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MARSLAND PRESS
Multidisciplinary Academic Journal Publisher

The Implication Of *Helicobacter Pylori* In Stomach Ulcers And The Sensitivity And Specificity Of Some Methods Used In Its Diagnosis.

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ABSTRACT

A total of 152 ulcer patients were used in the analysis of *Helicobacter pylori* to establish the implication of *Helicobacter pylori* as causative agent of these ulcers and accesses the specificity and sensitivity of the various test method used. The research work was carried out in the project laboratory of the Department of Microbiology, Chukwuemeka Odumegwu Ojukwu University, Uli and Faulty of Pharmaceutical Sciences Nnamdi Azikiwe University Agulu both in Anambra State, Nigeria. *Helicobacter pylori* serum antigen (HPsA), isolation using selective media, monoclonal faecal antigen enzyme immunoassay, and Biochemical and polymerase chain reaction (PCR) methods were utilized. Of the 152 samples, 62 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 the bacteria isolates positive for *Helicobacter pylori*. The sensitivity, specificity, positive predictive value, negative predictive value of the HPsA test are 65% (53.59-74.77), 91% (81-96.5), 90% (79.8-96.3), 67% (55.9-76) respectively. The sensitivity in the cultured method is 82% at 95% confidence interval (72.5-89.7), 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).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) and91%(80-96.9) at 95 % confidence interval; respectively. This study shows that *Helicobacter pylori* are the major causative agent of gastric, duodenal, colon and peptic ulcers but is not the only causative agent of ulcer. Some other factors like long term use of antiflammatory drugs; hyperacidity has shown that *Helicobacter pylori* are not the only causative agent of ulcers.

[Umeaku C.N. (Ph.D), Alachi, Mathias Ogbole. **The Implication Of *Helicobacter Pylori* In Stomach Ulcers And The Sensitivity And Specificity Of Some Methods Used In Its Diagnosis.** .*Nat Sci* 2023,23(8):24-30].ISSN1545-0740(print);ISSN2375-7167(online).http://www.sciencepub.net/nature 03.doi:10.7537/marsnsj210823.03.

Keywords: **Implication; *Helicobacter Pylori*; Stomach; Ulcer; Diagnosis**

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 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).

RESEARCH PROBLEM

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). Researches 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.

AIM OF THE STUDY

This study aims to

1. Estimate the implication of *Helicobacter pylori* in stomach ulcer and
2. Evaluate and compare the sensitivity and specificity of the various laboratory typing methods.

Objectives of the study

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, and *H. pylori* supplements and defibrinated sheep blood (selective media) to isolate the organisms

To use molecular biologic method analyses to further identify the isolated organisms.

Methodology

A total of 152 ulcer patients were used for this research. The samples were collected from the Federal Medical Centre Owerri, the Medical Centre of the Alvan Ikoku Federal College of Education Owerri using sterile containers. Medical records of patients were obtained 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. Blood and faecal samples were used. *Helicobacter pylori* serum antigen (HPsA) test was done using the *H.pylori* strip, Blood samples were taken from each of them left for 5 hours for the serum to separate and HPsA is done by dipping the strips in the serum, double band indicates positive reaction while single band indicates negative reaction. The cultured test was carried out using Columbia agar with *Helicobacter pylori* supplement (Dent agar) and defibrinated sheep blood (*H. pylori* selective media) was used to culture the organisms in a gas jar for three days for growth of milk- white translucent colonies). For the monoclonal faecal antigen enzyme immunoassay which detects *H. pylori* antigen in stool specimen, were performed according to the manufacturer's recommendation of using optical density of 0.5 as cut off value. The stool antigen test is an enzyme immune assay which uses monoclonal mouse anti-*H. pylori* antibodies absorbs to micro wells as captured antibody. 50ul of supernatant of diluted stool sample (0.1g in 0.5ml sample diluents) and there after 50ul conjugated monoclononal 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, 10ul of a stopping solution were added and incubated for 10 minutes and the results were read by spectrophotometer (450/630nm) double wave length. The cultured positive organisms were 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 the urea broth and was incubated at 37^{o}c in water bath; a positive red colour seen in minutes indicated *H. Pylori.* The catalase test was also carried out by placing a loopful of *Helicobacter pylori* colonies in a drop of 3% of H_2O_2 on a glass slide. Positive reaction was seen by instant formation of the bubbles of oxygen. The oxidase reaction test was carried out by transferring some portion of *Helicobacter pylori*

colonies by a wire loop into a piece of a filter paper soaked with oxidase reagent. A deep black reaction at the point of contact was seen which indicated the presence of the enzyme. The Cultured positive organisms were further identified by molecular test Polymerase Chain Reactions (PCR). Amplification was conducted in a total volume of 25 µL. The reaction mixture contained 12.5 uL, 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 µM 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 55^0c 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:

GlmM-F AAGCTTTTAGGGGTGTTAGGGGTTT

GlmM-R AAGCTTACTTTCTAACACTAACGC

U1-CCAGCAGCCGCGGTAATAC

U2-ATCGGTACCTTGTTACGA

RESULTS

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

	Plasma/serum(HpsA) Test	Cultured test	Monoclonal faecal antigen enzyme immunoassay
Positive	62	80	97
Negative	90	72	55

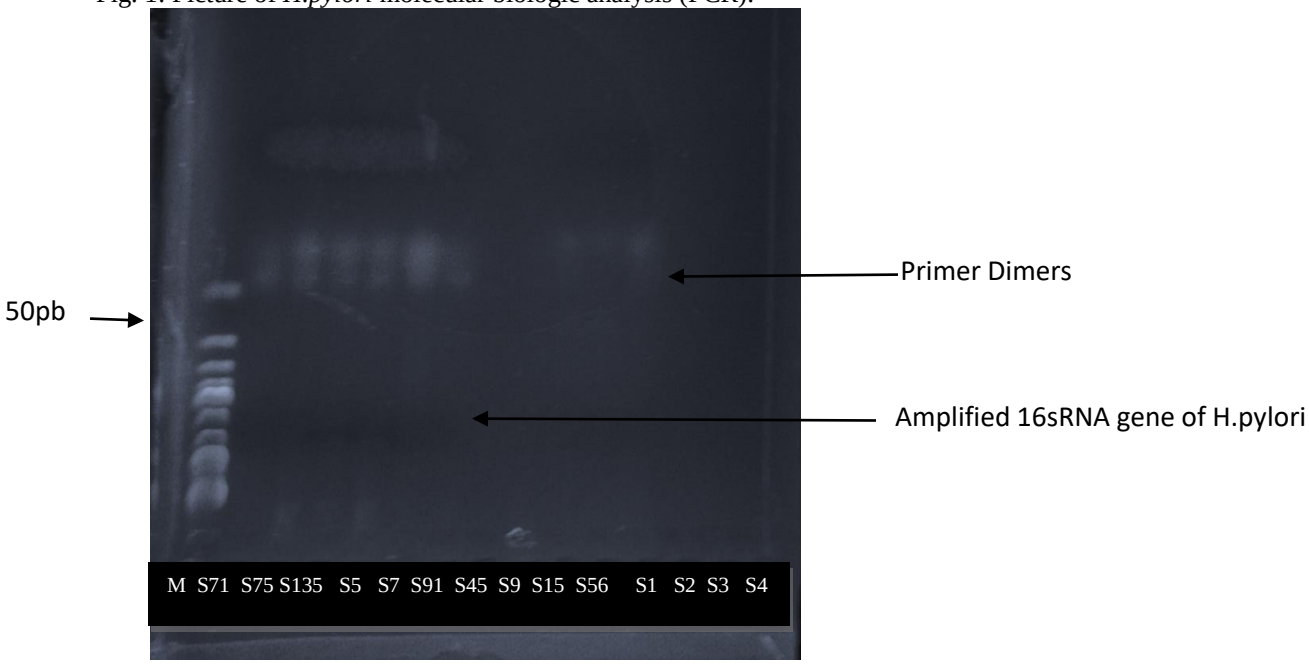
Table 2: showing the sensitivity, specificity, PPV, NPV and the accuracy of the test

	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)	3.6%
Serum/plasma	65% (53.59-74.77)	91% (81-96.5)	90% (79.8-96.3)	67% (55.9-76)	76%

Table 3: Percentage by age distribution

Age range	Plasma/Serum		Cultured test	Cultured test	MCFAEA	
	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%)

Fig. 1: Picture of *H.pylori* molecular biologic analysis (PCR).



KEY
M= Marker (DNA Ladder)
S= Specimen
S1= *Escherichia coli*
S2= Enterococcus*spp*
S3= Pseudomonas *spp*
S4= *Staphylococcus aureus*
S71-S56= H.pylori

DISCUSSION

Table 1 show that a total of 97(63.8%) ulcer patients test positive to *H. pylori*. It *is* positive when any of the method of the test detects *H. pylori* in any of the patients, with the *H. pylori* serum antigen (HPsA) having 62 (40.8%), cultured method having 80 (52.6%) and monoclonal faecal antigen enzyme immunoassay (97(63.8%). 55 ulcer patients test negative to *H.pylori*. The monoclonal faecal antigen enzyme immunoassay have the highest of 94%(86.8-98) at 95% confidence interval while the HPsA have the lowest of 65%(53.59-74.77).

Table 1 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 2 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* that 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 3 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 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 of 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 to detect 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 l(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 nonsteriodal 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 2 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 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 2 also shows the sensitivity, specificity, positive predictive value and negative predictive value of the monoclonal faecal faecal enzyme immunoassay to be 94%(86.8-98),75%(62.5-84.4),82%(73-89) and91%(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 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* as evidenced in the Picture (fig 1) while all the other isolates came negative as we had only primer dimers at the end of the run. 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.*

CONCLUSION

This research have been able to prove that *H. pylori* is the major cause of ulcers base on the results from the laboratory typing of this organism and the pathogenic strains responsible for causing the disease have been be identified. However it have also been established that *H. pylori* is not the so causative agent of ulcer.

ACKNOWNEDGEMENT

With a deep sense of honour and gratitude I wish to appreciate God Almighty for the grace made sufficient for me to undertake this task.

I am very grateful to, Dr C.N. Umeaku for her patience and her ever willingness to teach and to correct me especially her motherly deposition towards her students also the computer operator Miss.Chizoba, for typing this work, my course mates for their assistance, management and staffs of Federal Medical Center Owerri ,the Medical director and staffs of the Medical Centre of Alvan Ikoku Federal College of Education Owerri for giving me access to their patients. My thanks also go to Mr Moses of the Faculty of Pharmaceutical Sciences Nnamdi Azikwe University Agulu, the Laboratory Technologist of Department of Microbiology Project Laboratory and all those used for this research work.

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2/25/2023