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 *hefD* (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[14,15].

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%/9. 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[10]. However in Nigeria Harrison et al[10] 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[10]. 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), 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 > 0.125µg/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

| Efflux pump genes | SEQUENCES | AMPLICON SIZE |
|-------------------|-----------|---------------|
| HP0605F (hefA)    | AGCCGTCGACTGAGAGGCAAAGCCAGGTGG   | 410bp |
| HP0605FR (hefA)   | ACGGCTTTCAGTTGCAATTGCTAGCAT   | 402bp |
| HP0971F (hefD)    | AGCCGTCGAGGAAACCGGTAATTGGG   | 327bp |
| HP0971R (hefD)    | AGCCGCTATAGGCCTTTGCTTATCAGAT   | 327bp |
| HP1327F (hefG)    | AGCCGCTCGAGGAAACTTGGTGTGGAT   | 327bp |
| HP1327R (hefG)    | AGCCGCTATAGGTTCCTGACCAATTTAGG | 327bp |

Source: Amsterdam et al[24]
Table 2 Susceptibility testing of H. pylori isolates by E-test

| Sample Identification Number | Amoxicillin Res ≥ 0.12 µg/Ml | Clarithromycin Res ≥ 0.5 µg/Ml | Metronidazole Res ≥ 8 µg/Ml | Tetracycline Res ≥ 1 µg/Ml |
|-----------------------------|-------------------------------|--------------------------------|----------------------------|---------------------------|
| N1-4A                       | 0.19                          | 0.016                          | 256                        | 1.00                      |
| N1-4C                       | 0.19                          | 0.016                          | 256                        | 0.016                     |
| N1-8A                       | 9                             | 0.19                           | 256                        | 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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| Gene   | MIC 1 | MIC 2 | MIC 3 | MIC 4 |
|--------|-------|-------|-------|-------|
| 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 | 0.23  | 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.
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). 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 (x²) analysis gave the association between antibiotic resistance and efflux pump genes hefA with p-values as follows 0.572, 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[19]. 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[18]. Elsewhere in Brazil, there have been reports of 42% metronidazole resistance, 29% amoxicillin, 7% clarithromycin and tetracycline in dyspeptic patients[8]. 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[15]. The absence of hefG genes had been attributed to the presence of a regulatory mechanism controlling the expression of the gene[21,22].

**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 hefA frequency | Efflux pump hefD 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 - χ² analysis testing association of Drugs’ resistance with efflux pump hefA and hefD

| Association | P-values |
|---|---|
| Amoxicillin and hefA | 0.572 |
| Clarithromycin and hefA | 0.014 |
| Metronidazole and hefA | 0.22 |
| Tetracycline and hefA | 0.575 |
| Amoxicillin and hefD | 0.004 |
| Clarithromycin and hefD | 0.735 |
| Metronidazole and hefD | 0.261 |
| Tetracycline and hefD | 0.506 |

**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.
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.7-9, 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)10. Huang et al.11 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 tetracycline12.

Similarly the discovery from the other studies by Bina et al.13 and Kutschke and Jonge14 also confirmed the presence of hefD and hefD in H. pylori isolates from Australia, Sweden, Argentina, the United States and Canada which was 34% in Nigerian isolates21-22. 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 genes15.

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, AII, LO, NDA, AO, IA, BM, NFN.

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