Helicobacter pylori 23S rRNA gene A2142G, A2143G, T2182C, and C2195T mutations associated with clarithromycin resistance detected in Sudanese patients

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Abstract

Background: Clarithromycin resistant *Helicobacter pylori (H. pylori)* strains represent a worldwide health problem. These stains are usually carrying mutations within the 23S rRNA gene associated with clarithromycin resistance. This study aimed to detect *H. pylori* and clarithromycin resistant associated mutations from Sudanese patients with gastritis symptoms.

Materials and Methods: Two hundred and eighty-eight gastric biopsies were collected using gastrointestinal endoscopy from patients with gastritis symptoms in different hospitals in Khartoum state. *H. pylori* was detected by PCR using primers targeting 16S rRNA and 23S rRNA. Then allele-specific PCR and DNA sequencing were used to screen for the presence of A2142G and A2143G point mutations.

Results: Out of 288 samples, *H. pylori* was detected in 97 (33.7%) sample. Allele-specific PCR detected the variant A2142G in 9/97 (9.3%) sample, while A2143G mutation was not found in any sample. The DNA sequencing revealed the presence of mutations associated with clarithromycin-resistance in 48% (12/25) of samples; the A2142G was present in one sample, A2143G in 5 samples, T2182C in 4 samples, and C2195T in 3 samples. There was no association of 23S rRNA gene point mutations with gender, age group and geographical distribution of patients.

Conclusion: This study revealed a high frequency (48%) of mutations associated with clarithromycin resistance using DNA sequencing of the 23S rRNA gene's V domain. This information should be taken into consideration before choosing optimal therapy for *H. pylori* eradication.

Introduction

*Helicobacter pylori (H. pylori)* is an exceptional bacterium in its ability to create permanent stomach colonization in untreated humans. Multiple factors contribute to the characteristic gut colonization, inflammation, alteration in the production of gastric acid, and tissue destruction caused by *H. pylori* ¹. The mechanisms by which *H. pylori* causes mucosal inflammation and damage are not well described but have both bacterial and host factors likely to be involved. Toxins and lipopolysaccharides produced by bacteria can damage the mucosal cells, and the ammonia released by the action of urease can also directly harm the cells ². In many developing countries, the infection rate has been reported to be as high as 70–80% ³. *H. pylori* is responsible for more than 80 percent of peptic ulcer diseases, and 95% or more of duodenal ulcers ⁴. Diagnostic testing is typically divided into invasive (endoscopic) and non-invasive approaches. The invasive diagnostic method involves endoscopic imaging, histology, rapid urease examination, culture, and molecular techniques. Non-invasive diagnostic tests include breathing tests for urea, antigen check for stools, and serological tests ⁵.

Elimination of *H. pylori* is based on successful treatment with a proton pump inhibitor (PPI), such as omeprazole, lansoprazole, and rabeprazole, and at least two antibiotics of clarithromycin (CLR), metronidazole (MTZ), amoxicillin (AMX), and tetracycline (TET). Combination therapy consisting of a PPI,
CLR, and either AMX or MTZ for up to 14 days is one of the common approved first-line regimens. As opposed to other macrolides, clarithromycin is used as an antibiotic against H. pylori due to its unusual acid stability. The antibiotic reversibly binds to domain II hairpin 35 and the domain V peptidyl transferase loop of the 23s rRNA molecule inside the ribosomal subunit of the 50s. This binding prevents protein elongation by releasing peptidyl-tRNA prematurely from the acceptor site and thus effectively blocks the synthesis of bacterial proteins. One mechanism by which H. pylori acquires antibiotic resistance is through vertical mutation transmission. Clarithromycin-resistant strains also carry mutations within the gene 23S rRNA. Several studies have shown that A/G point mutations in Domain V at positions 2142 and 2143 or a T/C mutation in Domain VI of the 23S rRNA gene cause clarithromycin resistance. The prevalence of antibiotic-resistant H. pylori is increasing worldwide. Antibiotics resistance is the main factor of failure of H. pylori eradication therapies. Clarithromycin resistance, in particular, has a major negative impact on the efficacy of the recommended first-line triple therapy of H. pylori. In Sudan, there is very limited data on the prevalence of clarithromycin-resistant H. pylori. The aim of this study was to determine the H. pylori resistance to clarithromycin in Sudanese patients with gastritis symptoms.

Materials And Methods

2.1. Collection of biopsy specimens

Gastric biopsies were collected from 288 patients, in which both the antrum and corpus had been sampled by endoscopy. Biopsies were collected by physicians from patients indicated for gastric endoscopy at different hospitals in Khartoum State (Omdurman Medical Military Hospital, Al-Amal National Hospital, Police Hospital, Ibn Sina Hospital, and Fedail hospital) at the period from June/2018 to January/2019.

2.2. Preservation and processing of specimens

The specimens were immediately placed in thioglycollate broth, which provides anaerobic conditions until processing. Manual grinding of biopsies took place using disposable material.

2.3. Bacterial identification

The DNA of Helicobacter pylori has been extracted from specimens of the gastric biopsies using the guanidine chloride method. Biopsies were grind by sterile blades and tips and then washed twice by phosphate buffer saline (PBS) to eliminate excess media. We add to the pellet 2 ml of lysis buffer, 10μl of proteinase K, 1 ml of guanidine chloride, and 300 μl of ammonium (NH4) acetate, vortexed and incubated at 65°C for 2 hrs. The mixture was cooled to ambient temperature, and then 2 ml of pre-cooled chloroform was applied, vortexed, and centrifuged for 5 min at 3000 rpm. The upper layer of the mixture was moved to a new tube, and 10 ml of absolute cold ethanol was applied, shaken, and held for 2hrs or overnight at -20°C. The tube was then centrifuged for 15-20 min at 3000 rpm, the supernatant was
carefully removed, and the tube was inverted for 5 minutes on a tissue paper. The pellet was washed with 70 percent ethanol 4 ml, centrifuged for 5 min at 3000 rpm. The supernatant was poured away, allowing the pellet to dry for 10 min. Then re-suspended into 50 μl of distilled water, briefly vortexed, and held overnight at -20°C. The extracted DNA was stored at -70°C until use.

2.4. Polymerase Chain Reaction (PCR)

Two primer sets were used for the detection of the bacteria, targeting 16S rRNA (532bp) and 23S rRNA (320bp). Allele-specific PCR was used for the detection of A2142G and A2143G point mutations using four primers called FP-1, RP-1, RP2142G, and FP2143G (Table 1). When the strain is wild type (wt), neither RP2142G nor FP2143G anneals with the template and polymerase chain reaction (PCR) amplification proceeds between FP-1 and RP-1, resulting in a 320bp amplicon. In the case of the presence of A2142G mutation, the PCR amplification primarily takes place between FP-1 and RP2142 G, which results in an amplicon of 238 bp. Similarly, in the case of the A2143G mutation, the PCR amplification goes between FP2143G and RP-1, resulting in an amplicon of 118 bp. The primers were dissolved according to manufacturer guidelines to prepare 10pmol/μl.

The first protocol used for amplification of 16S rRNA was as follows: initial activation at 94°C for 3 minutes, followed by 35 cycles at 94°C for 30s, 53°C for 30s, and 72°C for 45s, and a final extension at 72°C for 5 minutes (Table 1).

The second protocol used for amplification of 23S rRNA was as follows: initial denaturation at 95°C for 5 minutes followed by 35 cycles of denaturation at 95 °C for 15s, annealing at 60.5°C for 20s, and extension at 68°C for the 30s and a final extension of 2 min at 68 °C (Table 1).

Table (1): Primers sequences and PCR protocols used in this study

| Protocols | Primer name | Primer sequence (5´-3´)                                  | Amplicon size (bp) | References |
|-----------|-------------|----------------------------------------------------------|--------------------|------------|
| 1st       | 16s RNA     | GCTAA GAGA TCA GCC TAT GTCC                              | 532                | 18         |
|           |             | TGGCAATCAGCGTCAGGTAATG                                    |                    |            |
| 2nd       | FP-1        | TCGAAGGTTAAGAGGATGCAGTC                                   |                    |            |
|           | RP-1        | GACTCCATAAGAGCCAAGGCCCCTTAC                               | 320                |            |
|           | RP2142G     | AGTAAAGGTCCACGGGTATTCC                                     | 238                |            |
|           | FP2143G     | CCGCGGCAAGACAGAGAGA                                       | 118                | 19         |

2.5. DNA sequencing

A total of 25 samples of 23S rRNA (320bp) amplified genes were sent for sequencing (by BGI, business, China) for both strands of PCR products. The pairwise alignment was done for successful sequences by
BLAST, and then multiple sequence alignment was done by BioEdit software\textsuperscript{20}. The sequences were compared with the 23S rRNA reference (U27270).

2.6. Statistical analysis

The obtained data were analyzed using IBM SPSS statistics 20. The chi-square test was used to compare the correlations and associations between variables ($p$-value $\leq 0.05$ considered significant).

Results

3.1. Demographic data

One hundred and twenty-eight (44.4\%) were females, and one hundred sixty (55.6\%) were males from two hundred and eighty-eight enrolled patients. They were divided into two age groups: adolescents (10) and adults (276). One hundred seventy-five (60.8\%) specimens were collected from Khartoum city, and one hundred and thirteen (39.2\%) specimens were collected from Omdurman city.

3.2. Endoscopic findings

According to endoscopic findings by a physician, one hundred and ninety (66\%) patients were diagnosed as gastritis, twenty-nine (10\%) as a gastric ulcer (G. ulcer), twenty-five (9\%) as a duodenal ulcer (D. ulcer), fifteen (5\%) as esophagitis and twenty-nine (10\%) were of normal finding.

3.3. Genotyping of \textit{H. pylori}

Out of 288 specimens investigated for the presence of \textit{H. pylori} using primers targeting 16S rRNA and 23S rRNA by PCR, 97/288 (33.7\%) were \textit{H. pylori}-positive either by the presence of two genes or at least one of them. 16S rRNA was positive in 88(30.6\%) specimens, 23S rRNA in 62(21.5\%) specimens, and both genes were positive together in 53(18.4\%) specimens (Figure 1).

3.4. Association between the presence of \textit{H. pylori} with the epidemiological and endoscopic findings

There was no significant correlation between the presence of \textit{H. pylori} and epidemiological findings (Gender, age group, and geographical distribution of patients) in this study.

There is a significant association between the presence of \textit{H. pylori} and endoscopic findings ($P$-value=0.039). \textit{H. pylori} present in 66 patients with gastritis, 14 patients with D. ulcer, 8 patients with G. ulcer, two patients with esophagitis, and 7 were normal (Table 2).

Table (2): Association between the presence of 23s RNA and endoscopic findings
### 3.4. Detection of A2142G and A2143G point mutations by allele-specific PCR

The A2142G point mutation was detected in 9/97 (9.3%) specimens, whereas the second mutation (A2143G) was not detected at all samples.

### 3.5. DNA sequencing results

From twenty-five successfully sequenced samples, 12 (48%) showed mutations associated with clarithromycin resistance; One sample showed the presence of A2142G point mutation, and the A2143G was found in 5 samples. Two other mutations (T2182C and C2195T) were detected in 4 and 3 samples, respectively. The A2142G was detected in one sample labeled D20, A2143G mutation detected in 5 samples D19, D33, K2, K37, and M14, T2182C mutation was detected in samples F11, K37, M11, and C5. The C2195T was detected in 3 samples D3, D4, and D34 (Figure 2).

### Discussion

*H. pylori* infection is increasingly reported nowadays. Although patients are receiving treatment, the problem of antibiotic resistance still hinders their recovery. In particular, Clarithromycin is the most prescribed antibiotic by physicians, and resistance to it may lead to failure of treatment.

In this study, out of 288 patients with gastric pain, 66% were diagnosed as gastritis, 10% as gastric ulcer, 9% as duodenal ulcer, 5% as esophagitis, and 10% were normal patients. This finding is in agreement with other studies, found that gastritis is the most prevalent gastric disease.

A significant association was noticed between *H. pylori’s* presence and endoscopic findings, *H. pylori* present in high frequencies of 40% (10/25) among patients diagnosed with duodenal ulcers. This evidence explains why *H. pylori* implicated as a major etiologic factor in duodenal ulcers.

Although culture isolation has been the standard method for the detection of organism, but it may not be the most appropriate method for detection of *H. pylori* like organism due to cost, the special conditions...
required for specimen transport and growth, and the long interval between specimen harvest and test results, which delay treatment decision. According to Malfertheiner molecular technologies should be implemented as alternatives to traditional H. pylori identification. In this study, the prevalence of H. pylori infection was 33.7% (97/288), using PCR targeting both 16S RNA and 23S rRNA genes. The latest prevalence rates of H. pylori among gastric biopsies from Sudanese patients were 21.1% using PCR targeting only 16 S RNA gene, and 22.2% using culture. This variation could be attributed to that in our study, we targeting both 16S RNA and 23S rRNA genes.

H. pylori resist clarithromycin by specific mutations in the peptidyl transferase loop of the 23S rRNA molecule's V domain. Worldwide, the prevalence of clarithromycin resistant strains of H. pylori is 19.4%. Our study revealed a higher frequency (48%) of mutations associated with clarithromycin resistance using DNA sequencing of V domain of 23S rRNA gene. Generally, countries with an antibiotic resistance rate of more than 20% alter their treatment strategies. The point mutation A2142G detected in 9.3% (9/97) of specimens using allele-specific PCR. This percentage is a noticeable amount compared with Tran study in Vietnam, which found this mutation in about 3.6%, variation in the population may represent a critical factor.

Like Ghaith's study, point mutation A2143G was fallen to be detected by PCR; this could be justified according to Cheng, which is that there is only one nucleotide difference between wild-type DNA and point mutation in DNA sequence. Therefore, the unusual mutations between large excess wild-type alleles are difficult to detect by traditional gene variation assays. In contrast, both mutations A2142G & A2143G appeared by DNA sequencing technique, and they are already known to cause reduced affinity of the ribosome for CLA.

DNA sequencing also showed the presence of T2182C mutation in some specimens. According to Jung suggestion, this mutation is nonspecific, which does not play an essential role in clarithromycin resistance. Besides, point mutation C2195T was detected by sequencing, and according to Fasciana, it has no relation with clarithromycin resistance.

### Declarations

**Ethics approval and consent to participate**

The research was approved by the Khartoum state Ministry of health research department on 1/3/2018. Written consent was obtained from patients.

**Availability of data and materials**

The datasets used and analyzed during the current study are available at request.

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Not applicable

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

HNA, AMA, MMA, and EHO designed the study, HNA, AMA, MMA, EHO, DMZ, performed the experiments, analyzed the data, AAM collected gastric biopsies and performed clinical diagnosis, wrote the manuscript, HNA and MAA supervised the study and revised manuscript critically, all the authors read and approved the final version of the manuscript.

**Abbreviations**

AMX     Amoxicillin
Bp      Base Pair
CLR     Clarithromycin
DNA     Deoxyribo-Nucleic Acid
MTZ     Metronidazole
PCR     Polymerase Chain Reaction
PPI     Proton Pump Inhibitor
rRNA    Ribosomal Ribonucleic acid
SPSS    Statistical package for social science
TET     Tetracycline

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