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Evaluation of Invasive and Non-Invasive Methods of Diagnosing *Helicobacter pylori* Infection in Port Harcourt, Nigeria

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Several diagnostic approaches are available for the detection *of Helicobacter pylori* The aim of this study was to evaluate the non-invasive and invasive methods of diagnosing *H. pylori* infections. A total of three hundred (300) samples each for stool and blood were subjected to non-invasive techniques. The invasive method used in this study was the polymerase chain reaction method (PCR) targeting the 16SrRNA from stool samples while the non-invasive methods used were stool antigen test, urea breath, and serology test. The amplified 16SrRNA fragments of *H. pylori* from various samples on agarose gel showed their bands at 380 base pairs. Results obtained from this study showed that the PCR method recorded a 54% prevalence rate, the stool antigen test recorded

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a 13.7% prevalence rate while serology and urea breath test methods recorded a prevalence rate of 14.7% and 11.7% respectively. This study recorded a prevalence rate of 33.6% based on the study location, with females recording the highest prevalence rate in all the diagnostic methods used. The sensitivities of the various methods used against PCR were 90.3% for the stool antigen test method, 64.5% for the urea breath test, and 100% for the serology test method; while their specificities were 26.3% for both the stool antigen test and urea breathe test and 79% for serology test. The serology method showed a likelihood ratio of 4.76 while that of the stool antigen test and urea breath test were 2.354 and 0.462 respectively. In conclusion, this study has shown that the order of accuracy of diagnostic methods can be arranged as follows: PCR> serology > stool antigen test > urea breath test. However, the order might slightly change among related studies. Generally, the invasive method is preferred over the non-invasive method in developed countries while simultaneous use of more than one non-invasive method is encouraged in developing countries.

Keywords: Helicobacter pylori; polyclonal; agarose gel electrophoresis; serology test; urea breath test; stool antigen test.

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1. INTRODUCTION

The burden of disease conditions caused by *Helicobacter pylori (H. pyroli)* is enormous and has been a major challenge for Public Health. There are various techniques for detecting this organism but no single method has been generally accepted, thus, the organism is mostly either under-diagnosed or over-diagnosed in our routine laboratories. Healthcare givers have been attending to the patient with vague abdominal pain and which were treated as peptic ulcers.

Several diagnostic approaches are available for the detection *of H. pylori.* The choice of the approach is dependent on the factors such as accessibility of the test method, advantages and disadvantages, age of the patient, cost of each approach and need to perform endoscopy. The approaches are generally grouped into invasive and non-invasive techniques [1]. The use of a single technique is discouraged because there is no single technique that can satisfy on its own the required sensitivity and specificity in the identification of *H. pylori.*

Non-invasive methods have been marked as the preferred methods for laboratory diagnosis of gastric disorders caused by *H. pylori*, with stool antigen testing and urea breath testing being the current preferred tests [2]. The stool antigen test is an enzyme immunoassay that was established over a decade ago for the detection of *H. pylori* antigen in stool samples. This test passed through evaluation for the initial diagnosis and confirmation of clearance of *H. pylori* after treatment. The test employs the use of a polyclonal anti-*H. pylori* capture antibodies absorbed by microwells. The sensitivity and specificity as recorded by Gatta et al. [3] are 92.1% and 87.6% respectively. The stool antigen test only requires an optical spectrophotometer, which is usually present in the laboratory, with less cost for maintenance and has no special need for dedicated personnel.

The urea breath test is another type of noninvasive technique, regarded as the best private practice in the diagnosis of *H. pylori*. It involves the use of an expensive mass spectrophotometer. Routine urea breath protocol uses a test meal to setback gastric emptying and to permit time for even circulation of urea throughout the stomach. It was however observed that the absence of a test meal has no effect on the accuracy of the result [3]. The protocol formerly employed the baseline sample and sample collected 30 minutes after ingestion of urea; presently, a tablet formulation is available, making the breath sampling possible 10 minutes after ingestion with excellent accuracy and averting the interference from urease-producing bacteria in the oropharynx, which may cause false-positive result [3]. The sensitivity and specificity of the urea breath test method were 94.7% and 95.7% as reported by Allahverdiyev et al. [4].

The serology test is based on the detection of specific anti-*H.pylori* IgG antibodies in the serum of a patient. It was the first non-invasive method for *H. pylori* detection. It was unable to differentiate between active infection and a previous contact because antibody level remains the same in the blood for a long time, leading to false-positive results [3]. The use of this technique is better employed in the assessment of antibody detection against pathologic markers such as CagA and VacA proteins using western blot, ELISA, and recombinant immunoblot

approaches for research purposes. CagA-positive strain of *H.pylori* exposes the infected individuals to more severe inflammatory reactions, higher incidence of duodenal ulcer, severe gastric atrophy, intestinal metaplasia, and intestinal-type gastric cancer. The vacuolating toxin VacA is an 87-kDa protein secreted by the Helicobacter strain and activated at low pH. This protein is resistant to acid and pepsin, causes vacuolation in cell culture *In vitro*, and damages mouse gastric epithelium *In vivo* [5].

Invasive techniques include the urease test, Histology test, and culture test methods. The molecular technique is both invasive and non-invasive. The urease test is easy to perform rapid test method but the result is influenced by factors that antagonize the activity of urease such as the use of antimicrobial agents, bismuth-containing compounds, and proton pump inhibitors. Moreover, other microorganisms which produce urease may give false-negative results [6]. Histology has been measured as the gold standard for the diagnosis of *H. pylori* infection. This is because the technique provides additional and vital knowledge on the condition of mucosa e. g the presence of chronic or acute swelling, lymphoid aggregates, intestinal metaplasia, and granular degeneration. Although the site of specimen collection and the number of specimens collected affect the reliability of the result.

Culture media such as Brain Heart Infusion broth supplemented with glycerol, Columbia agar enriched with 7% sheep blood, and antibiotic-containing selective mixture are available for isolation and sensitivity testing of *H pylori* [7]. This technique requires a lot of precautionary measures and certain controlled conditions such as incubation in an anaerobic jar under microaerophilic conditions (5-10% Oxygen and 10% carbon dioxide) and monitoring of bacterial growth. A positive culture is confirmed using a series of biochemical tests such as catalase, urease, and oxidase test [6]. Cultivation of *H. pylori* from gastric biopsies of a patient is the specific and most sensitive method of diagnosing disease conditions caused by this organism [4].

The molecular technique involves the use of polymerase chain reaction to detect *H. pylori* DNA in all forms of required specimens. Genes such as the 26k species-specific antigen gene, *glmM, ureA, ureB,* 16S RNA, vacA, and cagA genes are target genes for the molecular detection of *H. pylori* [6]. The sensitivity and specificity of PCR in the detection of *H. pylori* were 100% and 75% respectively as reported by Ramis et al, [6].

2. METHODOLOGY

2.1 Study Area

The study was done in Port Harcourt, Rivers State where clinical samples were collected and conventionally analyzed. The molecular analysis was done in Amassoma, Bayelsa State. Both cities are situated in the Niger Delta Region of Nigeria.

2.2 Study Design

The study was carried out among three hundred (300) randomly selected patients who were suspected to be infected with *H. pylori* and had visited Health centers in Rivers state (Rumuigbo, Okija, Police clinic, Rukpokwu and Akpajo) for a period of twenty months (April, 2017 to December, 2018). The patients were both males and females within all age range. Fifty control samples were also collected from apparently healthy individuals. All samples were collected under supervision.

2.3 Sample Size Determination

Determination of sample size was done using the formula [8].

$$n = \frac{z^2 pq}{d^2}$$

Where

n = sample size minimum
z = 95% confidence interval = 1. 96
P = proportion of the target population
q = 1.0 – p
d = with, degree of accuracy (95% interval) = 0.05%
P = 28% = 0.28

$$\frac{(1.96)^2 \times 0.28 \times 0.72}{(0.05)^2}$$

=309.79
=300 samples

2.4 Sample Collection

Stool specimens were collected in containers devoid of media, preservatives, animal serum or

detergents to avoid any of these additives interfering with the test result. The stool collected was stored at the temperature of 4^0 C. Blood samples were collected from each participant, processed, and serum was used for serology.

2.5 Inclusion and Exclusion Criteria

Participants selected for the study were patients attending health centers in Port Harcourt. Those who were experiencing intestinal discomfort were not on any antibiotic or non-steroidal anti-inflammatory drug two weeks before sample collection, and willingly gave their consent were selected. Control samples were also collected

2.6 Experimental Procedures

2.6.1 Stool antigen test (quick profile kit, USA)

Procedure: Test materials and samples were brought to room temperature. The test card was removed from the sealed foil pouch. The stool sample was diluted in a sample bottle containing buffer. An aliquot of diluted stool sample was added to the sample well of the test card. The sample was allowed to flow through a label pad containing *H.pylori* antibody combined with red-colored colloidal gold. The result was taken at 15 minutes and recorded.

Two pink-colored bands on the test line region and that of the control line region indicated a positive result while a negative result was indicated by a pink-colored band on the control line region only.

2.6.2 Urea breath test (kibion diagnostics, Germany)

Procedure: Each patient was given a 50mg 13C-urea capsule with 10ml of water to swallow as a single dose. This quickly shuts the duodenal sphincter to contain the stomach substance. They were then instructed to blow through a breath card until the color indicator changed. This served as a baseline sample. After 30 minutes, the blowing exercise into the second breath card was repeated to obtain a post portion test. The two samples were sent for carbon dioxide isotope investigation by mass spectrometry.

2.6.3 Serology tests (dialab, Austria):

Procedure: 5 ml of blood was collected and allowed to clot. The serum was separated and frozen at 4^0C. Serological assay for IgG antibodies against *H. pylori* was performed using a commercial *H. pylori* kit IgG ELISA kit (Dialab, Austria) according to the instructions given by the manufacturer. The test serum, buffer, and positive and negative control were brought to room temperature. The test cassette was placed on a flat clean surface and 2 drops of serum were vertically transferred using a dropper into the specimen well and a drop of buffer was added. The result was read after 10 minutes.

2.7 Polymerase Chain Reaction (Nested PCR)

All positive samples obtained from non-invasive techniques used were subjected to the Polymerase chain reaction technique (invasive method). A total of fifty (50) stool samples from participants whose samples recorded positive results in urea breath, stool antigen, or serology were used for PCR.

2.7.1 Extraction of DNA

Extraction was achieved utilizing a ZR Feacal/Soil DNA mini prep extraction kit provided by Inqaba South Africa. Substantial development of the unadulterated culture of the contagious confines was suspended in 200 microlitres of isotonic support into a ZR slamming dot Lysis tubes, and 750 microlitres of lysis arrangement were added to the cylinder. The cylinders were anchored in a dot mixer fitted with a 2ml cylinder holder together and handled at most extreme speed for 5 minutes. The ZR slamming globule lysis tube was centrifuged at 10,000xg for 1 minute.

Four hundred (400) microlitres of supernatant were exchanged to a Zymo-Spin IV turn Filter (orange best) in a gathering tube and centrifuged at 7000 xg for 1 minute. One thousand two hundred (1200) microlitres of contagious/bacterial DNA restricting support was added to the filtrate in the gathering tubes bringing the last volume to 1600 microlitres, 800 microlitres were then exchanged to a Zymo-Spin IIC section in an accumulation tube, and centrifuged at 10,000xg for 1 minute, the flow through was disposed of from the accumulation tube. The rest of the volume was exchanged to the equivalent Zymo-turn and spun. Two hundred (200) microlitres of the DNA Pre-Wash cradle were added to the Zymo-turn IIC in another accumulation tube and spun at 10,000xg for 1 minute pursued by the option of 500 microlitres

of contagious/bacterial DNA Wash Buffer and centrifuged at 10,000xg for 1 minute.

The Zymo-turn IIC segment was exchanged to a clean 1.5 microlitres axis tube, and 100 microlitres of DNA elution cradle was added to the section lattice and centrifuged at 10,000xg microlitres for 30 seconds to elute the DNA. The ultra-unadulterated DNA was then put away at -20 degrees for other downstream reactions.

2.7.2 Quantification of DNA

The removed genomic DNA was measured utilizing the Nanodrop 1000 spectrophotometer. The hardware was introduced with 2 ul of sterile refined water and blanked utilizing ordinary saline. Two microlitres of the removed DNA were stacked onto the lower platform, the upper platform was conveyed down to contact the extricated DNA on the lower platform. The DNA concentration was estimated by tapping on the "measure" button.

2.7.3 16S rRNA amplification

The 18S rRNA genes were enhanced utilizing nested PCR, Nest I PCR response volume was 30ul utilizing HP1F 5'-GGTCTCAGCAAAGAGTCCCT-3'and HP1R 5'-CCCACCAAGCATTGTCCT-3'primers on an ABI 9700 Applied Biosystems. The PCR mix included: the X2 Dream Taq Master blend provided by Inqaba, South Africa (Taq polymerase, dNTPs, MgCl), extract of DNA template at a concentration of 0.4 microlitres, and water. The PCR conditions were as per the following: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; toughening, 56ºC for 30 seconds; augmentation, 72ºC for 30 seconds for 35 cycles and last expansion, 72ºC for 5 minutes. The Nest II PCR was likewise completed at 30ul last volume utilizing HP2F5'-AGGATGCGTCAGTCGCAAGAT-3' and HP2R 5'- CCTGTGGATAACACAGGCCAGT-3' for 35 cycles utilizing 0.5ul of the Nest I item as a format. The Nest II PCR conditions were as per the following: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; toughening, 60ºC for 30 seconds; augmentation, 72ºC for 30 seconds for 35 cycles and last expansion, 72ºC for 5 minutes.

2.8 Agarose Gel Electrophoresis

The product was resolved on a 1% agarose gel at 130V for 25 minutes and envisioned on a blue transilluminator.

2.9 Statistical Analysis

The statistical analysis of data generated in this study was achieved using Statistical Package for Social Science version 23. The results were compared and interpreted using Pearson Chi-square, Excel, and percentage tables.

3. RESULTS

3.1 Prevalence Rate of *H. pylori* in Port Harcourt as Detected by Different Methods

This study showed that out of 300 participants examined for *Helicobacter pylori* infection using the stool antigen test method, 41 (13.7%) were positive. The same participants were also examined using urea breath and serology techniques with positive test results of 35 (11.7%) and 44 (14.7%) respectively. All positive samples obtained from non-invasive techniques were subjected to the polymerase chain reaction (invasive method) where 54% (27 out of 50) positive result was recorded. Hence, the prevalence of *Helicobacter pylori* in Port Harcourt using stool antigen method, urea breath, serology, and PCR is 13.7%, 11.7%, 14.7%, and 54% respectively. This is shown in Table 1.

Table 1. Prevalence rate of *H. pylori* in Port Harcourt as detected by different methods

Methods	Number Positive (%) N=300
Stool Antigen test	41(13.67)
Urea Breath test	35 (11.67)
Serology test	44 (14.67)
PCR (n = 50)	27 (54)

3.2 Gender Characterization of the Subjects and Frequency based on the Methods Used

Considering the gender characterization and frequency of *H. pylori* using different methods, 13 out of 21 male subjects tested using PCR were positive and 14 out of 29 female subjects tested were positive. The result obtained from the stool antigen test method showed that 18 out of 185 males tested were positive while the rest were negative and 23 out of 115 female subjects tested were positive. Results obtained from the urea breath test and serology showed that 13 and 19 male subjects were positive while 22 and

25 female subjects were positive for the different methods. This is demonstrated in Table 2.

Table 2. Gender characterization of the subjects and prevalence based on methods used

Gender	Male Positive	Female Positive	Total
PCR	13	14	50
Stool Antigen test	18	23	300
Urea Breath test	13	22	300
Serology test	19	25	300

3.3 Comparison of Predictive Values for the Non-invasive Techniques against PCR as Gold Standard

The sensitivity, specificity, positive predictive values, negative predictive values, and likelihood ratios of all the test methods used in this study using PCR as a gold standard were shown in Table 3. The sensitivity of the various methods was 90.3 (stool antigen test), 64.5 (urea breath), and 100 (serology test), the likelihood ratios were 2.354, 0.462, and 4.760 respectively.

3.4 Agarose Gel Electrophoresis of Amplified 16S rRNA of *H. Pylori*

Plate 1. shows the amplified 16S rRNA fragment of *H. pylori* from various samples on the agarose gel. Lanes 1, 2, 4, 6, 8, 10, 14, and, 16 show the 16S rRNA bands at 380 base pairs (bp). Lane L represents the 1000 bp DNA ladder.

4. DISCUSSION

The prevalence rates of *H. pylori* using stool tests, urea breath tests, serology tests, and PCR among the study population in this study were 13.7%, 11%, 14.7%, and 54% respectively. The study has shown a low prevalence rate considering the non-invasive methods used. The rate is considered low compared to the study done by Ayodele et al. [9] in the same location which recorded a 19.5% prevalence rate using the serology method only. Similar studies were done in Port Harcourt and other parts of the country with a higher prevalence rate. Early in 2022, the prevalence rate of *H. Pylori* was found to be 55% at Rivers State University Teaching Hospital Nigeria [10]. The higher prevalence in this area could be due to some risk factors peculiar to the researched location. The risk factors include poor personal hygiene, poor food hygiene, poor environmental sanitation, overcrowding, and poor quality water supply.

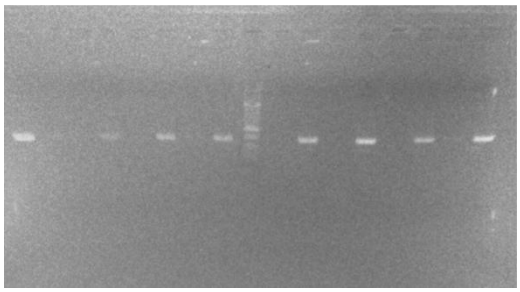

Plate 1. Agarose gel electrophoresis of the amplified 16S rRNA fragment of *H. pylori* from the various samples

This study recorded a relatively low prevalence rate using a non-invasive technique. The reasons could be due to the Public Heath enlightenment program in urban cities, and curtailed risk factors. It could also be a result of the methods used not being able to detect the organism under study because PCR showed a higher prevalence rate of 54%. Moreover, low bacterial load in the stomach, recent use of antimicrobials, or bleeding can lead to false-negative results when these non-invasive techniques are used.

The polymerase chain reaction has not been considered in routine diagnosis and detection of *H. pylori* as a practical tool due to cost implications and availability of the facility but now because of its convenience and reliability, a lot of laboratories are embracing this technology [11]. The higher prevalence rate in this study using PCR could also be because PCR is a molecular-based technique that aids specific detection of nucleic acid. It has been reported that the technique is very suitable for the detection of *H. pylori* when this organism is present in very small amounts, then the organism growth rate is difficult to identify by other methods [12], nevertheless, detection using this method is also affected by contaminants present in stool sample which if not properly removed before amplification, yields false-negative results [12].

Another study also reported that the detection of *H. pylori* using PCR produces false-positive results due to its propensity to detect clonal DNA from non- *H. pylori* organisms, especially in environmental samples [2].

Table 3. Comparison of predictive values for the non-invasive techniques against pcr as a gold standard

	Stool Antigen test(n=50)	Urea Breath test(n=50)	Serology(n=50)
Sensitivity (%)	90.3	64.5	100
Specificity (%)	26.3	26.3	79
PPV (%)	66.7	58.8	81
NPV (%)	62.5	31.3	66
LR	2.354	0.462	4.760

Key: PPV = Positive predictive value; NPV = Negative predictive value; LR = Likelihood ratio

The sex distribution of this study result showed that the prevalence rate of *H. pylori* infection in males was higher than what was obtained from that females. This record was in conformity with the result reported by Ayodele et al. [9]. However, this result did not agree with the observation made by Agi et al., [10] and Zhang et al. [13]. These researchers reported a higher prevalence rate in females than in male counterparts.

The higher prevalence in males could be a result of a carefree lifestyle noted in most males in terms of adequate healthcare and preventive measures. However, the higher prevalence rate was observed in males only in the PCR method. The results obtained from this study using other non-invasive techniques (stool antigen test, used breath test, and serology) showed a higher prevalence rate in females compared to that in males. Although most comparative studies did not pay attention to the sex distribution of *H. pylori* using different techniques, it is pertinent to consider this factor because it is of great value in making choices for diagnostic methods. A comparative study among different diagnostic methods according to the results from this study showed that the presence of *H. pylori* was as follows 13.7% for a stool antigen test, 11.9% for a urea breath test, 14.5% for serology test, and 54% for PCR method.

This study considered PCR as the gold standard method and thus the predictive values for non-invasive methods were calculated and compared against PCR. The predictive values include sensitivity, specificity, positive predictive, negative predictive value, and likelihood ratio.

For the stool antigen test method, the result showed a sensitivity value of 90.3%, specificity (26.3%), a positive predictive value of 66.7%, a negative predictive value of 62.5%, and a likelihood ratio of 2.354. The sensitivity recorded is very high although not the highest among the methods used. The sensitivity is in concordance with the study done in Saudi Arabia which recorded a sensitivity of 94% [11]. The difference in the antigens may affect the sensitivity as this method is influenced by the condition or nature of the stool sample i.e. watery stool contains a diluted portion of the antigen; bismuth and antibodies. These observations suggest that the stool antigen test has the potential to diagnose *H. pylori*. However, its specificity did not conform to various studies [11]. These studies reported higher specificity than the one obtained from this study. A stool antigen test has been employed in a few studies to control the effectiveness of eradication therapy.

When compared to the urea breath test; it is more economical and showed a better likelihood ratio (LR). Although the LR of this technique was high, it was lower than that obtained from the serology test. The likelihood ratio of the stool antigen test is affected by the temperature, the interval between stool sample collection, and the measurement procedure [14]. The method may be underused because of its cost-intensive in some regions, especially in developing countries.

The sensitivity, specificity, and likelihood ratio of the urea breath test in this study were 64.5%, 26.3%, and 0.462% respectively. These results were very poor compared to other methods. However, the reports were not in accordance with that reported in several studies which rated the urea breath test as one of the best non-invasive or indirect methods in diagnosing *H. pylori* [15]. The method is dependent on the ability of *H. pylori* to digest urea into carbon dioxide which is then absorbed from the stomach and excreted or discharged in the breath [14]. Hence, it could be that patient compliance, time, and interpretation of the test outcome affect the results obtained using this technique. There is also concrete proof to implicate that use of PPI (proton pump inhibitors) less than two weeks before testing, use of antibiotics less than 4 weeks prior to diagnosis, bleeding or precancerous conditions lead to false-negative

results in using urea breath test; thus powering the sensitivity, specificity, and accuracy of used breath method [14].

The serology test method in this study showed the highest sensitivity of 100%, specificity of 79%, and likelihood ratio of 4.760. Among the non-invasive methods used, the serology test was the best method comparable to the PCR. The test method is inexpensive widely available, and highly sensitive though not very specific. Although its sensitivity is high and agreed with studies done by other researchers, none recorded 100% sensitivity [13,15,16].

One major advantage of this technique is that its reliability level is low as it detects antibodies months after treatment of *H. pylori,* thus it is not recommended for checking the eradication of *H. pylori* [13].

5. CONCLUSION

Both invasive and non-invasive tests for *H. pylori* play a vital role in its management. Based on the results generated in this study, the accuracy of diagnostic methods for *H. pylori* can be arranged in the order as follows: PCR> serology > stool antigen > urea breath test. However, the order might slightly change among related studies; generally, the invasive method is preferred over the non-invasive method in developed countries while stool antigen is preferred in developing countries. None of these methods can be considered the generally accepted method alone.

PCR is a useful method noted for its high sensitivity, especially in specimens such as faeces but faeces are known to contain PCR inhibitors which limit the direct application of PCR to faecal specimens. PCR requires special laboratory facilities for its use and is not generally available as the method required for eradication control and management of *H. pylori* for both short and long term is still needed. Therefore, this study has recommended that non-invasive methods should not solely rely on the diagnoses of *H. pylori.* Secondly, simultaneous utilization of invasive and non-invasive methods should be encouraged.

CONSENT AND ETHICAL APPROVAL

Oral informed consent was also obtained from the participants after properly educating them on the need for the study. Ethical approval was obtained from the Department of Planning Research and Statistics, Ministry of Health, Port Harcourt.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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