29}\). Strain 4 showed 50% ClaR and 50% ClaS population with a different RAPD pattern, suggesting that a mixed infection of at least two strains co-existed in the stomach. Strains 2 and 5 had the same RAPD pattern, with the ClaR population contributed only 5% of the population. One of the strains was eradicated while one was not. This supported the hypothesis that acquired resistance may actually represent emergence of resistant organisms that were already present in the stomach rather than actual development of resistance \(^{30}\). Recently, another study reported that treatment failure could be due to either a single strain that had acquired resistance to clarithromycin, or due to presence of different strain types of *H. pylori* \(^{31}\). Hence from our finding and others, the outcome of treatment did not depend only on the acquired clarithromycin resistance. Pre-treatment work up for clarithromycin susceptibility may be necessary to understand better the clinical correlation with in vitro susceptibility. More studies need to be done in regard to clarithromycin, and in conjunction with metronidazole susceptibility.

Dual resistance to metronidazole and clarithromycin might become a major problem in *H. pylori* eradication. Although in the present study and in some other studies \(^{32,33}\), high eradication rates were achieved by treatment regimens containing both metronidazole and clarithromycin even for metronidazole resistant strains, the rate decreased dramatically for ClaR strains. In our
study, six of 87 patients (7.2%) had dual resistant infections, despite their absence of prior treatment for *H. pylori* infection. This dual resistance would certainly affect the success of treatment, with only 66.7% of patients being eradicated. In countries where metronidazole resistance is common, it may be advisable not to use the combination of metronidazole and clarithromycin as the first line treatment, to avoid the emergence of more dual resistant strains.

Furazolidone has shown promising results in first line treatment, with the absence of furazolidone resistance so far. However, the drug should not be used in areas endemic for G-6-PD deficiency, like Hong Kong and South China, for the high prevalence of G-6-PD deficiency was reported in 4.4% of males and 0.35% of females 

In conclusion, our data indicated that around half of *H. pylori* infections in Hong Kong were metronidazole resistant, and that clarithromycin resistance was also disturbingly common. The high prevalence of dual resistance needs special attention and new therapeutic approaches.
ACKNOWLEDGEMENT

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REFERENCES


Figure Legends

Figure 1. Error-rate bounded analysis of metronidazole Etest MIC versus agar dilution MIC for 87 *H. pylori* strains.

Figure 2. Error-rate bounded analysis of clarithromycin Etest MIC versus agar dilution MIC for 87 *H. pylori* strains.

Figure 3. Detection of the point mutation at 2144 (A to G) in 23S rRNA gene by *BsaI* digestion of PCR products. Lane M: 100 bp DNA ladder (Gibco BRL, Gaithersburg, MD, USA). Lane P: point mutation positive control; Lane N: point mutation negative control. Lane 1-5: strains still resistant to clarithromycin after preservation and transportation. Strains 1-3 each had A2144G point mutation. Strain 4 was a mixed infection with some colonies having A2144G point mutation and some colonies don’t. Only 5% of colonies from strain 5 were confirmed as resistant to clarithromycin. DNA was prepared from the ClaR colonies and the A2144G mutation was detected by digesting with *BsaI* repeatedly (data not included in this figure). Lane 6-8: strains sensitive to clarithromycin after preservation and transportation, no A2144G point mutation was found.

Figure 4. RAPD fingerprinting of ClaS and ClaR colonies from strains 2, 4, and 5. M: 1kb DNA ladder (Gibco BRL, Gaithersburg, MD, USA); S: isolates from ClaS colonies; R: isolates from ClaR colonies. Strains (2, 4, 5) corresponding to that of figure 3. Different RAPD patterns were found for strain 4, meaning that this patient had a mixed infection of two different strains with one ClaS and one ClaR. Identical RAPD patterns were found for ClaS and ClaR isolates from strains 2 and 5.
<table>
<thead>
<tr>
<th>No. of Patients</th>
<th>Metronidazole</th>
<th>Clarithromycin</th>
<th>Resistance Rate (%)</th>
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<tr>
<td>39</td>
<td>S</td>
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<tr>
<td>6</td>
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S=susceptible, R=resistant

**Table 1.** Results of susceptibility test of 83 *H. pylori* isolates to metronidazole and clarithromycin
Table 2. Prevalence of metronidazole and clarithromycin resistance of 83 *H. pylori* isolates according to their clinical information

<table>
<thead>
<tr>
<th>Clinical Information</th>
<th>metronidazole resistance (%)</th>
<th>p value</th>
<th>clarithromycin resistance (%)</th>
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**Table 3. H. pylori eradication rate and metronidazole / clarithromycin resistance**

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<th>Eradication rates</th>
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<th>Mtz resistant</th>
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<td>33/35 (94.3%)</td>
<td>28/31 (90.3%)</td>
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<td>Cla resistant</td>
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<td>4/6 (66.7%)</td>
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<td>Total</td>
<td>34/36 (94.4%)</td>
<td>32/37 (86.5%)</td>
<td>66/73 (90.4%)</td>
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Mtz: metronidazole  
Cla: clarithromycin
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<td>3</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
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<th>&lt;0.25</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>16</th>
<th>32</th>
<th>64</th>
<th>128</th>
<th>&gt;256</th>
<th>Etest MIC (ug/ml)</th>
</tr>
</thead>
</table>

Figure 1
\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Figure 2}
\end{figure}