COMPARATIVE METHODS OF DETECTION OF *HELICOBACTER PYLORI* IN INDIVIDUALS SUFFERING FROM STOMACH ULCERS

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ABSTRACT

Helicobacter pylori are gram negative microaerophilic bacteria; it is present in appropriately half of the world population thus, one of the most frequent and persistent bacterial infection associated with stomach ulcer and gastric cancer. Various diagnostic method exist to detects infection and the choice of one method or and another depends on several factor such as accessibility, advantages and disadvantages of each method, cost and age of patients. A total of 304 samples were collected from 152 ulcer patients and were used for this research. Medical records of 200 ulcer patients of which 100 patient records were obtained from the Federal Medical (F.M.C) Centre Owerri, and another 100 were obtained from the Medical centre of Alvan Ikoku Federal College of Education (A.I.F.C.E) Owerri and questionnaires were issued to them. They were categorized into 8 groups according to their ages. The answers confirmed their status as ulcer patients. 100 faecal samples were collected from F. M. C and 52 faecal samples were also collected from Medical Centre of A.I.F.C.E. in a pre sterilized sealed containers using the method described by Dan Higgins (2008)*. 100 blood samples were also collected from the same patients in F.M.C and 52 from A.I.F.C.E using the needle and syringe protocol and were taken to the laboratory immediately at room temperature for the examination. The research work was carried out in the project laboratory of the Department of Microbiology, Chukwuemeka Odumegwu Ojukwu University, Uli and Faculty of Pharmaceutical Sciences Nnamdi Azikiwe University Agulu both in Anambra State, Saint Zico reference Laboratory Owerri, Imo State Nigeria. Helicobacter pylori isolation was done using selective media, (Columbia agar, Dent agar and defibrinated sheep blood), monoclonal faecal antigen enzyme immunoassay, H. pylori serum antigen test, biochemical test and polymerase chain reaction (PCR) methods were utilized. 62 samples were positive by HPsA method, 80 were positive by the culture method, 97 were positive by the monoclonal faecal antigen enzyme immunoassay and the Molecular biologic test confirmed 75 bacteria isolates positive for Helicobacter pylori. The sensitivity, specificity, positive predictive value, negative predictive value of the HPsA tests is 65% (53.59-74.77), 91% (81-96.5), 90% (79.8-96.3), and 67% (55.9-76) respectively. The sensitivity, specificity, positive predictive value, negative predictive value of the specificity of the cultured method is 82% (72.5-89.7), 85% (74-92.6), 87.5% (78-93.8), and 79% (80.99-96) at 95% confidence interval respectively. The sensitivity, specificity, positive predictive value and negative predictive value of the monoclonal faecal enzyme immunoassay are 94% (86.8-98), 75% (62.5-84.4), 82% (73-89) and 91% (80-96.9) at 95 % confidence interval; respectively. This shows that the monoclonal faecal antigen enzyme immunoassay test is better for simple non invasive H. pylori diagnosis.*

1. Introduction

Helicobacter pylori are gram negative microaerophilic bacteria found in the stomach. They are widely dispersed in nature and are present in appropriately in one half of the world population. More than half of the world population harbour *Helicobacter Pylori* in their upper gastrointestinal tract (Blaser, 2006). This organism can reside as common commensals in the upper gastrointestinal tracks of man and animals and may be present in other part of the body, such as the eye causing diseases that ranges from chronic gastritis, gastric ulcers to duodenal ulcers and peptic ulcer (Corticelli *et al.*, 2006). It is linked to the development of duodenal ulcers and stomach cancer. However, over 80% of individuals infected with the bacterium are asymptomatic and may play an important role in the natural stomach ecology (Yamaoka Yoshio, 2008). Worldwide, *Helicobacter pylori* have one of the highest global prevalence for a pathogen. Chronically infected can reach up to 90% by adulthood (Tonkic et al., 2012). Rates of distribution vary according to country as infection rates can decrease with improvements in industrialization and socioeconomic condition (Bardwin, 1997).The age at which this bacterium is acquired seems to influence the possible pathologic outcome of the infection: people infected with it at an early age are likely to develop more intense inflammation that may be followed by atrophic gastritis with a higher subsequent risk of gastric ulcer, gastric cancer, or both. Acquisition at an older age brings different gastric changes more likely to lead to duodenal ulcer (Brown, 2000). Infections are usually acquired in early childhood in all countries (Kuster et al., 2006). A meta-analysis conducted in 2009 concluded that the eradication of *Helicobacter Pylori* reduce gastric Cancer risk in previously infected individuals, suggesting the continued presence of *Helicobacter Pylori* which constitute a relative risk factor of 65% for gastric Cancers. In fact; despite that, the incidence of *Helicobacter Pylori* infection has decreased substantially in western countries (developed countries) but persist in developing countries. The situation in Nigeria is even worrisome in the sense that 90% of the population that have this organism are not aware of their *H. Pylori* status *(*Aderemi *et al*., 2012). A study, (Prevalence of a maker of active *H. pylori* infection among patients with type 2 diabetes mellitus) conducted in Lagos, Nigeria in 2012 also show that *H. pylori* is associated with patients with type 2 diabetes mellitus *(*Aderemi *et al*.,2012). Another study conducted in 2003 also shows that *H*. *Pylori* infection is also associated with a higher risk of Malt lymphoma of the stomach and other cancer such as colorectal and gastric cancer (Tsuji et al., 2003). Researchers have shown that inflammatory response as the result of *H*. *Pylori* infection can result to atrophy of the stomach lining and eventually lead to ulcer in the stomach. *Helicobacter pylori* colonization is associated with a lower incidence of childhood asthma (Chen et *al.*, 2008). *Helicobacter pylori* infection is also associated with a 1–2% lifetime risk of stomach cancer and a less than 1% risk of gastric MALT lymphoma. In other to determine the source and route of infection of this organism and the pattern of its transmission, laboratory typing is necessary. Laboratory typing is a useful tool in clinical epidemiology for defining the source and route of infection, for studying the persistence and reinfection rates, clonal selection in the host and bacterial evolution (Schreiber *et al.*, 2004). Untreated virulent infection with this organism may lead to more complicated disease like adenocarcinoma hence this research.

2. Aim

This study aims to compare some methods of diagnosis of *Helicobacter pylori* in individuals suffering from stomach ulcers

Objectives

To Issue questionnaires to ascertain the ulcer status of the patients, to obtain medical records of the patient to know the state of the ulcer patients. To use the Columbia agar, *H. pylori* supplements and defibrinated sheep blood (selective media) to isolate the organisms and to use molecular biologic method analyses to further identify the isolated organisms.

3.0 Materials and Methods

3.1 Materials

The stool and blood samples used for this study were taken from ulcer patients in Federal Medical centre and the Medical centre of Alvan Ikoku Federal College of Education both in owerri, Imo State, Nigeria

3.2 Methods

The stool samples were collected using the procedure as described by Dan Higgins (2008) and the blood sample was collected using W.H.O guideline for venepuncture and bleeding. A total of 304 samples were collected from 152 ulcer patients and were analysed. Medical records of 200 ulcer patients of which 100 patient records were obtained from the Federal Medical (F.M.C) Centre Owerri, and another 100 were obtained from the Medical centre of Alvan Ikoku Federal College of Education (A.I.F.C.E) Owerri and questionnaires were issued to them. They were categorized into 8 groups according to their ages. Group one (1-10years), Group two (11-20years), group three (21-30years), Group four (31-40years), Group five (41-50years), Group six (51-60years), Group seven (61-70years) and Group eight (70 and above). The answers confirmed their status as ulcer patients. 100 faecal samples were collected from F. M. C and 52 faecal samples were also collected from Medical Centre of A.I.F.C.E. in a pre sterilized sealed containers using a clean head pan (disposable receiver) using spoon attached to the lid and filling the container to one-third.100 blood samples were also collected from the same patients in F.M.C and 52 from A.I.F.C.E using the needle and syringe protocol and were taken to the laboratory immediately at room temperature for the examination. *Helicobacter pylori* serum antigen (HPsA) test was done using the *H. pylori* strip. Blood samples collected were left for 5h for the serum to separate out. The HPsA is done by dipping the strips in the serum, and observed for 2 minutes then the reading was taken. Double band revealed positive reaction while the single band revealed negative reaction. The cultured test was carried out using Columbia agar media with *Helicobacter pylori* supplement (Dent agar) and defibrinated sheep blood (*H. pylori* selective media).The *H. pylori* selective media was prepared by measuring 39grams of Columbia agar in to a clean conical flask containing 500mls of distilled water using a weighing balance, it is then dissolved by putting the conical flask on bursen burner and swirling at interval until it is completely dissolved. It is then autoclave at 121^{o}C for 15 minutes, and left to cool to 50^{o}C. 2mls (one vial) of H*. pylori* supplement was added followed by 35mls of defibrinated sheep blood. It was properly mixed together and poured into Petri dish for it to solidify.The organism was inoculated using sterile wire loop to scope little portion of the stool samples and streaking on the solidified *H. pylori* media in the petri dish and incubating in an anaerobic jar for 48 hours at 37^{o}C**.** Small round milky-white colonies on agar plate was seen in a microaerophillic environment with cushion shape top and raised edge (in anaerobic jar).0.5 to 2.0 mm, translucent to yellowish colonies in old cultures was also seen. After 3 to 5 days of incubation the bacteria looked pleomorphic, with spiral and irregular curved rods. Monoclonal faecal antigen enzyme immunoassay which detects *H. pylori* antigen in stool specimen was

performed according to the manufacturer's recommendation of using optical density of 0.50 as cut off value. This test (stool antigen test) is an enzyme immune assay which uses monoclonal mouse anti-*H. Pylori* antibodies absorb to micro wells as captured antibody.50µl of supernatant of diluted stool sample (0.1g in 0.5ml sample diluents) was added in the wells, there after 50µl conjugated monoclonal antibody solution were added to the wells and incubated for one hour at a room temperature on a shaker. The unbounded materials were removed by washing four times with a washing buffer. After washing, 10µl of a stopping solution were added and incubated for 10 minutes and the results were read by spectrophotometer of absorbent of 450nm and 630nm (450/630nm) double wave length. The isolated organisms were further identified by biochemical test such as the urease test which was carried out by inoculating a loopful of the organism (*Helicobacter pylori*) into 0.25ml of urea broth and incubating at 37^{o}C in water bath. A positive red colour was seen in minutes. The Catalase test was carried out by placing a loopful of *Helicobacter pylori* colonies in a drop of 3% hydrogen peroxide (H_2O_2) on a glass slide. Positive reaction was seen by instant formation of bubbles of oxygen. The oxidase reaction test was carried out by transferring some portion of *Helicobacter pylori* colonies with platinum loop into a piece of a filter paper soaked with oxidase reagent. A deep black reaction at the point of contact was seen. The isolated organisms were further identified by molecular test. Polymerase Chain Reactions (PCR): DNA extraction was done using boiling method. 24h broth cultures of the organisms were used for the DNA extraction. One and half millilitres of the broth cultures were transferred to 2mls eppendorf tube. Centrifugation was done at 12,000 rpm for 1 minute, after which the supernatant was decanted and vortexed for another 1 minute. The vortexed residue was heated using the heating block at 95^{0}C for 10 minutes. This was followed by final centrifugation at 12,000 rpm for I minute. The supernatant (DNA) was collected and stored at -20^{0}C until further use for PCR. Amplification was conducted in a total volume of 25 µL. The reaction mixture contained 12.5 µL, 2X ready PCR mix (Thermo Scientific) and consisted of 1.25 U Taq-Pol, 75 mM Tris-HCL (pH 8.8), 1.5 mM $MgCl_2$, and 0.2 mM of each dNTP. The reaction mixture contained 12.5 µL master mixes, 1.0 uM of each forward and reverse primers, 1 µg DNA template, and 8.5 µL RNase free water to a total volume of 25 µL. The amplification was carried out in a thermal cycler (Applied Biosystem, USA) according to the following program: an initial denaturation step at 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55^{0}C for 1 min, and a final extension step at 72°C for 5 min. Amplified PCR products were resolved by agarose gel electrophoresis using 2% agarose in Tris Acetate-EDTA (TAE) buffer containing 0.5 µg/mL of ethidium bromide. Molecular size ladder of 1kb (Thermoscientific, Germany) was used to determine the size of the bands. The gel was viewed and photographed on a Gel-Doc System (Bio-Rad, USA). The primers used for the amplifications were obtained from Inqaba(South Africa), shown below:

***Helicobacter pylori* genus specific primers (both forward and reverse primers)**

GlmM-F AAGCTTTTAGGGGTGTTAGGGGTTT

GlmM-R AAGCTTACTTTCTAACACTAACGC

4. Results

Table 1: Summary of the results of the various methods of *H. pylori* laboratory diagnosis carried out.

	Plasma	Cultured test	monoclonal antigen test
Positive	62	80	97
Negative	90	72	55

Table 2: Table summarising the Percentage by age distribution of the entire group used for this test.

Age	Plasma				cultured test				MCFAEA			
Range	positive		Negative		positive		Negative		positive		Negative	
1-10 yrs	3	(1.97%)	17	(11.2%)	8	(5.3%)	12	(7.9%)	10	(6.58%)	10	(6.58%)
11-20 yrs	4	(2.6%)	16	(10.53%)	8	(5.3%)	12	(7.9%)	10	(6.58%)	10	(6.58)
21-30	6	(3.94%)	14	(9.2%)	8	(5.3%)	12	(7.9%)	10	(6.58%)	10	(6.58)
31-40	8	(5.3%)	12	(7.9%)	10	(6.58%)	I0	(6.58%)	11	(7.2%)	9	(5.92%)
41-50	13	(8.56%)	7	(4.6%)	13	(8.56%)	7	(4.6%)	16	(10.53%)	4	(2.6%)
51-60	12	(7.9%)	8	(5.3%)	12	(7.9%)	8	(5.3%)	14	(9.2%)	6	(3.94%)
61-70	9	(5.92%)	11	(7.2%)	12	(7.9%)	8	(5.3%)	15	(9.87%)	5	(3.29%)
70 above	6	(3.94%)	6	(3.94%)	9	(5.92%)	3	(1.97%)	10	(6.58%)	2	(1.32%)

5. Statistical analysis

Descriptive statistics (sensitivity and specificity with confidence intervals and positive and negative predictive values) of the stool tests (serological strip methods, cultured and monoclonal faecal antigen enzyme immunoassay) were calculated and the statistical analysis was performed using SPSS (Statistical package for social sciences version 21) and the exact binomial and Poisson confidence interval (revised 05/25/2009). The monoclonal faecal antigen enzyme immunoassay was done according to the manufacturer's guideline, an optical density of ≥ 0.50 is the cut off point, therefore it was considered as positive and < 0.50 was considered as negative. This differentiated between those that tested positive to

Helicobacter pylori and those that are negative. The 152 patients used for this research were grouped into 8 groups according to age, in all 20 patients were examined in each age group except in the group of 70 years and above where 12 patients were examined. The age group were tabulated as seen below.

Table 3: Table showing the sensitivity, specificity, PPV, NPV and the accuracy of *H. pylori* test

	Sensitivity	specificity	PPV	NPV	Accuracy
MCFAEA	94% (86.8-98)	75% (62.5-84.4)	82%(73-89)	91% (80-96.9)	86%
Culture test	82% (72.5-89.7)	85% (74-92.6)	87.5% (78-93.8)	79% (80.99-96)	83.6%
Serum/plasma	65% (53.59-74.77)	91% (81-96.5)	90% (79.8-96.3)	67% (55.9-76)	76%

NB: PPV – Positive predictive value, NPV – Negative predictive value

6. Discussion

Table 1 show that a total of 97(63.8%) ulcer patients test positive to *H. pylori*. monoclonal faecal antigen enzyme immunoassay which is the most sensitive method. 55 ulcer patients test negative to the monoclonal faecal antigen enzyme immunoassay. It *is* positive when any of the method of the test detects *H. pylori* in any of the patients, the *H. pylori* serum antigen (HPsA) has 62 (40.8%) and cultured method has 80 (52.6%). The cultured method reveals 70 *H. pylori* negative patients while 90 patients test negative the HPsA method. The monoclonal faecal antigen enzyme immunoassay have the highest sensitivity of 94% (86.8-98) at 95% confidence interval while the HPsA have the lowest of 65%(53.59-74.77).This means in *H. pylori* non invasive test the most reliable method still remain the monoclonal faecal antigen enzyme immunoassay. Table 1 also show the results of the phenotypic analysis using the serological strip method of detection of *Helicobacter pylori*, the cultured method and the monoclonal faecal method of *Helicobacter Pylori* diagnosis in the 8 different age groups. The serological strip method although very fast, shows the least sensitivity in *Helicobacter pylori* diagnosis since it could confirm only 62 (40.8%) positive *Helicobacter pylori* patients out of the total of 152 patients used for the analysis as seen in table 1. In table 1 also, the cultured method of diagnosis detected 80 (52.6%) positive *Helicobacter pylori* patients and therefore more sensitive and more reliable than the serological method. However the most sensitive is the monoclonal faecal antigen enzyme immunoassay which tested 97 (63.8%) patients positive to *Helicobacter pylori* and is therefore more reliable especially for non invasive test in children. Table 3 shows that the sensitivity of the serological strip method is 65% at 95% confidence interval (53.59-74.77) which means that serological test have a low sensitivity in young

children and cannot be considered non invasive test in children (Oliveira *et al*., 1999). while 90 patients (59.2%) were negative to *Helicobacter pylori,* although *Helicobacter pylori* may be present in the children, it cannot be pick up by the strip at this early stage. This could be that the organisms may be very few in the blood stream and concentrate in the gastric region where it multiply and usually asymptomatic. Table 2 show the analysis of patients from age one to ten (10) years which have only three (3) (1.97%) patients that test positive to *Helicobacter pylori* using the strip serological method. The remaining seventeen (17) (11.2%) patients test negative. Comparing the strip method of diagnosis with the cultured method, it is been shown that there is an improvement in the age range of one to ten (1-10) years. This was able to diagnose 8(5.8%) positive *Helicobacter pylori* patients; this could be that the predilection sites of this bacterium in children as it is more in the gastric region than the blood stream. *Helicobacter pylori* colonize the gastric epithelial cells (Ruiz-Bustos *et al*., 2001). However the monoclonal faecal antigen-enzyme immunoassay is sensitive and detects 10(6.6%) *Helicobacter pylori* positive patients in the same range. Many studies have describe the use of ELISA-base HpSA stool antigen kits with either polyclonal or monoclonal antibodies for diagnosis of *Helicobacter pylori* infection diagnosis (Malfertheiner *et al*., 2002).Table 3 shows that infection is least in children between the ages of 1-10 as indicated by the (ELISA base HpSA stool antigen) have 10(6.58%) positive patients as compare to 11(7.2%), 10(6.5%) and 11(7.2%) *Helicobacter pylori* positive patients in ages 11-40 years confirming that infection are usually acquired in early childhood in all countries (Kuster *et al.,* 2006) but are asymptomatic; however infection rate in children in developing countries like Nigeria is higher probably due to poor sanitary condition perhaps combined with lower antibiotic usage for related pathologies. Table 3 shows that ages 41 and above have higher prevalence of *Helicobacter pylori* infection base on this research. The higher prevalence among these groups of the elderly reflects higher infections in the past when the individuals were children rather than more recent at a later stage of individual (Kusters et al., 2006). *Helicobacter pylori* are always almost acquired in early childhood and usually remain throughout life unless a specific treatment is given (Rothenbacher *et al.,* 2000). The age at which this bacterium is acquired seen to influence the possible pathologic outcome of the infection. People infected with these *Helicobacter pylori* at an early stage are likely to develop more intense inflammation at later stage that may be followed by atrophic gastritis with higher subsequent risk of gastric ulcer, gastric cancer or both. Acquisition of older age brings different gastric changes more likely to lead to duodenal ulcer (Brown, 2000).However, the high prevalence rates seen to be declining in patients of age 70 years and above in the various methods of diagnosis so far carried out in plasma/serum strip methods, the patients that test positive were 6 (3.94%), cultured method was 9 (5.92%) and 10 (6.58%) in monoclonal faecal antigen enzyme immunoassay method. The is could be due to the facts that older people depends more on medicines including antibiotics for healthy living and other ailments that comes with aging and antibiotics tends to suppress or even eliminate this bacterium from the body. *Helicobacter pylori* eradication rates were higher for seven day antibiotics regimen containing lansoprazole, amoxilli and clarithromycin(LAC) (Liu *et al*.,2010). Table 2 shows the sensitivity of the rapid serum strip method of *Helicobacter pylori* diagnosis to be 65% at 95% confidence interval (53.59-74.77), this means most ulcer patients test negative to the test method even though they have the disease, however the specificity of 91% at 95% confidence interval (81-96.5) shows that most of the patients are free from the ulcer. The table also shows that the positive predictive value is 90% at 95% confidence interval (79.8-96.3). This means that most of patients that test positive to *Helicobacter pylori* were correctly diagnosed as positive suggesting that the organism is the major cause of ulcer in patients. The table show the negative predictive value of this test to be 67% at 95% confidence interval (55.9-76) which means that not all the ulcer patients that tested negative to *Helicobacter pylori* was correctly diagnosed as negative and cannot be concluded as not having the organism. However, this shows *Helicobacter pylori* is not the only cause of ulcer but that they are other extraneous cause such as long

term use of non-steriodal anti-inflammatory drugs (NSAIDs) such as aspirin and ibuprofen, hyperacidity which may be related to genetics, lifestyle such as stress, smoking and certain food (Shannon, 2015).The low sensitivity in the rapid serum strip method of diagnosing *Helicobacter pylori* could be due to the fact that serological test method show a low sensitivity in children and cannot be considered as a non invasive test in children (Oliverira *et al*.,1999). Table 3 also show the result for *Helicobacter pylori* cultured test. The sensitivity is 82% at 95% confidence interval (72.5-89.7), this show an improvement in sensitivity since more ulcer patients test positive to *Helicobacter pylori* infection. However the specificity is high which may not necessarily classified most of the ulcer patients as free from *Helicobacter pylori* infection but still show that not all ulcers are cause by this organism. The specificity of this test is 85% at 95% confidence interval (74-92.6).The positive predictive value is 87.5% at 95% confidence interval (78-93.8) and negative predictive value is79% at 95% confidence interval (80.99-96) with accuracy of 83.6%. Table 3 also shows the sensitivity, specificity, positive predictive value and negative predictive value of the monoclonal faecal enzyme immunoassay to be 94% (86.8-98),75% (62.5-84.4), 82% (73-89) and 91% (80-96.9) at 95 % confidence interval; respectively with diagnostic accuracy of 86%.This is a more excellent diagnostic method of laboratory typing of *Helicobacter pylori* than the other two methods. It then means that of the total number of ulcer patients tested for the *Helicobacter pylori* infection, 94% are sensitive to *Helicobacter pylori* showing that *Helicobacter pylori* is the cause of both gastric, duodenal and colon ulcer. In the molecular biologic analysis, all the isolates accessed for *Helicobacter pylori*, were positive with the amplification of 16sRNA gene of *Helicobacter pylori* .The *Helicobacter pylori* PCR test was done using *Helicobacter pylori* genus specific primers. All were positive with the amplification of the 16SRNA gene of *Helicobacter pylori*

7. Conclusion and recommendation

In summary, some detection methods were carried out for the analysis of *Helicobacter pylori* to compare it diagnosis in ulcer patients. Monoclonal faecal antigen enzyme immunoassay tests are better, simple and easier to perform than the other non invasive *H. pylori* test carried out.

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