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Plasmid Analysis and Curing of Multidrug Resistant of *Helicobacter pylori* Isolated from Ulcer Patients in Ondo State, Nigeria

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Authors' contributions

This work was carried out in collaboration among both authors. Authors MAB and AKO designed the study, Author MAB carried out the research, Author AKO supervised the research, Author MAB performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors MAB and AKO managed the analyses of the study. Author MAB managed the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

Plasmid profile of multidrug resistant *Helicobacter pylori* isolated from ulcer patients in Ondo State, Nigeria was investigated. It was observed that the *H. pylori* isolated from ulcer patients in the course of the research were resistance to certain antibiotics. Consequently on the emergence of multiple drug resistance exhibited by the *H. pylori,* a total of 9 different antibiotics were tested against the *H. pylori* isolated from various locations to determine their *in-vitro* susceptibility pattern. The findings of the *in-vitro* antibiotics susceptibility testing are quite astonishing due to a high incidence of multiple drug resistance. Majority of the *H. pylori* showed high level of resistance against some specific number of antibiotics. The multidrug resistance observed in this study seems to be plasmid mediated. Moreover frequent use of over-the-counter antibiotics and abuse of non steroidal anti inflammatory drugs (NSAIDs) administration and abuse of drugs of sub – standard chemical quality has been identified as a major problem. The results from this investigative study have shown that resistance in *H. pylori* was plasmid based.

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1. INTRODUCTION

Helicobacter pylori, previously known as *Campylobacter pylori*, is a Gram negative, microaerophilic bacterium usually found in the stomach. It was identified in 1982 by Australian scientists Barry Marshall and Robin Warren, who found that it was present in a person with chronic gastritis and gastric ulcers, conditions not previously believed to have a microbial cause. It is also linked to the development of duodenal ulcers and stomach cancer. However, over 80% of individuals infected with the bacterium are asymptomatic, and it may play an important role in the natural stomach ecology [1].

Helicobacter pylori is one of the primary causes of upper gastrointestinal diseases, including dyspepsia, peptic ulcer diseases, heartburn, gastroesophageal reflux disease and even malignant transformation. It is the most common infectious human pathogen, infecting more than 50% of the populations worldwide (approximately 30% of children and 60% of adults), and is associated with 70% of benign gastric ulcers and 90% of duodenal ulcers [2,3].

H. pylori is a common type of bacteria that grows in the digestive tractand has a tendency to attack the stomach lining. It infects roughly 60 percent of the world's adult population which majority are asymptomatic. *H. pylori* infections are usually harmless, but they're responsible for the majority of ulcers in the stomach and small intestine.

Helicobacter pylori can inhibit various areas of the stomach, particularly the antrum where it causes gastritis (a chronic low-level inflammation of the stomach lining) and is strongly linked to the development of peptic or gastric ulcer, duodenal ulcer, gastric carcinoma and mucosa-associated lymphoid tissue lymphoma [4]. Approximately 50% of the world population is infected making it the most widespread infection in the world [5]. However, only about 10-20% of infected person become symptomatic [6]. Although, infection with *H. pylori* occurs worldwide, prevalence varies greatly among nations and among population groups in the same nations. Its occurrence is more common in developing countries with over 80% among middle-aged adults as compared to 20-50% in developed countries (Western Europe, Australia, Asia, North America) [6]. Infections are usually acquired in early childhood in all countries [7].

By the late 19th and early 20th centuries, several investigators had reported the presence of spiral microorganisms in the stomachs of animals. Soon afterward, similar spiral bacteria were observed in humans [8], some of whom had peptic ulcer disease or gastric cancer.

Antibiotic resistance is an ever increasing problem associated with the treatment of most microbial infections including *Helicobacter pylori* infection which is shown to be the major cause of gastritis and peptic ulcer disease [9]. Chronic infection with *H. pylori* is an accepted risk factor in the development of gastric cancer [10].

The aim of this study was to examine plasmid profile analysis and curing of multidrug-resistant *Helicobacter pylori* isolated from ulcer patients in Ondo State, Nigeria.

2. MATERIALS AND METHODS

2.1 Description of Study Location/Area

This research work was conducted in different hospitals across Ondo State, Nigeria. The eighteen (18) local government areas in Ondo State includes Akure South, Akure north, Ondo west, Ondo East, Akoko North west, Akoko North East, Akoko South East, Akoko South West, Owo, Ose, Ore, Ilaje, Ifedore, Odigbo, Okitipupa, Ikare, and Idanre. Ondo state covers an area of 15,195.2 sqaure kilometers and lies at latitude 7°10′ North and longitude 5° 05′ east. A total of 355 ulcer patients which include infants (1 – 9 years), 10-19, 20-29, 30-39, 40-49, 50-59 and 60 years and above were included in this study. Patients on ulcer medication or any other antibiotics within 7 days prior to specimen collection were excluded. Informed consent was obtained from the suspected patients and asked to fill a questionnaire provided prior to specimen collection. Ethical approval for the study was obtained from the Ethics and Research Committee of the various government hospitals across Ondo State.

2.2 Collection of Samples

The Clinical Specimens were obtained from suspected cases of ulcer patients from the selected hospitals in Ondo State, which includes;

State Specialist Hospital Akure, Miteda Hospital Akure, and Ikaramu-Akoko Health Centre between June 2014 to February 2016 and were retrospectively studied. Three gram (3 g) of stool samples were collected and put onto the *Helicobacter pylori* portagerm, a semisolid agar medium and transported in a cold ice packs bag at 4°C. Prior to this, the patients had been tested positive to *Helicobacter pylori* infection using urease breath test and stool antigen test kits respectively.

2.3 Isolation of Bacteria

In isolating bacteria from the clinical specimens collected, one ml of each samples were aseptically collected and aseptically transferred into sterile McCartney bottle containing 9ml of sterile distilled water. Serial dilution were made to the fifth dilution factor and plated out on nutrients agar, Eosin methylene blue agar and Columbia blood agar plates. All the plates were incubated and observed for growth after 24 hrs at 37°C. Colonies were counted and recorded as colony forming units per gram (cfu/g).

2.4 Preparation and Isolation of *H. pylori* Inoculum

The stool samples collected were homogenized with peptone buffer solution (PBS) (250 mg in 1 ml PBS), then sieved the slurry with 0.25 mm Millipore filter and centrifuged at 20,000 xg for 30 minutes. The pellets were washed in PBS while the resulting pellets were plated out on Dent's medium reconstituted with Columbia blood agar, incubated at 37°C under microaerophillic conditions for 3-7 days.

The pure stock cultures of each isolates were scrapped from the plates and transferred onto Muller Hinton agar slants and maintained at 4^0C. Active cultures for experiments were prepared by transferring a loopful of cells from the stock cultures to bottles of Muller Hinton broth. This was incubated for 72 hrs at 37°C under microaerophillic conditions and maintained the inoculums at 4°C.

2.5 Identification of Bacteria

Appearance of the colony isolated on the agar media was studied. Characteristics observed includes: shape, edge, colour, elevation after 24 hrs of incubation and 72 hrs for *H pylori*. Staining reaction and other biochemical tests were done in accordance with the method from Holt and Krieg [11] and Olutiola et al. [12] and identification of bacterial isolates was carried out to species level using Cowan and Ste]el [13] method.

3. ANTIBIOTICS SUSCEPTIBILITY TEST

3.1 Standardization of Inoculums

One percent (1%) of solution of H_2SO_4 was prepared by adding 1 ml of concentrated sulphuric acid to 99 ml of distilled water, after which the solution was mixed properly. Also, 1% solution of barium chloride was prepared by dissolving 0.5 g dehydrated barium chloride (BaCl. $2H_2O$) in 50 ml of distilled water. A 0.6 ml aliquot of Barium Chloride solution was added to 99.4 ml of the 1% sulphuric acid solution and it was then thoroughly mixed together. The solution was transferred in to covered tube of the same type used for both the control and the test inoculum. The solution was kept at 4°C [14].

3.2 Antibiotic Sensitivity Test

The antibiotic sensitivity profile was investigated in order to compare the sensitivity of the microorganisms to the different conventional antibiotics. The disc diffusion method described by Bauer et al. [15] was used to determine the susceptibility and resistance of the organisms to the antimicrobial drugs.

3.3 Plasmid Analysis

A 1.5 ml of an overnight culture of *H. pylori* was pipetted onto eppendorf tube, placed in a micro-centrifuge and spinned to pellet cells for 1-2 minutes at 7000 rpm. The supernatant was gently decanted leaving the sediment. To the sediment, 20 µl of Tris-EDTA (TE) was added to each sample and agitate well and close the tube. A 10 µl of distilled water was added to make it in liquid form. Later, 200 µl of SDS solution was added to each sample and rock very well, while leaving for 5 minutes at room temperature. To the samples, 150 µl of 3.0 M sodium acetate with PH 5.2 were added and vortexed for 2-5 seconds to mix completely. The mixture was spinned for 5minutes in micro-centrifuge to pellet debris and chromosomal DNA. The supernatant was decanted and transferred to a fresh eppendorf tube and incubated in ice (Freezer) for 30 minutes at -15°C. The supernatant was mixed well with 400 µl of cold absolute ethanol,

incubated in ice again at -15°C for 1 hour and spinned for 5 minutes to pellet plasmid (white pellet). The supernatant was discarded and dried in vacuo for 2-3 minutes and was re-suspended by adding 20 µl of Tris-EDTA (TE) buffer for further use.

3.4 Plasmid Curing

A 5 µl aliquot of Acridine orange (0.10 mg/ml) was added to 5 ml of Luria Beriaru (LB) broth. Plasmid containing cultures were inoculated to LB broth having acridine orange. It was incubated in a shaker incubator for 24 hours. Thereafter, the culture was swabbed into the Mueller-Hinton agar plates. The antibiotics disc was inserted into the plates and the plates were incubated for 72 hours at 37°C under microaerophillic conditions [16,17,18].

3.5 Post Curing Sensitivity Testing

The plasmid cured isolates were subjected to those antibiotics disks to which they were previously resistant. A 1ml aliquot of 18 hours broth culture that had been adjusted to the 0.5 McFarland standards was dispensed into sterile petri dishes. Molten sterile Mueller- Hinton agar was aseptically poured into the plates and were gently rotated for the organisms to be homogenously distributed in the medium. The agar was allowed to solidify, after which a sterile cork borer of 6mm in diameter was used to cut uniform wells in the agar plates. The wells were later filled with .5ml of each extract. In addition, 20% Tween 20 was used as the negative control while chloramphenicol served as the positive control. The experiment was conducted in triplicate. All the plates were incubated at 37°C for 72 hours. Clear zones around the wells were measured in millimetre. The diameter of zones of inhibition was measured using meter rule in millimeter and the zones were compared to standard antibiotics chart [19].

3.6 Statistical Analysis Of Data

Data obtained were subjected to one way analysis of variance, while the means were compared by Duncan's New Multiple Range Test at 95% confidence interval using Statistical Package for Social Sciences version 16.0. Differences were considered significant at $p \leq 0.05$.

4. RESULTS

Plate 1. is showing the Agarose gel electrophoresis of plasmid DNA of *H. pylori* isolated from ulcer patients

Table 1. is showing Antibiotic sensitivity patterns of *H. pylori* isolated from ulcer patients

Table 2. Post Curing antibiotic sensitivity patterns of *H. pylori*isolated from ulcer patients was presented in Table 2.

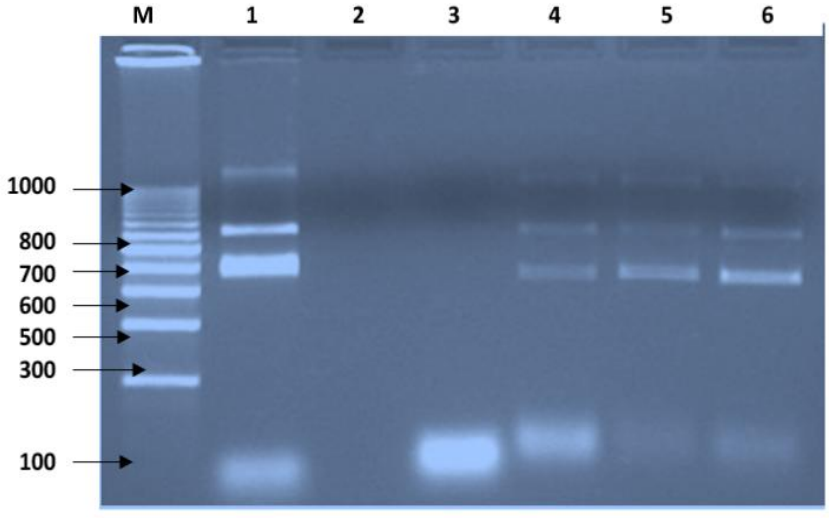

Plate 1. Agarose gel electrophoresis of plasmid DNA of *H. pylori* from patients

M; Maker, Lanes; 1 H. pylori isolated from SSH Akure, lane 2 H. pylori isolated from Miteda Hospital, lane 3 and 4 H. pylori isolated from IkaramuAkoko, lane 5 and 6 H. pylori isolated from SSH Ondo

Table 1. Antibiotic sensitivity patterns of *H. pylori*

Orgs	SXT	CH	CPX	AM	AU	GN	PEF	OFX	S
A_1	0.00±0.00^{a}	0.00±0.00^{a}	1.67±0.33^{bc}	5.33±0.33^{e}	3.33±0.33^{d}	1.33±0.33^{b}	2.33±0.33^{c}	0.00±0.00^{a}	0.00±0.00^{a}
A_2	1.67±0.33^{a}	6.67±0.33^{c}	12.33±0.33^{d}	14.33±0.33^{e}	13.33±0.33^{de}	4.33±0.33^{b}	12.33±0.33^{d}	13.33±0.33^{e}	1.33±0.33^{a}
B_1	2.33±0.33^{b}	0.00±0.00^{a}	3.33±0.33^{c}	0.00±0.00^{a}	5.33±0.33^{d}	0.00±0.00^{a}	9.33±0.33^{f}	7.33±0.33^{e}	00.0±0.00^{a}
B_2	0.00±0.00^{a}	7.33±0.33^{d}	0.00±0.00^{a}	12.67±0.33]^{f}	2.33±0.33^{b}	5.33±0.33^{c}	9.33±0.33^{e}	2.33±0.33^{b}	0.00±0.00^{a}
C_1	0.00±0.00^{a}	3.33±0.33^{b}	0.00±0.00^{a}	0.00±0.00^{a}	4.33±0.33^{c}	9.33±0.33^{d}	10.67±0.33^{e}	2.67±0.33^{b}	0.00±0.00^{a}
C_2	0.00±0.00^{a}	8.33±0.33^{d}	0.00±0.00^{a}	2.67±0.33^{b}	2.33±0.33^{b}	6.33±0.33^{b}	6.67±0.33^{c}	8.33±0.33^{d}	0.00±0.00^{a}

Data are presented as Mean ± S.E (n=3). Values with the same superscript letter(s) along the same row are not significantly different (P<0.05); Legend: A_1 = H. pylori isolated from SSH, Akure, A_2 = H. pylori isolated from Miteda Hosp. Akure; B_1 and B_2 = H. pylori isolated from IkaramuAkoko Health Centre; C_1 and C_2 = H. pylori isolated from SSH, Ondo; SXT = Septrin; CH = Chloramphenicol; CPX =Ciprofloxacin; AM =Amoxicillin; AU =Augumentin; GN = Gentamycin; PEF =Pefloxacin; OFX =Ofloxacin; S = Streptomycin

Table 2. Post Curing antibiotic sensitivity patterns of *H. pylori*

Orgs	SXT	CH	CPX	AM	AU	GN	PEF	OFX	S
A_1	9.33±0.33^{b}	14.33±0.33^{d}	12.33±0.33^{c}	12.33±0.33^{c}	16.67±0.33^{f}	15.33±0.33^{e}	12.33±0.33^{c}	11.33±0.33^{c}	6.33±0.33^{a}
A_2	1.67±0.33^{a}	6.33±0.33^{c}	12.33±0.33^{d}	14.67±0.33^{e}	13.33±0.33^{d}	4.33±0.33^{b}	12.33±0.33^{d}	14.33±0.33^{e}	1.67±0.33^{a}
B_1	12.33±0.33^{d}	12.33±0.33^{d}	9.67±0.33^{c}	7.33±0.33^{b}	12.00±0.00^{d}	5.67±0.33^{a}	12.33±0.33^{d}	15.33±0.33^{e}	7.33±0.33^{b}
B_2	10.33±0.33^{c}	8.67±0.33^{b}	18.33±0.33^{g}	16.33±0.33^{f}	16.67±0.33^{f}	14.33±0.33^{d}	17.33±0.33^{f}	15.33±0.33^{e}	3.33±0.33^{a}
C_1	7.33±0.33^{c}	15.33±0.33^{f}	7.67±0.33^{c}	5.67±0.33^{b}	9.33±0.33^{d}	10.33±0.33^{d}	18.33±0.33^{g}	13.33±0.33^{e}	1.33±0.33^{a}
C_2	9.33±0.33^{bc}	15.33±0.33^{f}	8.33±0.33^{b}	9.67±0.33^{c}	13.33±0.33^{d}	8.33±0.33^{b}	17.33±0.33^{g}	14.33±0.33^{e}	2.33±0.33^{a}

Data are presented as Mean ± S.E (n=3). Values with the same superscript letter(s) along the same row are not significantly different (P<0.05); Legend: A_1 = H. pylori isolated from SSH Akure; A_2 = H. pylori isolated from MitedaHospAkure; B_1 and B_2 = H. pylori isolated from IkaramuAkoko; C_1 and C_2 = H. pylori isolated from SSH Ondo; SXT = Septrin; CH = Chloramphenicol; CPX =Ciprofloxacin; AM =Amoxicillin; AU =Augumentin; GN = Gentamycin; PEF =Pefloxacin; OFX =Ofloxacin; S = Streptomycin

5. DISCUSSION

The aim of the study was to present plasmid profile analysis and curing of multidrug resistant *Helicobacter pylori* isolated ulcer patients in Ondo State, Nigeria. It was observed that the *H. pylori* isolated from ulcer patients in the cause of the study were resistance to antibiotics. Consequently on the emergence of multiple drug resistance exhibited by the *H. pylori,* a total of 9 different antibiotics were tested against the *H. pylori* isolated from various locations to determine their *in-vitro* susceptibility pattern. The findings of the *in-vitro* antibiotics susceptibility testing were quite astonishing due to a high incidence of multiple drug resistance. Majority of the *H. pylori* showed high level of resistance against a number of antibiotics.

This has posed a medical challenge and this might occurred due to the rate at which organism multiply and change their genetic materials or acquire new genes resulted from improper or drug abuse by the patients from prior or low compliance level . This organism exerts biochemical resistance either by preventing the entry of the drug or by rapidly extruding the drug or by enzymatically inactivating the drug or altering its molecular target [14]. Almost all the isolates were resistance to Septrin, Chloramphenicol, Ciprofloxacin, Amoxicillin, Augumentin and Streptomycin which corroborates the findings ofAboderin et al. [20] who submitted that *H. pylori* isolates are resistance to Amoxicillin. The antibiotic resistance pattern encountered in this study exhibited significantly high resistance to Septrin, Chloramphenicol Streptomycin and Amoxicilin with moderate resistance to Augumentin, Gentamycin and Ofloxacin. This is in agreement with the findings of Smith et al. [21] who also reported most of the tested isolates to be moderate resistance to Augumentin and Ofloxacin.

The multi–drug resistance observed in this study seems to be plasmid mediated moreover frequent use of over- the- counter antibiotics and abuse of non steroidal anti inflammatory drugs (NSAIDs) administration and abuse of drugs of sub – standard chemical quality has been identified as a major problem. Penfold et al. [22] first reported 48% of clinical isolates of *Campylobacter pylori* to possess plasmids, while 8-32% positivity for plasmids in fresh clinical isolates of *H. pylori* has been reported subsequently [23]

This affects the bioavailability of the agents with its attendant biological clinical and epidemiological consequences [24]. This situation and the ever increasing trend of antimicrobial resistance among *H. pylori* infection have continued to prompt calls to reduce unnecessary antibiotic use and improve treatment protocols in order to maximize the life span of these drug. The introduction of highly active broad spectrum antibiotics with triple therapy of drugs viz; Clarithromycin, Amocixillin and proton pump inhibitors (PPI) has improved the situation in Ondo State, Nigeria with regards to the treatment of *H. pylori* infections.

Plate 1 revealed the plasmid profile of the antibiotics resistance patterns of the *H. pylori* isolated from various locations. Out of six (6) *H. pylori* isolates, five (5) isolates contain plasmid bands which might be responsible for the resistant exhibited by these isolates to antibiotics used. This is in accordance with the findings of Thomas et al.[25] who reported that plasmid mediated mechanism may increase the likelihood of horizontal spread.

Plasmid had been documented to have encoded genes that provide resistance to antibiotics in Competitive environmental niche [26] .Multidrug resistance has been ascribed to other factors such as integrons. These factors, which could mediate resistance, were first described by Stoke and Hall in 1989. They are a group of apparently mobile elements that can contain one or more antimicrobial resistance genes, and have been found in a variety of bacteria, hence they are thought to be largely responsible for the dramatic increase in multidrug resistance. Its therefore, not surprising that integrons carrying antimicrobial resistance genes that have detected in plasmid and transposons as well as in chromosomal DNA of some bacteria [27]. Comparisons of the Sensitivity pattern of the five (5) out of six (6) isolates containing plasmid before, however, after curing it can be concluded that resistance were majorly due to plasmid. Although, resistance after curing may also due to chromosome or efflux pump mechanism [28] or other factor like mutation of the bacteria. All the five isolates cured were carrier of 1 to 4 plasmids each with molecular weight 100bp, 700bp, 800bp and 1000 bp when compared with Hind III lambda as a molecular marker. Kroll et al.[26] reported that plasmid mediated mechanisms may increase the likelihood of horizontal spread of resistance.

The compliance level of ulcer medication on the occurrence of *H .Pylori* infection among the patients was examined. The finding shows that 86(24.2%) out of 355 strictly adhere to their medication, 152 (42.8%) averagely adherence to their medication, 111(31.2 %) of the patients had weak compliance level while 6(1.7%) of the patients had poor adherence level.

This could be explained by the fact that this habits had called for multiple drugs resistance as revealed in the study and this is in accordance with the findings of Smith et al.[21].

6. CONCLUSION

This investigation has been able to investigate plasmid profile analysis and curing of multidrug resistant *Helicobacter pylori* isolated from ulcer patients in Ondo State, Nigeria. *Helicobacter pylori* have high multiple resistances to antibiotics in the course of the research and after the resistant *Helicobacter pylori* were subject to plasmid curing the microorganisms were susceptible to antibiotics.

CONSENT

Written consent was obtained from the suspected patients and asked to fill a questionnaire provided prior to specimen collection.

ETHICAL APPROVAL

Ethical approval for the study was obtained from the Ethics and Research Committee of the various government hospitals across Ondo State.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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