Research Article

Helicobacter pylori Mutations Conferring Resistance to Fluoroquinolones and Clarithromycin among Dyspeptic Patients Attending a Tertiary Hospital, Tanzania

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1. Introduction

Helicobacter pylori is a gram negative bacterium, spiral shaped, microaerophilic, and motile with polar flagella, belonging to the genus Helicobacter. H. pylori can lead to gastritis, peptic ulcer diseases, and gastric cancers [1]. Invasive and noninvasive tests can be used to diagnose H. pylori infection; however, culture and molecular tests are methods which can detect the presence of organism as well as the resistance patterns of the H. pylori strains [2]. Polymerase chain reaction (PCR), which selectively amplifies the target gene, is a quick, highly sensitive, and specific test to diagnose H. pylori infections [3]. In developing countries, culture and molecular methods are not routinely done. Molecular method is among the methods used for the detection of H. pylori and determination of mutation which confers antimicrobial resistance even at a concentration so low that could not be detected by the culture [4].
H. pylori infection can complicate the chronic atrophic gastritis which is the precancerous stage to adenocarcinoma [5, 6]. In the recent published global cancer statistics, gastric cancer was ranked third for cancer-related mortality worldwide and fifth for incidence [7]. H. pylori eradication prevents and slows down the progression of nonatrophic chronic gastritis to atrophic gastritis, hence reducing gastric cancer risk [8]. Therefore, early treatment of patients with H. pylori may decrease gastric cancer incidence and its associated mortality [8].

The treatment of H. pylori consists of triple therapy (PPI + clarithromycin + either amoxicillin or metronidazole) which can be used in areas with known low clarithromycin resistance, while nonbismuth quadruple concomitant regimen (a proton pump inhibitor, amoxicillin, metronidazole, and clarithromycin) is recommended as first line together with bismuth quadruple therapy (PPI + bismuth + metronidazole + tetracycline) in areas of high clarithromycin resistance [9–11]. Efficacy of these regimens is compromised by drug resistance which is increasing in Africa [12].

Clarithromycin resistance in the first line triple therapy regimens is the main cause of H. pylori eradication failure [13, 14]. In the H. pylori treatment, clarithromycin is one of the important drugs in the standard therapy of H. pylori, while the quinolone is the key drug in the second line therapy [15]. Worldwide, the prevalence of clarithromycin and fluoroquinolone resistance is 19.74% and 18.94%, respectively while in Africa the prevalence of clarithromycin has been found to range from 0% to 100% and that of fluoroquinolones from 0% to 32% [12, 16].

Several mutations have been detected at peptidyl transferase-encoding region in V domain of the H. pylori 23S rRNA, which is a component of the large subunit (50S) of the bacterial ribosome. This domain is the most common binding site for antibiotics that inhibit translation like clarithromycin. Therefore, 23SrRNA is used to diagnose H. pylori and at the same time detect antibiotic resistance (mutations associated with antibiotic resistance). Most of the known point mutations are A to G transition mutations [17, 18] but three mutations are A to G transition mutations [17, 18] but three

2. Materials and Methods

2.1. Study Design and Study Population. This was a cross-sectional study among dyspeptic patients undergoing upper gastrointestinal (GI) endoscopy at the endoscopy unit of the Bugando Medical Centre, from August 2014 to August 2016. All adult dyspeptic patients referred for upper GI endoscopy as part of their workup for their dyspeptic symptoms with no history of antibiotic treatment for H. pylori within the past 30 days were included in the study. Dyspepsia was defined according to the ROME criteria [24]. During the upper GI endoscopy procedure from one patient, two biopsies were taken from both antrum and fundus. Biopsies for every patient were stored in a single container with 70% ethanol. A total of 208 tissue samples were obtained from 208 patients.

2.2. DNA Extraction, Amplification, and Sequencing of the Clarithromycin and Quinolone Resistance-Determining Regions

**DNA Purification from Tissues.** Two biopsies (antrum and fundus) were ground using a tissue homogenizer (Ultra-Turax; Labo-Moderne, Paris, France). The genomic DNA was extracted using a QiAamp DNA minitissue extraction kit (Qiagen SA, Courtaboeuf, France) according to the manufacturer’s instructions [25]. The H. pylori detection and clarithromycin mutations: amplification of a 267 bp fragment of the H. pylori 23S rRNA was performed by Real-Time PCR (Light Cycler- Roche) using oligonucleotides HPY-S: 5-AGGTTAAGAGGATTGCCTCAGTC and HPY-A: 5-CGCGATGATATTCCCATTAGCAGT (GenBank accession no. U27270) as previously described [26]. The PCR was carried out in 15μl volume containing Ampli Taq DNA polymerase 1U, PCR buffer 1X, deoxynucleoside triphosphate (dNTP) 200μM, PCR water 7μl, and 0.2μM of each of the primers [26]. After an initial denaturation step at 95°C for 10 minutes, 40 PCR cycles were performed with 95°C for 10 seconds (denaturation), 60°C for 10 seconds (annealing), and 72°C for 20 seconds (extension). Melting curve analysis was performed for each sample. PCR products were purified using the Qiagen PCR-purification kit [25] and sequenced (Seqlab, Göttingen). Mutations within the 23S gene were detected by DNA sequence alignment with the wild type allele using the Geneious software package [version 8.0.4 available from www.geneious.com (Biomatters, Ltd.)].

**GyrA Genes Amplification for Fluoroquinolones Mutations.** Using oligonucleotides GyrA-1 (TTAGCTTATCTCATGCCTGTCGCTC-GGCCTGCTCAGTC) and GyrA-2 (GCGACGGCTTGGTTAGAATA), a 428 bp GyrA fragment was amplified from genomic DNA by Real-Time PCR (Light Cycler, Roche) as previously described...
Table 1: Patterns of mutations among 54 patients/samples with point mutations.

| Antibiotic tested | Tested samples | Mutation type   | All point mutations identified | Heterozygote H. pylori strains | Homozygote H. pylori strains |
|-------------------|----------------|-----------------|-------------------------------|-------------------------------|-----------------------------|
| Clarithromycin    | 188            | A2143G          | 30/54                         | 13/30                         | 17/30                       |
|                   |                | A2142G          | 20/54                         | 14/20                         | 6/20                        |
|                   |                | A2142C          | 1/54                          | 0                             | 1                           |
|                   |                | A2143C          | 1/54                          | 0                             | 1                           |
|                   |                | A2143G + A2142G | 2/54                          | 1/2                           | 1/2                         |

Figure 1

(a) (b) (c)

[27]. The PCR was carried out in 20\(\mu l\) volume containing 4 \(\mu\) (Ampli Taq DNA polymerase 1U, PCR buffer 1X, deoxynucleoside triphosphate (dNTP) 200\(\mu M\), 5 \(\mu l\) DNA, 7 \(\mu l\) H\(_2\)O, and 2 \(\mu l\) of each primer (5 \(\mu M\)). After an initial denaturation step at 95°C for 10 minutes, 40 PCR cycles were performed with 95°C for 10 seconds (denaturation), 55°C for 10 seconds (annealing), and 72°C for 20 seconds (extension). PCR products were purified using the Qiagen PCR-purification kit [25] and subjected to DNA sequencing (Seqlab, Göttingen). Mutations within the quinolone resistance-determining region (QRDR) of the \(H.\) pylori GyrA gene (GenBank accession no. AE000583) [28] were detected by DNA sequence alignment with the wild type allele using the Geneious software package [version 8.0.4 available from www.geneious.com (Biomatters, Ltd.)].

This molecular work was done in the Department of Medical Microbiology, University of Göttingen, Germany. Data were entered in Excel sheet and summarized using percentages. The total numbers of samples tested for clarithromycin and fluoroquinolones mutations were used as denominators.

3. Results

Out of 208 biopsies from nonrepetitive patients examined for \(H.\) pylori by PCR method, 188/208 (92.2%) were PCR positive. Mutations conferring resistance to clarithromycin were detected in 54/188 (28.7%) of patients. The mutations detected were A2143G (30) [Figure 1], A2142G (20), A2142C (1), and A2143C (1). Two samples had double mutations A2142G +A2143G and 22/188 (11.7%) samples had both wild type and mutants (Table 1).

On the other hand, out of 188 patients with positive \(H.\) pylori PCR, 131 (69.7%) were analyzed for gyrA mutations that are known to confer fluoroquinolones resistance. Fluoroquinolone resistance mutations were detected in 77/131 (58.8%) samples; these included N87I (20) [Figure 2], N87K (7), D91G (8), D91N (15), D91Y (11), and A92T (20.8%) Table 2. Nine (11.7%) samples out of 77 had both wild type and mutants or had heterozygote \(H.\) pylori strains (Table 2).

A total of 20/77 (25.9%) who had mutation in gyrA gene had also point mutation in \(H.\) pylori 23S rRNA gene implying that 20/54(37%) of samples with clarithromycin mutations had also quinolones mutations.

4. Discussion

Worldwide, the prevalence of primary \(H.\) pylori resistant to clarithromycin is 19.4% [16]. In European countries, a high prevalence has been reported, ranging from 12.5% to 23.5% [19], while in Africa the overall clarithromycin resistance was 29.2% [12]. In our study, we have observed the presence of clarithromycin mutations that predict drug resistance in 28.7% of patients who were not on eradication therapy. These findings are in line with other studies [12, 29, 30]. However, the observed prevalence of clarithromycin mutations is low compared to the prevalence found in certain parts of Asia (India and Vietnam), whereby the prevalence of 43% and that of 85.5%, respectively, were observed [31, 32], and it is higher compared to study done in Congo Brazzaville [33]. The high prevalence of clarithromycin mutations in developing countries could be linked to the overuse of macrolides for treatment of diarrheal diseases in developing countries [34].
In 23S rRNA gene, most of the known point mutations conferring resistance to clarithromycin are A to G transition mutations [18]. Of these, three point mutations, namely, A2142G, A2143G, and A2142C, are responsible for 90% of primary clarithromycin resistance in *H. pylori* [19]. This was confirmed in the present study, whereby A2143G, A2142G, and A2142C formed the majority of clarithromycin mutations detected [17, 19, 35, 36]. It should be noted that the observed mutations have different therapeutic outcome; the presence of A2143G significantly reduced the eradication rate of the *H. pylori* compared to other mutations [36, 37]. Of note, clarithromycin resistance mutations detected in this cohort significantly predicted treatment failure as documented in our previous publication [38]. In areas with high prevalence of clarithromycin resistance of >15%, the recommended regimens are the Bismuth quadruple therapy and concomitant therapy for 14 days according to the Canadian guidelines and 10 days according to the North American and European guidelines [9, 11, 15]. Other mutations which can be found in other parts of the world which have been found to confer clarithromycin resistance in *H pylori* strains are the T2289C, T2190C, T2182C, A2223G, C2195T, C2245T, C2694A, G2141A and G2224A, A2146C, A2146G, and A2147G [39–42].

Regarding fluoroquinolones resistance rates, 3.9% prevalence has been reported in Europe and 17.4% in Africa [43]. The highest rate in Africa has been reported in Congo Brazzaville [12, 33, 44]; however in the current study, about 59% of patients studied carried known fluoroquinolones resistance mutations. Quinolone resistance to *H. pylori* has been associated with second line treatment failure [20]; therefore these findings are alarming because a significant proportion of patients on the second line regimen might have treatment failure. This could be explained by the fact that in Tanzania quinolones are commonly used in the treatment of urinary tract infections, typhoid fever, infectious diarrhea, and genital discharge syndrome, hence selecting for *H. pylori* resistant strains. The alternative for treatment of *H. pylori* for the patients who fail second line is bismuth quadruple therapy [45] which is not commonly available in lower health facilities.

The main fluoroquinolones resistance mutations have been detected in gyrA gene at the codon positions 87, 88, 91, and 97 [46, 47]. As in other studies, in the current study the commonest mutations were in gyr87 [12]. In our study, the mutation A92T in the gyrA gene was detected for the first time in *H. pylori*. This mutation has been reported in *Neisseria gonorrhoea* [48]. In that study, the resistant isolates to gepotidacin (topoisomerase type II inhibitor) were found to have an additional A92T mutation. In our study, other mutations which have been identified in other parts of the

### Table 2: Patterns of mutation among 77 patients/samples with point mutations.

| Antibiotic tested | Tested samples | Mutation type | All point mutations identified | Heterozygote *H. pylori* strains | Homozygote *H. pylori* strains |
|-------------------|----------------|---------------|--------------------------------|---------------------------------|-------------------------------|
| Fluoroquinolones  | 131            | N87I          | 20                             | 5/20                            | 15/20                         |
|                   |                | N87K          | 7                              | 3/7                             | 4/7                           |
|                   |                | D91G          | 8                              | 2/8                             | 6/8                           |
|                   |                | D91N          | 15                             | 1/15                            | 14/15                         |
|                   |                | D91Y          | 11                             | 1/11                            | 10/11                         |
|                   |                | A92T*         | 16                             | 2/16                            | 14/16                         |

* Unknown mutation
world were not detected; these mutations include N87H, N87Y, and D91A. This could be explained by the fact that the distribution of mutations depends on the phylogeographic tree differences of *H. pylori* due to gene content diversity which can be due to either gene loss or gene recombination in multiple strains [49].

5. Conclusion

A significant proportion of dyspeptic patients attending tertiary hospital in Tanzania are infected with *H. pylori* strains harboring clarithromycin or fluoroquinolones resistance mutations. Detection of more than 50% of strains with fluoroquinolones resistance mutations makes the *H. pylori* second line treatment questionable in our setting. There is a need of surveillance of *H. pylori* resistance patterns in Tanzania to provide data that can guide empirical treatment to reduce associated morbidity and mortality of *H. pylori* infections. The correlation between A92T fluoroquinolone mutation and phenotypic resistance in *H. pylori* requires further investigations.

Data Availability

The data belongs to the CUHAS University and Bugando Hospital; a permission is required to make them freely available.

Additional Points

Highlights. (i) 90% PCR positive for *H. pylori*. (ii) High prevalence of gyrA mutations. (iii) High frequency of A2142G and N87I. (iv) A92T unknown mutation in gyrA gene.

Ethical Approval

This study was approved by the Ethics and Research Committee of CUHAS/Bugando with an updated clearance for publication number CREC/066b/2015.

Consent

During and after study period, informed consent form was filled by all patients and all information collected was kept strictly confidential.

Conflicts of Interest

There are no conflicts of interest to declare.

Authors’ Contributions

All authors have made substantial contributions: Hyasinta Jaka, Nele Rütgerodt, and Stephen E. Mshana did the conception and design of the study, analysis and interpretation of data, and drafting of the article. Hyasinta Jaka, Nele Rütgerodt, Uwe Gross, and Wolfgang Bohn did the acquisition of data, laboratory work, and clinical work. Hyasinta Jaka, Andreas Mueller, Christa Kasang, and Stephen E. Mshana did the analysis of data and drafting of article and revised it critically for important intellectual content while Christa Kasang and Stephen E. Mshana approved the final version to be submitted.

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