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Molecular Detection of Antibiotic Resistance of *Helicobacter pylori* from Gastric Biopsies in Abidjan (Côte d'Ivoire)

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Authors' contributions

This work was carried out in collaboration between all authors. Author DTFB designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors CGMV and GN managed the analyses of the study. Author DTFB managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To determine the genes of resistance to amoxicillin, clarithromycin and metronidazole of *Helicobacter pylori* in gastric biopsies in Côte d'Ivoire.
Place and Duration: The study was performed at the department of gastroenterology of Cocody Hospital and University Center, at the laboratory of Bacteriology-Virology and at the molecular biology platform of Pasteur Institute of Côte d'Ivoire from August 2015 to December 2016.
Methodology: The rapid urease test was performed in endoscopy room and 98 positive biopsies

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were retained for the study. Gastric biopsies were collected and transported within a maximum of 4 hours. DNA extraction was followed by Polymerase Chain Reaction (PCR) amplification.
Results: The rdxA / frxA, 23S rRNA and pbp1 genes conferring resistance to metronidazole, clarithromycin and amoxicillin respectively were identified in 12.2% (12/98), 26.5% (26/98) and 58.2% (57/98). Cross-resistance genotypes to these three antibiotics were detected in 8.2% (8/98) of the samples.
Conclusion: These results show a high level of resistance of *Helicobacter pylori* to amoxicillin and presence of cross-resistance to the three commonly used antibiotics. These results support the need for an evaluation of *Helicobacter pylori* current therapeutic protocol in Côte d'Ivoire.

Keywords: Helicobacter pylori; resistance gene; gastric biopsies; Abidjan.

1. INTRODUCTION

Helicobacter pylori (*H. pylori*) is responsible for pathologies of gastroduodenal mucosa such as gastritis, ulcer, MALT (Mucosa associated lymphoid tissue) lymphoma and gastric adenocarcinoma [1-3]. Half of world's population is affected by this infection. *H. pylori* occurs in all regions of world with high prevalence in developing countries [4]. In Côte d'Ivoire, diagnosis and treatment of *H. pylori* infection is not common practice due to lack of resources and information. Eradication of *H. pylori* is not subject of specific recommendations. Thus, to date, eradication treatment is based on an empirical combination of proton pump inhibitors (PPI) and two antibiotics taken from amoxicillin (AML), clarithromycin (CLR) and metronidazole (MTZ), justified by availability of antibiogram data and the relatively low cost of these molecules. The research of African countries in terms of *H. pylori* antibiotic resistance are rarely published [5]. However, the susceptibility of *H. pylori* to these antibiotics differs from one country to another [6,7]. Thus, scientists propose eradication strategies according to their personal results. In case of Côte d'Ivoire, these data are currently not available. The current global problem in management of *H. pylori* infection is resistance to recommended standard eradication treatment [8]. Since no studies have been carried out in Côte d'Ivoire on the resistance of *H. pylori*, we have researched resistance of this bacterium. The aim of this study is to determine the genes of resistance to amoxicillin, clarithromycin and metronidazole of *H. pylori* in gastric biopsies.

2. MATERIALS AND METHODS

2.1 Gastric Biopsies

A total of 98 positive urease rapid test biopsy specimens were collected at Hospital and University Center of Cocody between August 2015 to February 2016 and were transported to Pasteur Institute's Bacteriology-Virology Laboratory of Côte d'Ivoire within a maximum of 4 hours. Gastric biopsies were then stored in dry tubes at -80℃.

2.2 Ethics

All patients underwent a socio-demographic questionnaire (age, sex, occupation) and medical history validated by Ethics Committee of Pasteur Institute of Côte d'Ivoire. Written consent was also given by each patient before endoscopy.

2.3 Extraction of *H. pylori* DNA

Extraction of *H. pylori* DNA was performed according to DNA extraction protocol of NucliSENS® kit (bioMérieux, France) with some modifications. Biopsies were ground in 0.3 ml of 1X PBS buffer with Potter grinder into a sterile tube and then suspended in 500 µl of buffer containing Tris-HCl 10 mM, EDTA 1 mM pH 8.0, Proteinase K 1 mg /ml and incubated at 60℃ for 24 h. DNA was extracted in 500 µl of lysis buffer containing 20 mM Tris, 2 mM EDTA, 150 mM NaCl, 1% SDS and Proteinase K 100 µg/ml for 1 h at 60℃. 1 ml of phenol-chloroform-iso-amyl alcohol mixture (25:24:1) was added and centrifuged at 13000 rpm for 15 min. Aqueous phase (upper phase) was collected and 1/ 10th of 3M sodium acetate and 500µl of absolute ethanol were added and incubated 1 at -80℃ for 1 h or overnight at -20℃. The pellet obtained was washed with 70% ethanol and dried at 65℃ for 15 min. Pellet obtained was eluted in 60 µl of buffer and DNA was stored at -20℃.

2.4 Resistance Gene Genotyping

PCR was performed in a volume of 50 µl containing 0.75 µl of each 10 mM primer, 3 µl of

genomic DNA, 1 µl of 10 mM dNTPs, 3 µl of 25 mM $MgCl_2$, 5 µl of each colorled and colorless buffer 5 ×, and 0.3 µl of Taq polymerase (Promega, USA). Amplification was performed in an automaton thermocycler (ABI 9700 96 Well PCR, Applied Biosystems GeneAmp, USA). Primer sequences used in this study and the conditions for gene amplification are summarized in Table 1.

2.5 Statistical Method

Data were entered and described using software called Epi-info version 3.5.4. (CDC, USA). These data were then transcribed into an Excel database to facilitate a single and varied analysis. Statistical tests were interpreted at significance level corresponding to an alpha risk of 5%. Qualitative variables were compared using Pearson Chi-2 test or Fisher's exact test when one of variables was less than 5.

3. RESULTS

3.1 Description of Patients with Positive Urease Rapid Test Biopsies

Patient population was predominantly female with 62.2% (61/98). Men accounted for 37.8% (37/98). Average age was 42.6 years with a minimum of 19 years and a maximum of 77 years. The most represented age group was that of patients aged between 31 and 40 years. Although some gene was detected more in one sex than in the other, their presence was not significantly associated with the age or sex of the patients (p> 0.05).

3.2 Metronidazole Resistance Gene

RdxA and frxA genes of *H. pylori* conferring resistance to metronidazole (MTZ) were detected in 12.2% (12/98). FrxA gene alone was detected in 5.1% (5/98) (Fig. 1A).

3.3 Clarithromycin Resistance Gene

23S rRNA gene of *H. pylori* conferring resistance to clarithromycin (CLR) was detected in 26.5% (26/98) (Fig. 1B).

3.4 Amoxicillin Resistance Gene

H. pylori pbp1 gene conferring resistance to amoxicillin (AML) was detected in 58.2% (57/98) (Fig. 1C).

3.5 Cross Resistance

Cross-resistance to three antibiotics was observed in 8.2% (8/98). Amoxicillin and clarithromycin had a cross-resistance of 18.4% (18/98). For amoxicillin and metronidazole, cross-resistance was 4.1% (4/98). There was no cross-resistance for metronidazole and clarithromycin.

3.6 Clinical Characteristics of Patients with Resistance Genes of CLR, MTZ and AML

Patients with resistance genes of MTZ, CLR and AML had a familial history of ulcer syndrome in majority of cases. None of these patients used tobacco (Table 2).

Table 1. Sequences of primers used and PCR conditions

Gene/allelic variant	Amplicon size (bp)	Oligonucleotide primer pair (5' to 3' sequences)	PCR cycling conditions	Reference
rdxA	749	RDX1(GCCACTCCTTGAACTTTAATTTAGG) RDX4(CGTTAGGGATTTTATTGTATGCTAC)	95℃: 1 min, 60℃: 1 min: 30 sec, 72℃: 1 min *	[9]
frxA	913	FRXA1(CGAATTGGATATGGCAGCCG) FRXA4(TATGTGCATATCCCCTGTAGG)		
23S *rRNA*	280	CLA1995FW(GTAACTATAACGGTCCTAAG) CLA2274REV(GAAACATCAAGGGTGGTATC)	95℃: 1 min, 50℃: 1 min: 30 sec, 72℃: 1 min *	[10]
pbp1	953	PBP1F(CACGAGCACCGGTAAGATTT) PBP1R(CGCTATCGTCTGTTCTTTTGGG)	95℃: 1 min, 60℃: 1 min: 30 sec, 72℃: 1 min	[11]

** All are 30 cycles (except pbp1, 35 cycles); With an initial denaturation step 95℃, 3 min (except pbp 1.5 min), and a final extension step 72℃, 5 min (except pbp1.7 min)*

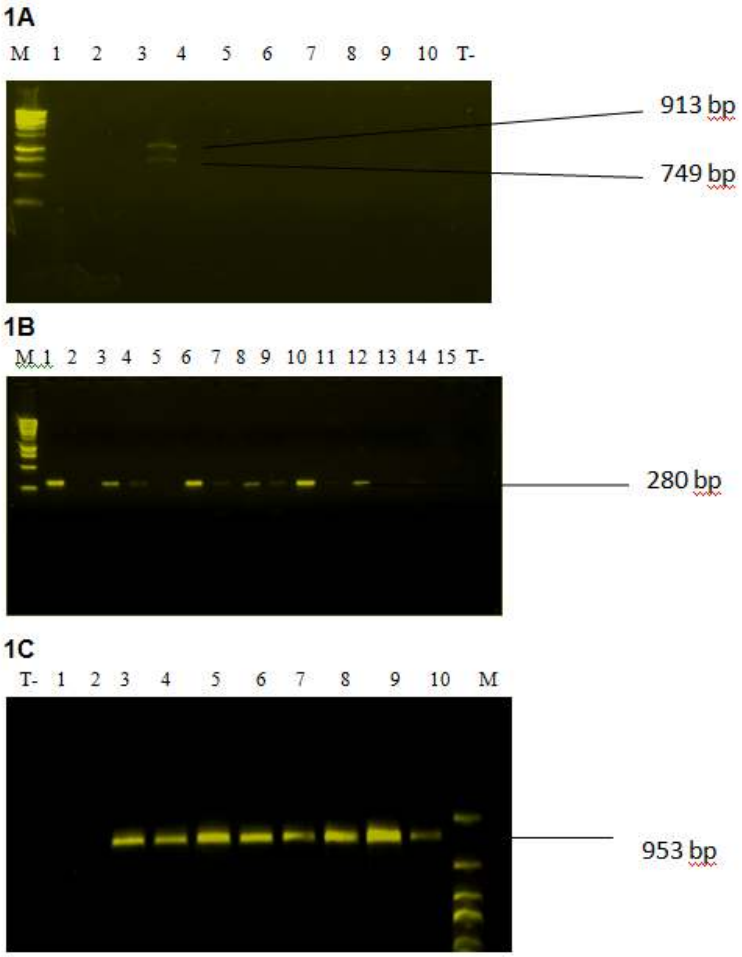

Fig. 1. Genotyping of rdxA/frxA, 23s RNA and pbp1 by PCR. A: rdxA/frxA gene. M: size marker, 200 bp. Line 3: rdxA/frxA positive gene. Line 1,2,4-10: rdxA/frxA negative gene. B: 23s RNA gene. M: size marker, 200 bp. Line 1,3,4,6-12: 23s RNA positive gene. Line 2,5,13-15: 23s RNA negative gene. C: pbp1 gene. M: size marker, 200 bp. Line 3-10: pbp1 positive gene. Line 1,2: pbp1 negative gene. T-: negative control without DNA. bp: base pairs

Table 2. Distribution of patients with genes according to clinical characteristics

	Genes			p-value
	MTZ (n=12)	CLR (n=26)	AML (n=57)	
Alcohol consumption	4 (33.3%)	7 (26.9%)	14 (24.6%)	0.48
Tobacco consommation	0	0	0	
Antecedent of fibroscopy	2 (16.6%)	5 (19.2%)	10 (17.5%)	0.5
Family history of ulcer syndrome	9 (75%)	17 (65.4%)	34 (59.6%)	0.33

Table 3. Distribution of resistance genes according to endoscopic aspect

Aspect endoscopique	Genes		
	MTZ (n=12)	CLR (n=26)	AML (n=57)
Gastropathy with gastric reflux	2(16.7%)	2(7.7%)	9(15.8%)
Erythematous gastropathy	7(58.3%)	15(57.7%)	30(52.6%)
Pangastropathy	3(25%)	7(26.9%)	15(26.3%)
Others[(1)]	0	2(7.7%)	3(5.3%)

[(1)]Others: Savary Miller's stage 2 esophagitis, congestive duodenopathy, cortistone without oesophagitis

3.7 Presence of Resistance Genes According to Endoscopic Aspect

Majority of resistance genes were observed in cases of erythematous gastropathy and pangastropathy. No cases of normal gastric mucosa were observed (Table 3).

4. DISCUSSION

One of main causes of therapeutic failure is resistance of microorganisms to anti-infectives used. In Côte d'Ivoire, it was important to know level of resistance of *H. pylori* to amoxicillin, clarithromycin and metronidazole mainly as regards antibiotic resistance of *H. pylori* poses a real public health problem.

We consider that both sexes are also affected by *H. pylori*, but studies have shown a male predominance in Côte d'Ivoire [12], while our study reports a female predominance of 62.2%. This contrast is surely related to sampling fluctuations when recruiting patients. Unlike developed countries, *H. pylori* infection is common in young adults in developing countries [13,14,15]. This is confirmed in our study with an average age of infected subjects of 42.6 years.

Percentage of resistance to metronidazole in Côte d'Ivoire (12.2%) is low compared with other developing countries (Senegal: 90% [5], Nigeria: 55% [7], South Africa: 95.5% [16]) and developed countries such as France (61%) [17], Canada (32%), England (25%) and North America (20%). Resistance to Mtz is currently the most common type of resistance found in *H. pylori* and is, along with other types of antibiotic resistance, a major cause of elimination failure [9,18]. These differences in resistance levels are both associated with the use of imidazole in the treatment of various parasitic diseases and gynecological disorders than non-controlled access of this molecule in some countries. Studies have shown a higher rate of resistance to metronidazole among South African patients because Mtz was one of the most widely used antibiotics in their healthcare system [19]. Therefore, low resistance of *H. pylori* to MTZ in this study compared to developed countries would probably be due to expensive cost of this molecule in Côte d'Ivoire and thus make it less accessible to population.

Percentage of resistance to clarithromycin (26.5%) places Côte d'Ivoire slightly above the average European and American rates, which are between 23 and 25% [8] but much higher than the rate in African countries (13%) where molecule was tested [4]. Frequency of resistances in different countries is related to use of macrolides, particularly in treatment of respiratory infections [20], as shown in study by De Koster et al. [21] in Belgium where resistance rate rose from 2.2% in 1990 to 11.1% in 1996. This rate of 26.5% in Côte d'Ivoire is certainly due to an earlier use of macrolides.

Amoxicillin appeared to be unaffected by problem of resistance. Indeed, highest resistance rate described in 2004 was less than 1% [8]. However, present study reports a high 58.2% resistance to amoxicillin. One of reasons would be overconsumption of this molecule in human medicine and self-medication observed in our populations probably due to accessibility and to low cost of this molecule. In fact, amoxicillin is used in Côte d'Ivoire as a first-line treatment for benign respiratory tract infections in adults and children. Studies in Côte d'Ivoire in community urinary tract infections reported a resistance rate of amoxicillin of more than 85% in strains of *Escherichia coli* [22]. Cross-resistance (8.2%) to three antibiotics studied clearly shows that self-medication, non-compliance with antibiotic protocols, antibiotic treatment in the probable absence of antibiotics, Standard antibiogram favor the emergence of bacteria resistant to one or more antibiotics. It becomes more than necessary to raise awareness of proper use of antibiotics. In addition, low cross-resistance to amoxicillin and metronidazole (4.1%) was observed and no cross-resistance to clarithromycin and metronidazole was identified in this study. This could guide protocol of treatment of *H. pylori* to the association of these two molecules (clarithromycin and metronidazole), especially in case of allergies to beta-lactams.

Alcohol and tobacco consumption were not significantly related to presence of genes studied ($p = 0.48$). We cannot attribute resistance to these factors because none of the patients used tobacco. Majority of patients had never undergone endoscopy before, few patients had already done (under 20%), this suggests that resistance detected in this study was primary and that we had new infections by *H. pylori*. Moreover, majority of gene carriers had a family history of ulcer syndrome, confirming interfamily dissemination *of H. pylori* [23]. However, contamination by same strain has not been demonstrated in this study.

No case of normal gastric mucosa was found in our study. Patients with genes were predominantly lesions of "endoscopic gastritis" (erythematous gastropathy and pangastropathy) characterized by inflammation and redness in gastric mucosa associated with presence and persistence of *H. pylori* in mucosa as described by Attia et al. [12]. This confirms pathogenesis of *H. pylori* in appearance of these pathologies [24]. Presence of antibiotic resistance genes used for treatment would confer to *H. pylori*, ability to foil all eradication attempts and persist in mucosa because presence of resistance genes was only observed in cases of pathologies and not in normal gastric mucosa [25,26,27]. Antibiotic resistance of *H. pylori* would favor the persistence of infection and its chronic evolution towards severe forms.

5. CONCLUSION

H. pylori rdxA/frxA, 23S rRNA and pbp1 genes conferring resistance to metronidazole, clarithromycin and amoxicillin respectively were identified in gastric biopsies. Due to high level of resistance to amoxicillin, classic tri-therapy of *H. pylori* eradication protocol in Côte d'Ivoire including amoxicillin, metronidazole and clarithromycin needs to be revised.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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