

Efflux Pump Mediated Antibiotic Resistance in Clinical Isolates of *Helicobacter pylori* From South West Nigeria

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ABSTRACT

INTRODUCTION: *Helicobacter pylori* is a spiral shaped gram

negative bacterium majorly causing peptic ulcers and gastric cancer in humans. The combinations of two or more antibiotics such as amoxicillin, clarithromycin, metronidazole or tetracycline with anti-secretory agents or bismuth have been used successfully for the treatment of *H. pylori*.

AIM: Increasing antibiotic resistance has been a contributory factor to treatment failures; as such evaluation of resistance mechanism will improve the management of *H. pylori* infection.

Methodology: The antibiotic resistance in *H. pylori* isolates from Nigeria was determined using E-test strips from Biomerieux SA, France and PCR for the efflux pump gene detection.

RESULTS: Resistance to metronidazole was found to be 93% (97/104), amoxicillin 42% (44/104), clarithromycin 39% (41/104) and tetracycline 27% (28/104). However 51% (53/104) of the isolates harboured multidrug efflux pump gene *hefA* (Hp605) and 34% (35/104) *hefD* (Hp 971). However, *hefG* (Hp 1327) was absent in all the isolates. There was significant association between clarithromycin resistance and *hefA* with p-value 0.014, there was also association between amoxicillin resistance and *hefD* with p-value 0.004.

CONCLUSION: The present study revealed that *H. pylori* antibiotic resistance is on the increase in Nigerian strains and the mechanism of resistance may possibly include the possession of multidrug efflux pump.

Key words: *Helicobacter pylori*, E-test, Resistance, Efflux pump

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INTRODUCTION

Helicobacter pylori is a spiral shaped gram negative bacterium^[1]. It is a major cause of peptic ulcer and gastric cancer in humans^[2]. The combinations of two or more antibiotics such as amoxicillin,

clarithromycin, metronidazole or tetracycline with anti-secretory agents or bismuth have been used successfully for treatment^[3,4,5].

Antibiotic resistance is increasing worldwide with different geographical patterns and different prevalence of *H. pylori*. The meta-analysis program conducted by the Surveillance of *H. pylori* Antimicrobial Resistance Partnership (SHARP) between 1993 and 1999 demonstrated that metronidazole resistance was 36.9%, clarithromycin 10.1% while amoxicillin 1.4%^[6]. In an investigation involving African countries from 1986 to 2017 antibiotic resistance was reported to be 75.8% for metronidazole, 72.6% amoxicillin, 48.7% tetracycline while it was 29.2% for clarithromycin^[7]. However in Nigeria Harrison *et al*^[8] reported an elevated metronidazole resistance rate of 99.1%, amoxicillin 33.3%, clarithromycin 14.4% and tetracycline 4.5%. Increased antibiotic resistance rates have been a contributory factor to treatment failures and multiple gene mutations are reported to play a role in high level of antibiotic resistance^[9]. Different resistant strategies in bacteria to antibiotics have been reported which includes possession of active efflux pumps which excrete toxic chemical compounds and drugs out of the cell^[10].

Opinions are divided over the actual mechanism by which this organism became resistant to antibiotics. These range from production of β -lactamase^[11], mutation or alterations in penicillin binding proteins^[12,13], possession of active efflux pumps which excrete drugs^[14] and decreased membrane permeability of antibiotics into the bacterial cells or combinations of these resistance strategies^[15]. This current study attempts to evaluate the presence of multidrug efflux pump as a resistance mechanism in these isolates which could serve as target for reversing drug resistance in *H. pylori*.

METHODOLOGY

Study subjects

Four hundred and ninety-two outpatient subjects attending the endoscopic unit of Lagos State University Teaching Hospital (LASUTH), Lagos University Teaching Hospital (LUTH), University of Benin Teaching Hospital (UBTH) and Obafemi Awolowo University Teaching Hospitals Complex (OAUTHC) in Nigeria were recruited for the study after informed consent was obtained.

Collection and processing of samples

Biopsies were obtained from the stomach corpus and antrum of the patients and transported to the laboratory in Portagerm pylori (Biomerieux, Marcy l'Etoile, France) within two hours. The biopsies were cultured on serum plates prepared from GC agar (Difco) containing DENT antibiotic selective supplement (Oxoid, Basingstoke, United Kingdom) at pH 6.8 -7.0, vitamin mix (1%) and horse serum (9%). The cultures were incubated in a microaerophilic condition (85% N₂, 10% CO₂, 5% O₂) at 37°C for minimum of 3 days after which the serum plates were examined for possible growth of *Helicobacter pylori* strains. Rapid urease test was used for biochemical confirmation of the isolates^[16].

Determination of the antibiotic minimum inhibitory concentrations (mics) of *H. pylori* isolates

MIC test was carried out on the isolates using amoxicillin, clarithromycin, metronidazole and tetracycline E-test strips (Biomerieux, Marcy l'Etoile, France), incubated for three to four days at 37°C for visible colonies to form at microaerophilic atmosphere. Resistant values were taken with the break points as > 0.125 µg/mL, 0.5 µg/mL, 8.0 µg/mL and 1 µg/mL respectively determined by Eucast version 7.1 (2017)^[17].

DNA extraction and PCR analysis

DNA was isolated from one hundred and four *H. pylori* isolates using QIAGEN DNA kit (Germany) following the manufacturer's instructions. The extracted DNA was subjected to polymerase chain reaction (PCR) using FIREPOL® DNA polymerase (Solis BioDyne, Tartu, Estonia) for the efflux pump genes with different primer pairs (Table 1). The PCR cycling conditions were as follows: 95°C for 5 min, then 30 cycles at 95°C for 30 s, 60°C for 45 s and extension at 72 °C for 1min^[13].

Ethical approval

Ethical approval was obtained from NIMR- IRB (registration number IRB/14/285)

Statistical analysis

SPSS version 21 was used to analyze the association between efflux pump and antibiotic resistance using Pearson chi-square and influence of efflux pump on antibiotic resistance using linear regression with the significant level set at $p < 0.05$.

RESULTS

The result of the antibiotic susceptibility testing showed that 93% (97/104) of the isolates were resistant to metronidazole, 42% (44/104) to amoxicillin, 39% (41/104) to clarithromycin and 27% (28/104) to tetracycline with resistant values equal or above the break points > 0.125 µg/mL, 0.5 µg/mL, 8.0 µg/mL and 1 µg/mL for amoxicillin, clarithromycin, metronidazole and tetracycline respectively (Table 2). Analysis of the presence of efflux pump genes revealed that fifty three of the one hundred and four isolates (51%) possessed multidrug efflux pump gene *hefA* (*Hp605*) while thirty five (34%) possessed *hefD* (*Hp971*). However, none of the isolates possessed efflux pump gene *hefG* (*Hp 1327*) (Figures 1 and 2).

The multidrug resistance analysis showed that nine (8.7%) of the one hundred and four isolates were resistant to the four antibiotic used in the MIC test analysis. Three of these nine isolates harboured *hefA* which is 5.7% of the entire isolates that harboured *hefA* (3/53), similarly three out of this nine isolates harboured *hefD* which is 8.6% of the entire isolates that harboured *hefD* efflux pump gene (3/35) (Table 3). Twenty three of the one hundred and four (22.1%) isolates were resistant to three of the antibiotics, sixteen out of these twenty

Table 1 Primers used for the detection of multidrug resistance efflux pump genes

Efflux pump genes	SEQUENCES	AMPLICON SIZE
HP0605F (<i>hefA</i>)	ACGCCTCGAGTAAAAGCGCAAGGGAATTG	410bp
HP0605FR (<i>hefA</i>)	ACGCTCTAGATTCGCTAATTTGGCCTAGCAT	
HP0971F (<i>hefD</i>)	ACGCCTCGAGAAACCAACGTGGAAACCAAA	402bp
HP0971R (<i>hefD</i>)	ACGCTCTAGACGCTTTGCAAAATCCAAGAAT	
HP1327F (<i>hefG</i>)	ACGCCTCGAGGAACITTTGGTGCGTTGGAT	327bp
HP1327R (<i>hefG</i>)	ACGCTCTAGATTTGCTCCACCAATTAGCC	

Source: Amsterdam et al^[24].

Table 2 Susceptibility testing of *H. pylori* isolates by E-test

Sample Identification Number	Amoxicillin Res $\geq 0.12 \mu\text{g}/\text{MI}$	Clarithromycin Res $\geq 0.5 \mu\text{g}/\text{MI}$	Metronidazole Res $\geq 8 \mu\text{g}/\text{MI}$	Tetracycline Res $\geq 1 \mu\text{g}/\text{MI}$
N1-4A	0.19	0.016	256	1.5
N1-4C	0.19	0.016	256	0.016
N1-8A	9	10	24	32
N1-8C	4	1.125	24	0.22
N1-18A	10	0.19	256	1.5
N1-18C	10	0.19	256	1.5
N1-24A	0.25	44	20	0.25
N1-24C	16	4	32	0.22
N1-25A	12	16	256	0.125
N1-25C	96	0.032	20	0.25
N1-26A	0.023	0.38	256	0.19
N1-26C	0.19	0.25	256	0.32
N1-29C	160	38	16	256
N1-31C	0.07	256	16	4
N1-45A	0.15	0.03	24	2
N1-45C	16	0.016	24	12
N1-96A	0.047	0.016	256	0.047
N1-96C	0.047	0.016	256	0.064
N1-99A	0.016	0.016	256	0.016
N1-99C	0.016	0.19	256	0.016
N1-102A	0.19	1.5	256	0.5
N1-102C	0.19	1.5	256	0.125
N1-103A	0.19	0.016	256	0.38
N1-103C	0.016	0.016	256	0.016
N1-107A	0.032	0.016	256	0.016
N1-107C	0.19	0.016	256	0.094
N1-123A	0.125	256	256	0.125
N1-123C	0.19	256	256	0.094
N1-125A	0.023	256	256	0.032
N1-125C	0.023	256	256	0.094
N2-1A	0.086	0.283	256	2.095
N2-1C	0.086	0.283	256	2.095
N2-15A	0.016	0.016	256	0.016
N2-15C	0.016	0.016	256	0.064
N2-16A	1.125	0.625	64	0.214
N2-16C	32.095	0.074	12.032	0.44
N2-18A	0.016	0.016	256	0.016
N2-18C	256	0.38	256	0.38
N2-26A	256	0.19	256	1.5
N2-85A	0.25	0.38	256	0.125
N2-85C	0.25	0.38	256	0.125
N2-87C	0.016	0.016	256	4
N2-88A	0.016	0.016	256	0.19
N2-96A	0.016	0.016	256	0.125
N2-96C	0.016	0.19	256	0.125
N2-97A	0.047	8	256	0.125
N2-97C	0.38	6	256	0.19
N2-98A	0.064	0.016	256	0.032
N2-98C	0.064	1.5	256	0.38
N2-106A	0.016	256	256	256
N2-106C	0.016	256	256	256
N2-107A	256	256	256	256

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N2-107C	0.016	256	256	0.016
N2-108A	0.019	0.016	256	0.064
N2-108C	0.094	0.016	256	0.125
N2-119A	256	256	256	256
N2-119C	12	256	256	16
N2-122A	0.125	16	256	0.023
N2-122C	0.125	256	256	0.023
N2-123A	0.016	0.016	256	0.023
N2-123C	0.016	0.016	256	0.016
N2-130A	0.019	256	256	0.25
N2-130C	0.019	256	256	0.25
N2-136A	0.25	0.016	0.016	0.016
N2-136C	0.016	0.016	0.016	0.016
N2-138A	256	256	256	256
N2-138C	256	256	256	256
N2-139A	256	256	256	256
N2-139C	0.016	256	256	256
N2-140A	0.032	256	256	0.032
N2-140C	0.016	256	256	0.016
N2-141C	0.016	0.016	256	0.064
N2-144A	0.016	0.016	256	0.125
N4-15A	0.016	0.016	256	0.064
N4-15C	0.125	0.016	256	0.094
N4-16C	0.019	0.25	16	0.25
N4-19A	0.016	1.5	256	0.07
N4-19C	0.016	0.09	256	0.21
N4-29C	0.38	0.14	0.19	0.32
N4-77A	0.064	0.016	256	0.094
N4-77C	0.064	0.016	256	0.094
N4-89A	0.016	0.016	0.25	256
N4-89C	0.016	0.016	0.25	256
N4-92A	0.016	0.023	256	0.016
N4-92C	0.016	0.023	256	0.016
N4-94A	0.016	0.016	256	256
N5-4A	256	0.016	256	256
N5-4C	256	0.016	32	0.158
N5-6A	0.016	0.016	256	0.5
N5-6C	0.016	0.016	256	0.5
N5-44A	256	0.125	256	0.125
N5-44C	0.023	256	256	0.125
N5-46A	0.064	0.016	256	0.38
N5-46C	0.064	0.016	256	0.38
N5-52A	0.064	0.023	256	0.75
N5-52C	0.016	0.023	256	0.5
N5-53A	0.023	8	256	0.38
N5-53C	0.125	0.016	256	256
N5-54A	0.016	0.5	4	0.25
N5-61A	256	256	256	1
N5-61C	0.064	6	256	0.016
N5-90A	256	256	256	0.38
N5-90C	0.016	0.016	256	256
N5-91C	0.38	0.016	0.016	0.016

† RES -RESISTANT break point, Epsilometer test - E-test.

Table 3 Multidrug Resistance Analysis and Frequency of Efflux Pump Genes *hefA* and *hefD*

Multidrug (Amoxicillin, Clarithromycin, Metronidazole and Tetracycline)	Frequency of resistant Isolate	Efflux pump <i>hefA</i> frequency	Efflux pump <i>hefD</i> frequency
Resistance to 4 drugs	8.7% (9/104)	5.7% (3/53)	8.6% (3/35)
Resistance to 3 drugs	22.1% (23/104)	30.1% (16/53)	8.6% (3/35)
Resistance to 2 drugs	32.7% (34/104)	34% (18/53)	34.2% (12/35)
Resistance to 1 drugs	35.6% (37/104)	28.3% (15/53)	48.6% (17/35)
Sensitivity to all drugs	0.96% (1/104)	1.9% (1/53)	0% (0/35)

Table 4 Pearson chi-square - χ^2 analysis testing association of Drugs' resistance with efflux pump *hefA* and *hefD*

Association	P-values
Amoxicillin and <i>hefA</i>	0.572
Clarithromycin and <i>hefA</i>	0.014
Metronidazole and <i>hefA</i>	0.22
Tetracycline and <i>hefA</i>	0.575
Amoxicillin and <i>hefD</i>	0.004
Clarithromycin and <i>hefD</i>	0.735
Metronidazole and <i>hefD</i>	0.261
Tetracycline and <i>hefD</i>	0.506

three isolates harboured *hefA* which is 30.1% of the entire isolates that harboured *hefA* (16/53), similarly three of these twenty three isolates harboured *hefD* which is 8.6% of the entire isolates that harboured *hefD* efflux pump gene (3/35) (Table 3). Thirty four of the one hundred and four (32.7%) isolates were resistant to two of the antibiotics at the same time, eighteen out of these thirty four isolates harboured *hefA* which is 34% of the entire isolates that harboured *hefA* (18/53). Similarly twelve of these thirty four isolates harboured *hefD* which is 34.2% of the entire isolates that harboured *hefD* efflux pump gene (12/35) (Table 3). Thirty seven of the one hundred and four (35.6%) isolates were resistant to one antibiotic, fifteen out of these thirty seven isolates harboured *hefA* which is 28.3% of the entire isolates that harboured *hefA* (15/53). Similarly seventeen of these thirty seven isolates harboured *hefD* which is 48.6% of the entire isolates that harboured *hefD* efflux pump gene (17/35) (Table 3). Only one isolate (0.96%) was sensitive to all the antibiotics, it harboured *hefA* but *hefD* was absent (Table 3).

Pearson chi-square (χ^2) analysis gave the association between antibiotic resistance and efflux pump genes *hefA* with p-values as follows 0.575, 0.572, 0.220 and 0.014 for tetracycline, amoxicillin, metronidazole and clarithromycin respectively. Similarly analysis of association between antibiotic resistance and efflux pump genes *hefD* gave p-values as follows 0.735, 0.506, 0.261 and 0.004 for association with clarithromycin, tetracycline, metronidazole and amoxicillin respectively (Table 4). The level of influence of the efflux pump genes on all the antibiotics determined by linear regression was $r = 0.079$ for *hefA* and $r = 0.091$ for *hefD*.

DISCUSSION

The information on the antibiotic susceptibility patterns of *H. pylori* in Nigeria is relevant as a guide for the treatment options and efflux pump resistance mechanism in *H. pylori* may give a clue to acquired multidrug resistance mechanism^[18].

It was observed in this study that there is an increase in resistance of the *H. pylori* isolates to amoxicillin, clarithromycin and tetracycline while metronidazole has decreased in resistance. This is in contrast to the observations in a previous study that reported metronidazole resistant rate of 99.1%, amoxicillin 33.3%, clarithromycin 14.4% and tetracycline 4.5% in *H. pylori* isolates from Nigeria^[8]. Elsewhere in Brazil, there have been reports of 42%

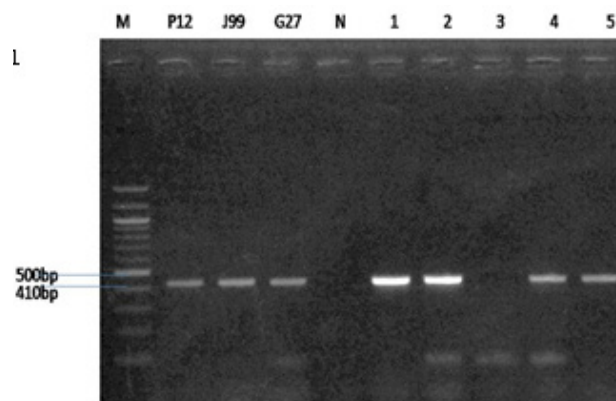


Figure 1 Agarose gel image of PCR products showing positive bands for *hefA* multidrug efflux pump gene, lane M: 100bp DNA marker, lane P12: positive control, lane J99 positive control, lane G27 positive, lane N: Negative control, lanes 1, 2, 4, 5 positive isolates for *hefA* gene, lane 3 negative for *hefA* gene.

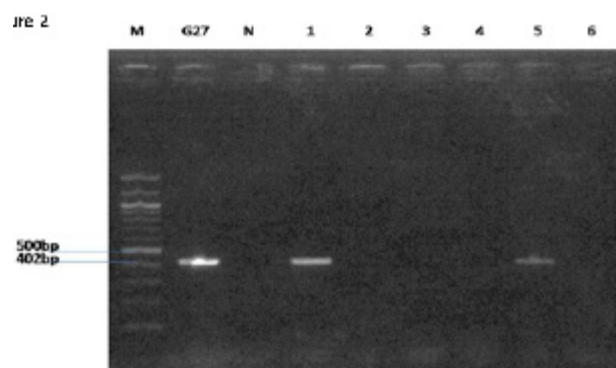


Figure 2 Agarose gel image of PCR products showing positive bands for *hefD* multidrug efflux pump gene, lane M: 100bp DNA marker, lane G27 positive, lane N: Negative control, lanes 1 and 5 positive isolates for *hefD* gene, lane 2, 3, 4 and 6 negative for *hefD* gene.

metronidazole resistance, 29% amoxicillin, 7% clarithromycin and tetracycline in dyspeptic patients^[19]. Findings around the world for a period of 5 years (2009-2014) revealed antibiotic resistance range from 30.5%-75.02% for metronidazole, 2% - 40.87% amoxicillin, 5.46%-30.8% clarithromycin, 0%-50% for tetracycline and all these findings suggested a progressive increase in resistance rate which may inhibit eradication therapy of *H. pylori* infections^[20].

This current study establish the presence of efflux pump genes in *H. pylori* strains from Nigeria, the *hefA* and *hefD* efflux pump genes have been identified in *H. pylori* isolates from Nigeria but *hefG* was absent, this finding is in agreement with the study carried out by Liu *et al*^[18] who reported that *hefA* and *hefD* genes were detected in multidrug resistant *H. pylori* isolates and they also fail to detect *hefG*^[18]. The absence of *hefG* genes had been attributed to the presence of a regulatory mechanism controlling the expression of the gene^[21,22].

The increased presence of efflux pump genes *hefA* (69.8%) and *hefD* (51.4%) in multidrug resistance *H. pylori* isolates is consistent with the findings of Liu *et al.*^[18], who reported increased detection of efflux pump genes, according to the author these genes play important role in multidrug resistance of *H. pylori* with statistical analysis showing efflux pump gene *hefA* was associated with clarithromycin (p -value = 0.014) and *hefD* with amoxicillin (p -value=0.004)^[18]. Huang *et al.*^[23] also confirmed that the presence of *hefA* genes can confer multidrug resistance on *H. pylori*. These authors treated selected multidrug resistant *H. pylori* with *hefA* genes with emodin, baicalin, schizandrin, berberine which inhibited the expression of *hefA*; they observed a significant decrease in the minimum inhibitory concentration value of *H. pylori* to amoxicillin and tetracycline^[23].

Similarly the discovery from the other studies by Bina *et al.*^[21] and Kutschke and Jonge^[22] also confirmed the presence of *hefD* in *H. pylori* isolates from Australia, Sweden, Argentina, the United States and Canada which was 34% in Nigerian isolates^[21,22]. Harrison *et al* reported the absent of point mutation of 23S rRNA in clarithromycin resistance however this study suggest the possible cause could be the presence of efflux pump genes^[8].

This current study, which is the first on efflux pump antibiotic resistance mechanisms in Nigerian *H. pylori* isolates, indicated that efflux pump may be an important mechanism of resistance and should be considered in *H. pylori* resistant mechanism.

The present study revealed that resistance of *H. pylori* to antibiotics used in the management of dyspeptic ulcer is on the rise in Nigeria and also establish for the first time that the mechanism of resistance may be due to the presence of a multidrug efflux pump system.

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Authors' contributions

Manuscript preparation and Clinical studies-JTF, Manuscript Editing and Clinical studies- AIA, SSI, NHA, Clinical studies - FMA, OC, UR, AIL, LO, NDA, AO, IA, BM, NFN.

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