Molecular detection of genotypic clarithromycin-resistant strains and its effect on the eradication rate of concomitant therapy in *Helicobacter pylori* infection

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Abstract

Antimicrobial eradication rates for *Helicobacter pylori* have been decreasing and the reason for treatment failure was found to be resistance to one or more of the antibiotics. Clarithromycin resistance to *H pylori* was associated with point mutations in the 23S rRNA gene and the PCR-RFLP method can detect these point mutations. The aim of this study was to determine the molecular detection of genotypic clarithromycin-resistant strains and its effect on the eradication rate of concomitant therapy in *H pylori* infection. The presence of *H pylori* DNA was confirmed by amplifying the UreC gene by polymerase chain reaction (PCR) and point mutations on 23S rRNA (A2142G and A2143G) were detected by PCR-RFLP. A total of 98 *H pylori*-infected patients were involved and among them, genotypic clarithromycin-sensitive strain was 93.9% and clarithromycin-resistant strain was 6.1%. All patients were found to have the A2143G point mutation but A2142G was not detected. Successful eradication rate of concomitant therapy was found to be 89.8% and unsuccessful rate was 10.2%. Among patients with the clarithromycin-resistant gene, only 16.7% had successful eradication and 83.3% had unsuccessful eradication. There was a statistically significant association between failure rate of concomitant therapy and detection of clarithromycin-resistant genes ($P < 0.01$). The presence of A2143G point mutation in the clarithromycin-resistant strain has a negative effect on the eradication rate of *H pylori* infection.

1 | INTRODUCTION

*Helicobacter pylori* infection, the most common chronic bacterial infection in the world, is linked to peptic ulcer disease and gastric cancer and is a key constituent of the human microbiome. They are unique bacteria ideally suited to live within the acidic environment of the human stomach, their spiral shape and multiple unipolar flagella allow them to manoeuvre freely through the gastric mucous layer in a microenvironment protected from low gastric pH. 

Among South-East Asian countries, the reported seroprevalence rate in volunteer blood donor was 35.9% in Malaysia, 31% in Singapore and 57% in Thailand. In Myanmar, the prevalence of *H pylori* infection among asymptomatic Buddhist monks was 65.4% by urea breath test, 78.6% in dyspeptic patients by rapid urease test and histology, 77.6% in dyspeptic patients by rapid urease test only and 30% by culture, respectively. One of the community studies reported that the overall seroprevalence of *H pylori* was 68.8%.
The antimicrobial eradication rates for H. pylori have been decreasing and the most likely primary reason for treatment failure were found to be H. pylori resistance to one or more of the antibiotics and patient compliance. The rates of antibiotic resistance for H. pylori are increasing throughout the world and they vary geographically and are higher in developing countries. Treatment success rates can vary among countries and regionally within countries, related to antibiotic resistance and local ecology. The variability of the clarithromycin-resistant rate of H. pylori seen in several regions emphasises the necessity to look at resistance rates in each geographical area to better guide treatment regimens. World Health Organization recently announced that the emergence of clarithromycin-resistant strains can cause global H. pylori treatment failure. In ASEAN countries the prevalence of clarithromycin resistance rate varied from 2% to over 30%.

The efficacy of current conventional clarithromycin-based triple therapy has decreased to an unacceptably low level worldwide (lower than 80%). An earlier study found that the clarithromycin resistance rate in Myanmar was 12.5% and a second study in 2015 showed an increased to 63.6% by using the Episilometer test in Myanmar.

In a recent intention-to-treat analysis, the eradication rate of both clarithromycin and levofloxacin was unacceptably low at 40% and 43.7%, respectively. Therefore, quadruple therapy is the method of choice for H. pylori eradication in Myanmar. As bismuth is locally not available, we used non-bismuth quadruple therapy (concomitant). The eradication rate of concomitant therapy was 92.2% in Myanmar, 77% in India and 91.7% in Spain.

Resistance to clarithromycin, an antibiotic in the treatment of H. pylori infection, is due to a lack of binding of the drug to the 23S rRNA of the bacterial ribosome, which is caused by the occurrence of point mutations in the variable domains of the peptidyl transferase of 23S rRNA. Point mutations in the 23S rRNA gene, mainly at positions 2142 and 2143 with a transition of A to G, are responsible for the resistance. The basic assay, first described in 1996, utilises a PCR-RFLP approach in which the region of the gene containing the mutations is amplified and then digested with restriction endonucleases that cut specifically at the mutation sites. A significant development was the invention that the PCR-RFLP assay might be successfully applied to the evaluation of clarithromycin resistance without culture by direct analysis of DNA extracted from gastric biopsies. Its culture, as well as antimicrobial susceptibility studies are difficult to perform as well as labour intensive.

Although the clarithromycin-resistant rate was variable in Myanmar, its effect on eradication rate of H. pylori infection has not been evaluated. This study set out to determine the molecular detection of genotypic clarithromycin resistance strains and its effect on the eradication rate of concomitant therapy in H. pylori infection. This study may have the potential to provide data for national survey for H. pylori infection.

2 MATERIALS AND METHODS

This hospital-based cross-sectional analytic study was conducted between October 2019 and October 2020 at the Gastroenterology Unit, No. (2) Military Hospital (500-bedded) and the Defence Services Medical Academy, Yangon. Inclusion criteria were patients who were found to have H. pylori infection detected by rapid urease test, age more than 18 years and both sexes. Patients who had taken proton pump inhibitors in the prior 2 weeks, antibiotics and bismuth in the prior 4 weeks, allergy to amoxicillin, tinidazole and clarithromycin and those who did not give informed consent were excluded.

2.1 Patient enrolment

Dyspeptic patients indicated for upper GI endoscopy who attended the outpatient clinic or admitted to the Gastroenterology Department were recruited. Two pieces of gastric mucosa from the antrum and the middle of the body were taken for detection of H. pylori by rapid urease test (proton dry test) from all enrolled patients. The tissue samples were put into a Pronto Dry well. The result was obtained after 20 minutes. An additional one-piece tissue biopsy from the antrum was also taken for detecting the clarithromycin-resistant gene by PCR-RFLP. If the patient with RUT test was negative, he or she was excluded from the study and the biopsy sample for molecular analysis was discarded according to the standard procedure of the Gastroenterology Department. If RUT turned out to be positive, biopsy for clarithromycin-resistant gene by RFLP was stored at −20°C in a freezer at the Post Graduate Common Research Laboratory. DNA was extracted within 12 hours followed by molecular tests for detection of the clarithromycin-resistant gene.

2.2 DNA extraction and genotyping

The DNA was extracted from biopsy specimens from the gastric antrum using a PureLink Genomic DNA Mini Kit (Invitrogen) and DNA quality and quantity were checked by gel electrophoresis and a Nanodrop spectrophotometer (BioSpec Nano).

A 294-bp fragment of the UreC gene of H. pylori was amplified with oligonucleotide primers Forward primer 5′-AACCTTTAGGGGTAGGGTTT-3′ and Reverse primer 5′-AAGCTTACTTTCTAACACCTAGC-3′. Amplification was performed with the Biometra TAdvanced thermal cycler (Analytik Jena) in a 25-μL reaction volume containing 100 ng of DNA, 0.5 μM of each oligonucleotide primer, 12.5-μL of GoTaq®Green Master Mix, 2X (Promega). PCR conditions were as follows: 40 cycles of PCR consisted of initial denaturation held at 95°C for 5 minutes, denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds, extension at 72°C for 30 seconds and a final extension at 72°C for 5 minutes. PCR products were checked by a 1.5% agarose gel.
For 23S rRNA gene amplification, total of 40 cycles of PCR consisted of initial denaturation held at 95°C for 5 minutes, denaturation at 95°C for 30 seconds, annealing at 56°C for 30 seconds, extension at 72°C for 30 seconds and a final extension at 72°C for 5 minutes. PCR product of 135 bp was amplified with the forward primer 5′-AGTGGAGGTGAAAATTCC-3′ and the reverse primer 5′-TAAGAGCCAAAGCCCTTAC-3′. These primers were designed from the published sequence of *H pylori* (GenBank accession number U27270.1).

For RFLP analysis, amplification products were digested with 10U of MboII and BsaI restriction endonucleases (New England Biolabs) according to the manufacturer’s instructions and subjected to electrophoresis in a 2% agarose gel.

### 2.3 Helicobacter pylori eradication therapy

All *H pylori*-positive patients were treated with concomitant therapy (amoxicillin 1G, clarithromycin 500 mg, tinidazole 500 mg, pantoprazole 40 mg, all given twice daily) for 14 days. The patients were offered medication and explained about the dose, timing and duration of all prescribed drugs. They were trained to write whether or not they took their prescribed medications and any adverse events they experienced. Patients were asked to visit the investigator on days 1 and 15. Thereafter, resolution of presenting symptoms and drug-related adverse effects were assessed and compliance was checked by calculating pill count. At the 4th week after *H pylori* therapy, successful *H pylori* eradication was detected by stool antigen test. Rapid urease test was not used for confirmation of *H pylori* eradication in our study because not every patient was not indicated to do recheck endoscopy.

### 2.4 Data analysis

Data were analysed using IBM SPSS version 22. The Fisher’s exact tests were calculated for finding the associations between clarithromycin-sensitive and -resistant *H pylori* strains, age, sex and endoscopic findings. The chi-squared test was used for comparing the eradication rates in clarithromycin-sensitive and -resistant *H pylori* strains after giving concomitant therapy.

### 2.5 Ethical considerations

Ethical clearance was obtained from the Ethical Committee of the Defence Services Medical Academy.

### 3 RESULTS

A total of 171 patients (Figure 1) were enrolled in this study. Among them, 102 patients (59.6%) were found to have *H pylori* infection and
69 patients were negative for *H pylori* infection and four patients were dropped out. Therefore, 98 patients were included. The mean age of the study population was 52.68 ± 12.31 years. Regarding sex distribution, 55 female patients (56.1%) and 43 male patients (43.9%) were included and female to male ratio was 1.2:1. Regarding the endoscopic findings, the majority (79.6%) were diagnosed with gastritis mostly antral predominant whereas minority were gastric ulcer (9.2%) and duodenal ulcer (7.1%) and normal finding (4.1%).

Regarding the distribution of genotypic clarithromycin-resistant and -sensitive *H. pylori* strains in the study population, 92 patients (93.9%) were found to have clarithromycin-sensitive strain and six patients (6.1%) had clarithromycin-resistant strain (Table 1). All patients (100%) were found to have the A2143G point mutation whereas none of A2142G point mutation was detected in this study. Among 92 patients of genotypic clarithromycin-sensitive status, the majority (53.3%) were found in the 41- to 60-year age group followed by over 60 years (28.3%) and the 18- to 40-year age group (18.4%). Among six patients of genotypic clarithromycin-resistant status, the most commonly found age group was between 41 and 60 years (66.6%). The association between genotypic clarithromycin strains and age distribution was not statistically significant in this study (P = 0.859).

In our study, among 92 patients of genotypic clarithromycin-sensitive strain, 45.7% were male and 54.3% were female. Among six patients of genotypic clarithromycin-resistant strain, 16.7% were male and 83.3% were female. The association between genotypic clarithromycin strains and sex distribution were not statistically significant (P = 0.226).

Among 92 patients with genotypic clarithromycin-sensitive strain, the majority (79.4%) were found to have gastritis followed by 8.7% with gastric ulcer, 7.6% with duodenal ulcer and 4.3% with normal endoscopic finding. Among six patients with genotypic clarithromycin-resistant strain, 83.3% had gastritis and 16.7% were found to have gastric ulcer. The association between genotypic clarithromycin strains and endoscopic findings was not statistically significant (P = 0.756).

Regarding *H pylori* eradication with concomitant therapy, 89.8% were found to have successful eradication and 10.2% had unsuccessful eradication. In clarithromycin-sensitive status of eradication, successful eradication rate after concomitant treatment was 94.6% and unsuccessful eradication was 5.4%. In clarithromycin-resistant status, only 16.7% achieved eradication. The association between the eradication rate of *H. pylori* infection after using concomitant therapy and the presence of clarithromycin-resistant strain was statistically significant (P < 0.01) (Table 2).

## Table 1: Distribution of genotypic clarithromycin-resistant and -sensitive *Helicobacter pylori* strains among study population (n = 98)

| Genotypic classification | Number of patients | Percent |
|--------------------------|--------------------|---------|
| Resistant strain         | 6                  | 6.1     |
| Sensitive strain         | 92                 | 93.9    |
| Total                    | 98                 | 100     |

## Table 2: Comparison of eradication rates in clarithromycin-sensitive and -resistant *Helicobacter pylori* strains after giving concomitant therapy (n = 98)

| Genotypic clarithromycin resistance status | *Helicobacter pylori* eradication | Fisher’s exact test | P value |
|-------------------------------------------|----------------------------------|---------------------|---------|
|                                           | Successful                       | Unsuccessful        | Total   |                     |
| Clarithromycin-sensitive                  | 87 (94.6%)                      | 5 (5.4%)            | 92 (100%) | 37.303              | <0.01 |
| Clarithromycin-resistant                  | 1 (16.7%)                       | 5 (83.3%)           | 6 (100%)  |                     |       |
| Total                                     | 88                               | 10                  | 98 (100%) |                     |       |

4 | DISCUSSION

The antibiotic resistance to *H pylori* infection has been increasing worldwide. The prevalence of *H pylori* infection is high at 77%5 and 48%21 in dyspeptic patients and 67% in asymptomatic monks.3 Our study showed *H pylori* infection rate was 59.6% which indicate that the infection is still prevalent in Myanmar.

Along with the high prevalence, antibiotics resistance to *H pylori* has been increasing particularly metronidazole and clarithromycin. Metronidazole resistance was also reported from 54% to 100% from 20056 to 2014.12 It is not surprising that metronidazole is the most commonly used self-medicating antibiotic, which is easily available as an over-the-counter medication. Therefore, metronidazole-containing triple therapy was no longer used for *H pylori* eradication in Myanmar. Previous studies reported an increase in clarithromycin resistance from 10.4% to 63.6% within a decade.6,13 These data are in line with ASEAN data where the clarithromycin resistance rate was varying from 43% in Cambodia to 2% in the Philippines.12 However, a recent study in 2018 using culture and molecular method showed no clarithromycin resistance in Myanmar.14 It is a fact that the different results of these studies on clarithromycin resistance should be a considerable factor for the current local guidance of *H pylori* eradication. The data indicates the trend towards quadruple therapy (bismuth-containing quadruple therapy or concomitant therapy) rather than clarithromycin-containing triple therapy for *H pylori* eradication.

In the previous Myanmar clarithromycin resistance studies, the earlier study in 20055 examined PCR-confirmed infections and the latter study in 201413 examined 200 gastric biopsies and found
H. pylori in 30 patients by Epsilometer test (E test). A culture-based method is difficult as H. pylori is a fastidious organism capable of growing only under a narrow set of conditions. Resistance to clarithromycin of H. pylori was associated with point mutations of the V domain of the 23S rRNA gene in the 50S subunit that decreases the affinity of drug binding to the ribosome, and PCR-RFLP method has been developed to detect these point mutations. PCR-RFLP method has expressed a reliable assay allowing cost-effective and rapid detection of clarithromycin-resistant strains. The diagnostic accuracy of PCR-RFLP was 94% in the study done by Kuo et al. Where expertise and facilities resources are available, the methods of molecular detection yield results on clarithromycin resistance within a day of endoscopy. It is considered to be a useful test before choosing optimal therapy for H. pylori eradication.

In our study, all of the study subjects were exhibited A2143G type of mutation in strains. A similar mutation was found in other studies and this point mutation may contribute to the treatment failure. None of the subjects with clarithromycin-resistant H. pylori strains were showed A2142G mutation, which is comparable to the Vietnam study. A low prevalence (2.9%) of genotypic clarithromycin resistance was reported as the use of macrolides, in general, has not been widespread in Malaysia. The lack of A2142G mutation in our study somehow can explain why resistance to clarithromycin is low in Myanmar. Therefore, continued use of clarithromycin as a first-line drug in the treatment and eradication of H. pylori infection might be considered. However, a multicentre study is still necessary to clarify the presence of other mutations among H. pylori infection in the country.

Age plays an important role in the distribution of antibiotic resistance. Ji et al found that the clarithromycin-resistant rate was 16.67% in patients below 20 years, and the rate increased from 14.29% in 2013 to 21.62% in 2014. The highest resistance rate was 23.02%, which was found in patients aged 71–80 years. In contrast, the highest rate was 66.6% in patients aged between 41 and 60 years in our study. The association between genotypic clarithromycin status and age distribution was not statistically significant in our study (P = 0.859) which is similar to the study done by Gehlot et al. This may be partially explained by a long history of clarithromycin consumption resulting in an extremely high rate of mutations.

In our study, female patients had a higher incidence of the A2143G clarithromycin-resistant genotype than male patients, a finding similar to that observed in the studies of Eghbali et al and Park et al. Females are preferentially infected with the H. pylori strain with the A2143G mutation, and thus the point mutation can contribute to the eradication failure. A difference in gastric physiology between males and females is another explanation.

Our study used clarithromycin as a concomitant therapy since the eradication rate of clarithromycin triple therapy was below 70% in a previous study. The eradication rate of concomitant therapy was 89.8%, which is similar to the findings of other two Myanmar studies where the eradication rate was 92.2% and 93%, respectively, and those of studies in different countries. Therefore, concomitant therapy of H. pylori infection appears to be safe, effective and well-tolerated with minor adverse effects.

In the present study, successful eradication rate after concomitant treatment was 94.6% and unsuccessful eradication rate was 5.4% in patients with clarithromycin-sensitive group, whereas successful eradication rate after treatment was only 16.7% and unsuccessful eradication rate was 83.3% in the clarithromycin-resistant group. Therefore, the presence of A2143G point mutation seems to play a main role in the effect of H. pylori eradication on concomitant therapy. The eradication rate of H. pylori infection with concomitant therapy was low in clarithromycin-resistant patients. Similarly, Ong et al found that the eradication rates of concomitant therapy fell to 71.2% when mutation with either A2143G, A2142G or both were found. The alternative regimen rather than concomitant therapy should be considered in individuals with point mutation to clarithromycin resistance.

In the present study, the low clarithromycin-resistant rate in H. pylori infection may be due to low macrolide exposure. Based on the study results, resistance rate of H. pylori to clarithromycin was low (only 6.1%) and eradication rate of concomitant therapy was high (89.8%), so this concomitant therapy should be a suitable choice as the first-line treatment of H. pylori infection in the future.

5 | CONCLUSION

In the present research, the rate of clarithromycin-resistant H. pylori genotype was low and was mainly associated with the A2143G point mutation. However, A2142G point mutation was not detected. According to our results, clarithromycin-containing H. pylori eradication therapy might still be recommended as empirical therapy in Myanmar.

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ETHICAL STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and ethical approval was obtained from the Ethics Review Committee of the Defence Services Medical Academy.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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