



## ASSESSMENT OF *HELICOBACTER PYLORI* IN GLAUCOMA DISEASE IN ABIA STATE OF NIGERIA

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### ABSTRACT

Glaucoma is a group of ocular diseases marked with elevated intraocular pressure IOP, reduced visual acuity, optic neuropathies and the corresponding visual field defects. Its etiology is unknown. The aim of this study was to assess *Helicobacter pylori* bacteria in glaucoma disease patients. The infection of *H. pylori* was associated with glaucoma by detecting serum IgG antibody against *H. pylori* qualitatively using immunochromatographic method; and also by isolating the organism from media cultures of fresh faecal samples of the glaucoma patients. In a total of 217 glaucoma patients, 212 (97.7%) were IgG serum positive; while only 25 (13.89%) of the 180 control was IgG positive  $P < .001$ . *H. pylori* was successfully cultured in 202 (95.3%) faecal samples of these 212 seropositives. The 202 isolates were all Gram negative and produced the enzymes catalase, oxidase and urease. They were highly motile; and poor in the hydrolysis of indoxyl acetate and hippurate (4.9% and 4.4% respectively). They poorly fermented sugar, 7.4% for sucrose and glucose and 12.3% for lactose. In the antibiotics susceptibility pattern, *H. pylori* was most resistant to vancomycin 66.8% (135 of the 202) and metronidazole 44.5% (90 of the 202); while *H. pylori* was most sensitive to tetracycline and ciprofloxacin by 94% and 91.5% respectively. The biochemical characteristics and the antibiotics resistant marker for claritromycin are chromosomal while the resistant marker for amoxicillin was plasmid located. In conclusion, *H. pylori* infection is associated with the risk for glaucoma development in the population. Clinicians who care for patients with *H. pylori* infection should also consider that *H. pylori* can cause not only digestive illness but also eye disease. This association offers a novel approach towards the care for glaucoma, the second leading cause of blindness.

**KEYWORDS:** Glaucoma; *H. pylori*, seropositive, biochemical characteristics, Antibiotics resistance, plasmid curing.

### INTRODUCTION

Glaucoma is a group of ocular disease marked with blockage and reduced outflow of the intra ocular fluid through the exit channel hence an elevated intra ocular pressure IOP. This leads to the compression of the retinal ganglion cells axons; and faulty blood flow to the retina/optic nerve head thus causing retinal ganglion cell death. These retinal ganglion cell deaths or losses present as cupping of the disc at the lamina cribrosa, visual field defects and reduced visual acuity.

Glaucoma is the second leading cause of blindness in the world, after cataracts, and the leading cause of irreversible blindness (1). The treatment for glaucoma medically so far, focuses on lowering intraocular pressure to a level unlikely to cause further optic nerve damage. Either by increasing outflows of aqueous from the anterior chamber or by reducing production of aqueous by the ciliary body; or the neuroprotectives for the retinal ganglion cells death. These treatments have been on managing the symptom/sign; but not on treating the condition- why the outflow channels get plugged up.

The Centers for Disease Control and Prevention estimates that two-thirds of the world's population is infected with *H. pylori*. Its infection causes more than 90% of duodenal ulcers and up to 80% of gastric ulcers.

*H. pylori* is implicated in a number of non-digestive conditions as well, including cardiovascular and cerebrovascular disorders and other vascular dysfunctions, such as migraine and Raynaud's disease; it has also been associated with diabetes. The organism is also involved in the pathology of the eye - specifically, glaucoma (2).

A relationship between *H. pylori* infection and glaucoma will provide novel therapeutic targets for this so far handicapping eye disease.

This study aims at the assessment of *Helicobacter pylori* infection in glaucoma disease.

### MATERIALS AND METHODS

#### Participants

Visual screening programme for glaucoma was conducted in ten autonomous communities of Uturu, Abia State South Eastern Nigeria, in the period of March 2012 through June 2013. In the visual screening programme, the following ocular features were accessed in the participant: typical glaucomatous optic neuropathy including rim thinning or notching in the inferior or superior temporal area of the optic nerve head or total glaucomatous cupping; corresponding typical visual field loss including paracentral or arcuate scotoma or a nasal step, diurnal intraocular pressure, anterior chamber angle by shadow test. Glaucoma was diagnosed in the population according to the International Society of Geographical and Epidemiological Ophthalmology classification (3,4,5). The visual fields were evaluated using Bjerrum tangent screen. The intra ocular pressure IOP was measured using Perkins applanation tonometer. A diurnal IOP persistently less than 21 mmHg (without medication) and with the glaucoma features were con-

sidered Normal Tension Glaucoma (NTG).

The participants in the control (sex/age matched), also underwent screening. Subjects with glaucoma, previous ocular surgery, and serious external or retinal disease were excluded from the control. The control subjects had a best-corrected visual acuity of or better than 20/25, and no findings suspicious of glaucoma in the disc or retinal nerve fiber layer in ophthalmoscopy.

#### Serologic Analysis

There were 217 glaucomatous patients and 180 sex/age matched control. With their consent, a fresh capillary blood (finger prick) of 50 µl was drawn from each subject (the glaucomatous and the control), and the Gamma immunoglobulin IgG antibody against *H. pylori* was analyzed qualitatively using immunochromatographic method (Chemtrue *H. pylori* one step rapid test; Chemtron Biotech Incorporated San Diego, USA).

#### Culture and Sensitivity

A fresh faecal specimen was submitted by the *H. pylori* seropositive glaucoma patients for *H. pylori* culture isolation, biochemical characterisation and antimicrobial susceptibility test.

*H. pylori* were isolated from fresh faecal specimen by faecal filtration technique (6). With the primary culture media as campy-Bap; a supplemented blood agar that supports the growth of *Campylobacter* and *Helicobacter* species due to its content of peptones, dextrose, yeast extract and blood. The incorporation of the antimicrobial agents, amphotericin B, cephalothin, polymyxin B, trimethoprim and vancomycin, suppresses the growth of the normal microbial flora in fecal specimens, thereby facilitating isolation of *Helicobacter* spp. and other cephalothin-resistant *Campylobacter* spp.

Colonies on the campy-Bap culture media were morphologically described. Pure cultures were prepared on 5% sheep blood agar incubated at 37°C for 48 hours under microaerophilic conditions, for the biochemical tests.

#### Antibiotics disk susceptibility test (Kirby-Bauer Disk Diffusion Method).

A suspension of the bacterial culture to be tested was made and the turbidity was adjusted to McFarland no.0.5 (7)

A sterile cotton swab was placed in the bacterial suspension and excess fluid was removed by pressing and rotating the cotton against the inside of the tube above the fluid level. The swab was streaked in three directions over the surface of the blood agar plate to obtain a uniform growth. The plate was allowed to dry for five minutes.

Using a sterile forceps the paper disks impregnated with these fixed standard concentrations of the antibiotics, as prescribed by The National Committee for Clinical Laboratory Studies NCCL [amoxicillin (20µg), metronidazole (10µg), erythromycin (5µg), kanamycin (30µg), clarithromycin (5µg), rifampin (5µg), tetracycline (30µg), ciprofloxacin (5µg), vancomycin (30µg), polymyxin B (300 IU) and trimethoprim (5µg)] were placed over the uniformly inoculated agar

plate. It was incubated within 15 minutes after applying the disks [the plates were incubated soon after placing the disks since the test is standardized under conditions where diffusion of the antibiotics and the bacterial growth commences at approximately the same time (1)]. Following an overnight incubation, the diameter of the zone of growth inhibition around each disk was measured to the nearest whole mm. Susceptibility breakpoints set by NCCL now called Clinical and Laboratory Standards Institute CLSI and large clinical trials were used (8,9)

#### Plasmid curing

Acridine orange was used for plasmid curing using the following procedure:

50µl of Acridine orange (0.10 mg/ml) was added to 5ml of peptone water.

The antibiotic resistant *H. pylori* culture was inoculated to peptone water having acridine orange and incubation for 24 hrs in a shaker incubator to cure.

The cured culture was swabbed in to the blood agar plates and culture for 24hrs at 37°C.

The cured culture was characterized by evaluating its morphology, and subjected to another phenotypic identification tests as recommended by Al-Suilami *et al.*, (6). These tests include H<sub>2</sub>S production, nitrate reduction, growth in 1% glycine and 3.5% NaCl, and resistance to nalidixic acid (30 µg disc) and cephalothin (30 µg disc). Additional tests were hippurate hydrolysis, indoxyl acetate hydrolysis, urease production, and Glucose fermentation.

#### Cured culture antibiotic plasmid Profile

A second antibiotic susceptibility test (disc diffusion method) was performed with Clarithromycin, amoxicillin and metronidazole considered as the resistant markers for plasmid curing. Incubation was at for 24 hrs at 37°C and a sensitivity result was recorded as plasmid encoded and a resistant result was interpreted as chromosomal crypted.

#### Statistical analysis

With the serologic test, the frequency of *H. pylori* in the glaucoma and control subjects was compared to find an association between *H. pylori* infection and glaucoma.

The 202 *H. pylori* culture positive glaucoma patients were distributed by the clinical characteristics of IOP, visual acuity, optic neuropathy and the visual field defects.

ANOVA was used to test the hypothesis visual acuity varied in the glaucoma types.

#### RESULTS

A total of 4,220 patients of age 40 years and above were clinically screened for glaucoma. Glaucoma was diagnosed by the clinical features of increased IOP, reduced vision, constricted visual field and cupping of the disc at the lamina cribrosa (Figure 1) in 217 (5.14%) of the patients. When classified, it was 150 (69.1%) primary open angle glaucoma POAG, 32 (14.7%) normal tension glaucoma NTG and 35 (16.1%) primary angle closure glaucoma PACG.

#### Serological test result in the glaucoma patients and controls

In the serologic rapid immunochromatography diagnoses for *Helicobacter pylori* performed at the screening field, (using the Chemtrue *H. pylori* one step rapid test; Chemtron Biotech Incorporated San Diego, USA) a positive result for IgG antibodies appeared within 5 minutes for 212 (97.7%) of the glaucoma patients and only in 25 (13.89%) of the age/sex matched control group (Table 1). The 212 *H. pylori* serologic positive glaucoma patients consist of 150 (70.7%) POAG, 32 (15.1%) NTG and 30 (14.1%) PACG.

#### The clinical characteristics of the glaucoma patients

In table 2, the sex distribution of glaucoma was almost even in POAG (48.57% in males and 51.43% in females); it was also the same pattern of near even distribution in NTG (53.12% in males and 46.84% in females). PACG showed a wider distribution between males (30%) and females (70%).

The elevated IOP in POAG and PACG showed a near even distribution in there populations. IOP of 20<30mmHg was distributed as 28.57% in the POAG and as 26.67% in the PACG. 30<40mmHg was as 32.14% in POAG and 33.37% in PACG and finally as 39.29mmHg in POAG and 40.00mmHg in PACG (P<0.005).

In the clinical characteristics of the glaucoma patients positive for *H. pylori*, in terms of laterality, POAG was bilateral (diagnosed in both two eyes) in 59.28%. NTG was diagnosed more often in both eyes 62.50% and PACG was most of the time 100% bilateral. Glaucoma was almost evenly distributed between males 94 (46.77%) and females 108 (53.40%) with the glaucoma type PACG occurring more in women 21 of the 30 (70%); while POAG and NTG occurred respectively as 51.43% and 46.87% P<0.25. The age group 40 < 50 had the least prevalence of glaucoma 10.71% of POAG, 15.62% of NTG and 16.67% of PACG. An older group, 50<60 had 21.42% of the POAG; 21.87 of the NTG and 23.33% of the PACG, the prevalence increased with age P<0.005. The better visual acuity of

“greater than 6/6 to less than 6/12” was least in the population of POAG (3.57%), while about 6.50% on the average in the populations of NTG and PACG.

The worst VA group with correction of “6/60 and worse” was seen also in the population of the POAG (48.85%). NTG and PACG had such poor VA as 31.25% and 30.10% of their populations P<0.025.

In the distribution of optic neuropathies, rim thinning was most in the POAG (32.14%), 15.63 in the NTG and 10.00% in the PACG. Notching in the inferior temporal occurred more than notching in the superior temporal. NTG and PACG recorded more notching than the PACG. Total cupping involved the PACG most by 40%, 37.50% of the NTG and less of the POAG (32.14%) P<0.005.

The more advanced field defects “nasal step”, “arcuate scotoma”, “double arcuate”, and “small island” were most frequent in the populations of NTG (75.00%) and PACG (70.00%); they were less in POAG (65.69%) P<0.0005.

#### The Culture and sensitivity tests results

Faecal cultures for the organism were prepared from the 212 *H. pylori* serological ELISA positive patients. On the Campy-Bap selective medium for the isolation of *campylobacter* and *helicobacter species*, the morphological appearance and the biochemical characterizations identified *Helicobacter pylori* and three other species *H. helmannii*, *H. felis* and *H. pullorum* as the isolates from the faecal specimens (Table 3).

In the 212 serological *Helicobacter pylori* IgG positive patients, *Helicobacter pylori* was successfully cultured in 202 of the patients (140 POAG, 30 PACG and 32 NTG); *H. helmannii*, *H. felis* and *H. pullorum* were also isolated in 24, 12 and 7 of the serological positives respectively.

The *H. pylori* colonies were small (1-2mm), circular and convex after 3-5 days (Plate 1). They had the following biochemical characteristics (Table 3): All the 202 *H. pylori* isolates were gram negative; they did not retain the crystal violet stain. The *H. pylori* appeared on Gram stained smears as curved or comma-shaped rods that demonstrate bluntly rounded ends, spiral or helical shapes were less evident. The *H. pylori* had up to six polar sheathed flagella which are essential for its motility.

Of the 202 colonies of the pure cultures of *H. pylori*, 195 (96.5%) were able to induce a weak (alpha) hemolysis on the supplemented blood agar. The agars around the colonies were dark and greenish.

The endospore protective structures used to survive extreme conditions were absent in all the isolates. They did not retain 5% malachite green for the vegetative cells appeared red.

All the *H. pylori* (100%) produced catalase that neutralizes the bactericidal effects of hydrogen peroxide by breaking it down into oxygen and water. This was evidenced by the rapid bubbling as oxygen was evolved (within 5-10 sec.).

*H. pylori* produce cytochrome c oxidase, an enzyme of the bacterial electron transport chain. Which catalyze the transport of electrons from donor compounds (NADH) to electron acceptors, usually oxygen. With the end-product of this metabolism as either water or hydrogen peroxide which will be broken down by catalase (7).

The cytochrome c oxidase oxidized the reagent tetramethyl-p-phenylenediamine that acts as an artificial electron donor for the enzyme oxidase to indophenols with a purple color end product. This was positive in all the 202 (100% of the) isolates.

Urease was produced by the *H. pylori* and they split urea in the presence of water to release ammonia and carbon dioxide. The ammonia combined with carbon dioxide and water to form ammonium carbonate which turns the medium alkaline. The test was also positive in all the 202 isolates (100%).

*H. pylori* was poor in nitrate reduction, when sulfanilic acid was added and then the α-naphthylamine, the media did not turn red. Meaning that most of the organism was unable to reduce the nitrate, or they were able to denitrify the nitrate or nitrite to produce ammonia or molecular nitrogen. On adding a small amount of powdered zinc to the tube, 192 (95%) of the isolates turned red; meaning the presence of unreduced nitrate.

The hydrolysis of indoxyl acetate also was poor. Only 10 (4.9%) of the *H. pylori* isolates was positive to the hydrolysis of indoxyl acetate.

In hippurate hydrolysis into glycine and benzoic acid, the glycine end product detected by the addition of ninhydrin reagent was in only 9 (4.4%).

*H. pylori* was more resistant to cephalothin (79.2%) and sensitive to nalidixic acid. It had limited growth in 1% glycine and 3.5% NaCl by 24.7% and 4.9% respectively.

*H. pylori* ability to produce tryptophanase, which hydrolyse the amino acid

tryptophan to produce indole was poor. It was only positive in 10 isolates (4.9%). Its ability to utilize thiosulfate anion as a terminal electron acceptor, reducing it to hydrogen sulfide was in 20 (9.9%) of the isolates. The isolates were highly motile and poor in carbohydrate fermentation. Sucrose, lactose and glucose in TSI media were fermented in 7.4%, 12.3% and 7.4% of the isolates respectively.

*Helicobacter felis* were rigid and spiral-shaped cells that measured 0.4µm wide and 5-7.5µm long. Spherical forms (diameter, 2-4µm) were present in older cultures. Endospores were not produced. The Cells were motile with a rapid corkscrew-like motion. Cells had tufts of 10 to 17 polar sheathed flagella of thickness about 25 nm that were positioned slightly off the centre at the end of the cell. No growth occurs in the presence of 1% glycine and 3.5% NaCl. They were asaccharolytic and no acid was produced from sucrose, lactose and glucose; it was only in 1 strain (8.3%). They (all the 12 isolates of *Helicobacter felis*) were positive for urease, oxidase, catalase and ten (83.3%) reduced nitrate. It was 16.6% (1 strain) that produced indole. Hippurate hydrolysis and H<sub>2</sub>S production were negative.

The *Helicobacter heilmannii* cells were tightly coiled spirals with up to nine turns, approximately 3.0–6.5mm long and 0.6-0.7mm wide. The coccoid cells predominate in older cultures. The cells were motile by means of tufts of up to 10 sheathed blunt-ended flagella at both ends of the cells. There was no growth in 3.5.5% NaCl or 1% glycine.

All the 24 isolates (100%) were positive for oxidase, catalase and urease tests. They reduce nitrate and hydrolyse hippurate. Indoxyl acetate hydrolysis was only in 3 (12.5%).

The *Helicobacter pullorum* cells were non-spore-forming, gently curved, slender, rod-shaped, 3-4µm in length. The cells had an unsheathed monopolar flagellum and a typical darting motility. They were microaerophilic and grow microaerobically at 37°C and 42°C. The colonies were pinpointed, non-pigmented, translucent and α-haemolytic.

All the 7 *Helicobacter pullorum* isolates were positive for oxidase and nitrate reduction. Most strains (57.1%) produce catalase. They were negative for hippurate and indoxyl acetate hydrolysis; while a strain (14.2%) was positive for urease production. The *H. pullorum* were intolerant to 3.5% NaCl. They were mostly sensitivity to nalidixic acid and (71.4%) were resistant to cephalothin.

In the antibiotics disc diffusion tests, the antibiotics susceptibility pattern of the 202 isolates of *H. pylori* (Table 3) shows that the *H. pylori* isolates were most sensitive to tetracycline, where 190 (94%) of the isolates had an inhibition zone that was greater than 21mm. Resistance to tetracycline was observed in only 8 strains with an inhibition zone that was less than 19mm; and an intermediate susceptibility with a zone of 21 to 19mm was observed for 4 strains.

Next to tetracycline was ciprofloxacin, 185(91.5%) of the *H. pylori* isolates were sensitive with an inhibition zone that was greater than 19mm. Ten (4.9%) isolates of the *H. pylori* was resistant to ciprofloxacin with an inhibition zone that was less than 17mm.

*H. pylori* was most resistant to vacomycin and trimethoprim, where 135(66.8%) isolates of the *H. pylori* had a zone of inhibition less than 13mm for vacomycin; and with trimethoprim, 125 (61.9%) isolates had a zone of inhibition that was less than 20mm. Forty seven (23.2%) and 52 (25.7%) strains were sensitive to vacomycin and trimethoprim respectively.

### The Plasmid cured

Clarithromycin, amoxicillin and metronidazole were considered as the resistant markers for plasmid curing. Four *H. pylori* isolates with multiple resistance to clarithromycin, amoxicillin and metronidazole randomly were selected and subjected to plasmid curing with Acridine orange.

The results of the disc diffusion after the plasmid curing indicated that all the four cured isolates of *H. pylori* were sensitive to amoxicillin (20µg) with zones of inhibition greater than 17mm; and resistant to clarithromycin (5µg) with zones of inhibition less than 15mm. Only two cured *H. pylori* isolates were sensitive to metronidazole (10µg) zones of inhibition greater than 31mm.

The results obtained from characterization of the cured *H. pylori* indicate that the cured *H. pylori* exhibited similar biochemical characteristics regarding all tests as well as the same morphology as the original *H. pylori* isolates.

### DISCUSSION

*Helicobacter pylori* infection has been implicated in ischemic heart disease, cerebrovascular disease, Raynaud's phenomenon, and migraine (10).

Kountouras et al, (2) were the first investigators to report an association between *H. pylori* and glaucoma, and they subsequently reported that *H. pylori* IgG antibody levels were elevated in the aqueous humors and sera of POAG and PXG patients (11). In addition, another study based on serologic testing showed a possible relationship between *H. pylori* infection and POAG (12). However, a study

by Galloway *et al.* (13) in a Canadian population demonstrated that *H. pylori* infection is not associated with POAG, PXG, NTG, or ocular hypertension. However some limitations in the study by Galloway *et al.* (13) were noted (14).

Our study agrees more with the previous authors, a significant positive association was analyzed between *H. pylori* infection and glaucoma. *H. pylori* IgG antibody was detected in 212 (97.69%) of the glaucoma patients and only in 25(13.89%) of the control,  $P < 0.001$ . This interpretation is that there is a less than 1 in 1000 chance that these results have occurred randomly.

*Helicobacter pylori* as its main reservoir is the stomach and associated with various upper gastrointestinal diseases *H. pylori* induce a strong systemic host immune response, and the release of various vasoactive and proinflammatory substances (15,16); it contributes to the development of diseases in extradigestive areas.

Normal-tension glaucoma (NTG) involves progressive glaucomatous optic neuropathy and corresponding visual field defects with the intraocular pressure (IOP) in the normal range. Since the IOP remains normal, mechanisms involving ocular blood flow or an autoimmune reaction are believed to play a role in the pathogenesis of NTG (17). Therefore *H. pylori* infection may be associated with the pathogenesis of NTG more than others, in which a high IOP is recognized as a major pathogenic factor. The study corroborated this, *H. pylori* serology positive was distributed as 93.33% in (140 of the 150) POAG, 85.71% in PACG and 100% (30 of the 30) in NTG.

If *H. pylori* can live in the trabecular meshwork, decreased outflow facility induced by inflammation may cause ocular hypertension and glaucoma. Some studies collected aqueous humor from anterior chamber to analyze the correlations. In one study *H. pylori* was detected at significantly higher levels in the POAG group (19). Once more our study recorded a 93.33% (140 of the 150) serum prevalence of *H. pylori* IgG in POAG. Another study showed significantly higher levels of *H. pylori* IgG antibody in the PXE group than in the POAG group (19). Kountouras *et al.* 2002 reported both POAG and PXE groups showed significant high levels of antibody though it was higher in the POAG group than in the PXE group. However, this mechanism of decreased outflow facility induced by inflammation in the trabecular is not expected in NTG patients. In NTG patients, *H. pylori* infection lead to optic disc damage by decreasing ocular blood flow, secreting toxic materials, and causing antibody-induced apoptosis attributed to inflammation in the retrobulbar area. Although this hypothesis can be proved with invasive retrobulbar tissue biopsies, these are difficult to perform in practice. The other possible hypotheses for the association between *H. pylori* infection and NPG as suggested (12), are the release of proinflammatory and vasoactive substances (15,16), platelet activation and aggregation, induction of increased levels of tissue factor-like procoagulants (13), development of cross-mimicry between endothelial and *H. pylori* antigens, and production of reactive oxygen species (16). It is also amazing that *H. pylori* infection has been reported to be associated with diverse nondigestive diseases, such as cerebrovascular disease, ischemic heart disease, and migraine, all which are associated with NTG (10). In the case history reports and 'condition of general health' virtually all of our NTG patients had vascular involvements.

As a result of angle closure by the peripheral iris, a more increased IOP from aqueous outflow obstruction solely is expected for PACG. However, the distribution of IOP in the study between POAG and PACG was similar and much more comparable (Table 2),  $P < 0.005$ . Our diagnosis of PACG was limited and was based on the shadow test estimation of the anterior chamber angle.

Endoscopic biopsy remains the gold standard for diagnosing *H. pylori* infection. However, this technique is complicated, requires special skills, is time consuming, and thus is inappropriate for screening large populations. In addition, it does not reveal the presence of a previous infection. The urea breath test is a possible alternative means of detecting *H. pylori* infection and is a reliable and noninvasive method. However, it is expensive, time consuming, produces a radioactive product, and detects only current infections and thus is not appropriate for large populations. The presence of IgG antibodies against *H. pylori* can be determined by standardized ELISA testing, which is inexpensive and rapid, and ELISA can detect exposure to *H. pylori* regardless of treatment. Moreover, ELISA is generally viewed to have high sensitivity and specificity (beyond 90%), despite the fact that some authors have reported suboptimal accuracies for ELISA (19). In the present study, it was important that previous and current infections be detected in a large population; thus we performed the rapid ELISA testing for *H. pylori* IgG in the field screening exercise.

In addition, culture and antibiotic sensitivity testing is valuable, realizing the increasing prevalence of antimicrobial resistance and its potential negative impact on the efficacy of many treatment eradication regimens. Hence the microbiological isolation of the organism in a culture media was performed as a confirmatory test for the organism and further for antibiotics sensitivity.

Some authors (19) have shown that sufficient culture of *H. pylori* will be obtained after transportation of the specimen in a medium such as Stuarts transport medium for up to 24hr at low temperature (about 4°C), whereas a higher temperature (about 20°C) decreases the number of positive cultures significantly. In

this study, a fresh faecal specimen (1 gram) was collected from each serological positive patient and immediately emulsified in 10ml of sterile saline, after 2 minutes of its settling, drops of the suspension were filtered and inoculated onto the Campy-Bap selective medium. *Helicobacter pylori* was successfully cultured in 95.2% (202 of the 212) serological positive. This is similar to the studies (20) where a more than 95% concordance between culture and histological detection of *H. pylori* were reported when the biopsies were inoculated on agar plates within 4 h after the biopsies are taken. In another study with similar culture conditions but a transportation time for biopsies of up to 24 hours, *H. pylori* was only cultured from about 80% of biopsies with helicobacter-like organisms detected in histological sections (21). Thus, a decrease in culture rate of about 15% was found when biopsies were transported or stored overnight. A long transportation time decreases the number of *H. pylori* especially after antibiotic therapy, and if the number of bacteria is low, culture may become false negative. This yield can be improved by prolonged incubation, up to 12 days (20).

*H. pylori* can grow on different solid media containing blood or blood products (21). Most studies have used Brucella agar or Columbia agar as the agar base. An amount of 7 to 10% blood improves the growth of *H. pylori* as compared with 5% blood. Horse blood may also improve the growth of *H. pylori* as compared to sheep blood (22).

Often *H. pylori* grow poorly or not at all on selective media containing antibiotics (20). Skirrows and Dents selective media seem to be the best available commercial selective media and have been used in several studies (6). There seem to be greater differences between horse and sheep blood agar, in favor of horse blood, than between horse blood agar with and without antibiotics (22). Our culture medium, Campy-Bap medium supports the growth of *Campylobacter* and *Helicobacter* species due to its content of peptones, dextrose, yeast extract and blood. The peptones supply nitrogenous compounds, carbon, sulfur and trace ingredients. Yeast extract is a source of the B vitamins. Sheep blood supplies additional nutrients. The incorporation of the antimicrobial agents, amphotericin B, cephalothin, polymyxin B, trimethoprim and vancomycin, suppresses the growth of the normal microbial flora in fecal specimens, thereby facilitating isolation of *Helicobacter* spp. and other cephalothin-resistant *Campylobacter* spp.

*H. pylori* usually is grown in jars with gas-generation kits (6,20) or a standard microaerobic atmosphere, in CO<sub>2</sub> incubators or anaerobic chambers with a microaerobic atmosphere. Most studies with standardized atmospheres for culture of *H. pylori* have used 2 to 5% O<sub>2</sub>, 5 to 10% (optimal closer to 10%) CO<sub>2</sub>, and 0 to 10% H<sub>2</sub> (6).

The *H. pylori* colonies were small (0.5 to 2 mm), translucent to yellowish colonies on the Campy Bap medium. In very young cultures *H. pylori* appeared as almost straight rods on microscopy. After 3 to 5 days of incubation the bacteria look pleomorphic, with irregular curved rods, several being U shaped. In old cultures, *H. pylori* appeared as degenerative coccoid forms that Gram stained poorly. Because of their small size, *H. pylori* colonies may be difficult to identify and isolate when there are few colonies and additional contaminating oral microbiota. Some contaminating microorganisms may grow as small colonies but differ usually from *H. pylori* in color.

The conversion and morphological change of spiral-shaped *H. pylori* into coccoid forms are achieved in various ways: (i) by nutrient deprivation (6), (ii) by exposure to anti-ulcer drugs and antibiotics (24), (iii) by extended incubation (24), (iv) by pH adjustment (19), and (v) by attachment to the gastric epithelium (23). Changes in the morphology of *H. pylori* in the culture plates over time were observed: after 3 days spiral forms dominated, after 6 days about half of the bacteria converted into U-shaped or coccoid forms, and after 10 days only coccoid forms were found (19). Morphological changes are induced faster with exposure to detrimental environmental circumstances (23).

Coccoid forms of *H. pylori* have been found to be closely associated with damaged mucous cells whereas spiral forms have been found in proximity to unchanged or less damaged cells (24). Coccoid forms of *H. pylori* are found more frequently and in larger numbers in the gastric mucosa of patients with gastric cancer than in patients with peptic ulcer disease (23). The study on glaucoma patients showed organisms that appeared as gram-negative curved bacilli in up to day two of culture, with morphological changes to coccoid form appearing over time. All the (202) isolates were positive for oxidase, catalase and urease tests. Its ability to split urea within 30 seconds distinguishes it from other *Helicobacter* species cultured from the fecal specimens. They were negative for hippurate, nitrate reduction tests and very poor in fermenting carbohydrates.

The antibiotics most widely used in the treatment of *H. pylori* infection include metronidazole, clarithromycin, amoxicillin and tetracycline (21). Over the last few years, many studies from different parts of the world dealing with antimicrobial resistance rates in *H. pylori* have been published. In the studies, the resistance rate of metronidazole has been the highest. These metronidazole resistance results varies from <10% to >80% in different geographical regions (20). This high resistance may be related to the high utilization of this antibiotic in these areas (25); where it is used in the treatment of diarrheal disease including parasitic and bacterial infections and as also the first line therapy in gastritis infected with *H. pylori* strains. (26). Thus a high resistance to metronidazole can

be expected. In this study the most resistance of 44.5% was also reported for metronidazole.

Erythromycin had the next highest resistance of 17.3%. Clarithromycin another macrolide 13.8% resistance was unexpected, since clarithromycin is not an over the counter drug used in Nigeria as erythromycin. This resistance could be related to cross-reactivity between erythromycin and clarithromycin, which implies that if a strain is resistant to one macrolide it becomes resistant to all others (21).

Resistance to amoxicillin was observed in 8.9% of the isolates. Much lower rates of about 2 to 3% resistance have been reported worldwide (21,27,28).

Colonization of the stomach with other b-lactam-resistant bacteria may lead to transfer of amoxicillin resistance to *H. pylori* by transformation or a conjugation-like mechanism (29).

Like the studies that have been done worldwide, tetracycline and ciprofloxacin resistance for *H. pylori* is very low 3.9% and 4.9% respectively.

Potential reasons for the less resistant *H. pylori* strains in the study point to the type of population enrolled. Based on the selection criteria, these patients had a much less previous exposure to antibiotics and had no previous *H. pylori* eradication treatment, a strong risk factor for the development of acquired resistance. Another possible explanation for the low resistance of *H. pylori* isolates as compared to other regions of the world is the difficulty procuring these antibiotics due to their high cost. In this study, much of *H. pylori* positive glaucoma patients were retired unemployed and from the rural with average incomes below the poverty level defined in Nigeria.

Antimicrobial resistance property can come up through acquisition of genetic material encoding enzymes that inactivate a particular antibiotic (30). Gene resistance markers in *H. pylori* can be presented in plasmid, chromosome or both. For instance, erythromycin resistance has been reported previously as by chromosomal genes, whereas chloramphenicol resistance is plasmid encoded. It has been reported that resistance to chloramphenicol, kanamycin and tetracycline in *Camp. jejuni* is plasmid-mediated, while resistance to rest of the antibiotics is chromosomally mediated (30). On the other hand, several reports illustrated that some virulence factors of *Campylobacter* are associated with existence of the plasmid in the bacterium (31, 32).

Plasmid curing is defined as a loss of plasmid from cell, which leads to loss of specific phenotypes such as drug resistance (33). The *H. pylori* isolates that had combined resistant to clarithromycin, amoxicillin and metronidazole invitro were subjected to plasmid curing to find out the location of the resistant markers of clarithromycin, amoxicillin and metronidazole.

The result obtained indicated that cured strain were sensitive to amoxicillin and resistant to clarithromycin. Therefore, it can be concluded that probably amoxicillin resistant marker in *H. pylori* is plasmid mediated, while clarithromycin resistant marker is chromosomally mediated. This finding is supported by Akinjogunla and Enabulele (2010).

Dasti *et al.* (33) reported a bacterium resistance to any antibiotics could be plasmid or chromosomally mediated. In this study two out of the four cured *H. pylori* were still resistant to metronidazole; indicating a plasmid and chromosomal mediation.

The results obtained from characterization of cured strains of *H. pylori* using phenotypic identification tests showed no difference between the cured and the original *H. pylori*. Therefore, it can be concluded that the most of phenotypic characters of *H. pylori* are associated with primary metabolism and its metabolites are chromosomally mediated.

According to Baserisalehi and Bahador (34) plasmid mediated antibiotic resistance markers in *campylobacters* and *helicobacters* cause transmission of resistance markers among these bacteria and finally reach the human population by direct contact and via food products of animal origin.

In studies on multiresistance (35, 36, 37), low rates of multiresistance are observed with combinations of antibiotic unrelated to one other. Combination of omeprazole (as proton pump inhibitor), clarithromycin (a macrolide) and amoxicillin (a B-lactam) have been used in patients of duodenal ulcer disease associated with *H. pylori* with excellent cure rates (38).

In one of the recent studies done in on tripple thereapy with omeprazole 20 mg orally twice a day, clarithromycin 500 mg twice a day and amoxicillin 1 gram orally twice a day administered to 129 histopathology positive cases of *H. pylori*. Out of these, 81 patients (63%) showed improvement of symptoms while 48 patients (37%) did not respond to treatment (24).

## CONCLUSION

*H. pylori* infection is associated with the risk for glaucoma development in the population. Anti *H. pylori* IgG was detected more in the glaucoma patients than

the control group. The organism was isolated in 212 faecal samples of the 217 glaucoma patients. The organism was positive to catalase, oxidase, urease tests; and they were also highly motile. They were most sensitive to tetracycline (94%) and ciprofloxacin (91.5%). *Helicobacter pylori* was most resistant to vancomycin (23.2%) and trimethoprim (25.7%). They genetic marker for amoxicillin resistance was plasmid located; while that of clarithromycin was chromosomal. Metronidazole was both plasmid and chromosomal. *Helicobacter pylori* eradication in the glaucoma patients leads to improvement of the glaucoma clinical features. Clinicians who care for patients with *H. pylori* infection should also consider that *H. pylori* can cause not only digestive illness but also eye disease.

### RECOMMENDATION

The age at which *H. pylori* 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. Infections are usually acquired in early childhood in all countries (39). Thus the higher prevalence among the elderly reflects higher infection rates when they were children rather than infection at later ages (39). Early screening and an eradication scheme for *H. pylori* in children population is recommended.

The infection rate of children in developing nations is higher than in industrialized nations. This is due to poor sanitary conditions.

Antibiotics resistance is on the increase; there are already many metronidazole and clarithromycin resistant strains in most parts of the world (21). Thus the indiscriminate use of these drugs of first line of treatment should be avoided.

### TABLES

**Table 1: Serology test result for H pylori in the glaucoma patients and control**

| Serology result | Glaucoma<br>n (%) | Control<br>n (%) |
|-----------------|-------------------|------------------|
| Positive        | 212 (97.7)        | 25 (13.89)       |
| Negative        | 5 (2.3)           | 155 (86.11)      |
| P< .001         |                   |                  |

**Table : Clinical characteristics of H. pylori positive glaucoma patients**

|                           | POAG<br>n (%) | NTG<br>n (%) | PACG<br>n (%) |
|---------------------------|---------------|--------------|---------------|
| <b>Sex</b>                |               |              |               |
| Male                      | 68 (48.57)    | 17 (33.12)   | 9 (30.00)     |
| Female                    | 72 (51.43)    | 15 (46.87)   | 21 (70.00)    |
| <b>Laterality</b>         |               |              |               |
| Unilateral                | 83 (59.28)    | 12 (37.50)   | 0             |
| Bilateral                 | 57 (40.72)    | 20 (62.50)   | 30 (100)      |
| <b>Age</b>                |               |              |               |
| 40<50                     | 15 (10.71)    | 5 (15.62)    | 5 (16.67)     |
| 50<60                     | 30 (21.42)    | 7 (21.87)    | 7 (23.33)     |
| 60<70                     | 45 (32.14)    | 8 (25.00)    | 8 (26.67)     |
| 70 and above              | 50 (35.71)    | 12 (37.50)   | 10 (33.33)    |
| <b>Best correction VA</b> |               |              |               |
| >6/6 <6/12                | 5 (3.57)      | 2 (6.25)     | 2 (6.67)      |
| 6/12 <6/24                | 15 (10.71)    | 5 (15.63)    | 5 (16.67)     |
| 6/24 <6/36                | 30 (21.42)    | 7 (21.87)    | 7 (23.33)     |
| 6/36 <6/60                | 30 (21.42)    | 8 (25.00)    | 7 (23.33)     |
| 6/60 and worse            | 60 (48.85)    | 10 (31.25)   | 9 (30.00)     |
| <b>Optic neuropathy</b>   |               |              |               |
| Rim thinning              | 45 (32.14)    | 5 (15.63)    | 3 (10.00)     |
| Notch inferior temporal   | 35 (25.00)    | 11 (34.37)   | 10 (33.33)    |
| Notch superior temporal   | 15 (32.14)    | 4 (12.50)    | 5 (16.67)     |
| Total cupping             | 45 (32.14)    | 12 (37.50)   | 12 (40.00)    |
| <b>Field defects</b>      |               |              |               |
| General constriction      | 9 (6.43)      | 1 (3.12)     | 1 (3.33)      |
| Blind Spot baring         | 9 (6.43)      | 2 (6.25)     | 2 (6.66)      |
| Seidel scotoma            | 15 (10.71)    | 2 (6.25)     | 3 (10.00)     |
| Paracentral scotoma       | 15 (10.71)    | 3 (9.37)     | 3 (10.00)     |
| Nasal step                | 22 (15.71)    | 6 (18.75)    | 4 (13.33)     |
| Arcuate scotoma           | 30 (21.42)    | 6 (18.75)    | 5 (16.67)     |
| Double arcuate            | 20 (14.28)    | 6 (18.75)    | 5 (16.67)     |
| Small island              | 20 (14.28)    | 6 (18.75)    | 7 (23.33)     |

P < 0.005

P < 0.0005

POAG =Primary open angle glaucoma  
NTG =Normal Tension glaucoma  
PACG =Primary angle closure glaucoma

**Table 3: Biochemical characteristics of the faecal isolates**

| Characteristics                 | Faecal Specimen Isolates |         |                      |         |                 |         |                    |         |
|---------------------------------|--------------------------|---------|----------------------|---------|-----------------|---------|--------------------|---------|
|                                 | <i>H. pylori</i>         |         | <i>H. heilmannii</i> |         | <i>H. felis</i> |         | <i>H. pullorum</i> |         |
|                                 | n                        | (%)     | n                    | (%)     | n               | (%)     | n                  | (%)     |
| 1 Gram                          | --                       | --      | --                   | --      | --              | --      | --                 | --      |
| 2 Endospore                     | --                       | --      | --                   | --      | --              | --      | --                 | --      |
| 3 α Hemolysis                   | 195                      | (96.5%) | 20                   | (83.3%) | 12              | (100%)  | 5                  | (71.4%) |
| 4 β Hemolysis                   | --                       | --      | --                   | --      | --              | --      | 1                  | (14.2%) |
| 5 Catalase                      | 202                      | (100%)  | 24                   | (100%)  | 12              | (100%)  | 4                  | (57.1%) |
| 6 Oxidase                       | 202                      | (100%)  | 24                   | (100%)  | 12              | (100%)  | 7                  | (100%)  |
| 7 Urease                        | 202                      | (100%)  | 24                   | (100%)  | 12              | (100%)  | 1                  | (14.2%) |
| 8 Nitrate reduction             | 10                       | (4.9%)  | 24                   | (100%)  | 10              | (83.3%) | 7                  | (100%)  |
| 9 Indoxyl acetate hydrolysis    | 10                       | (4.9%)  | 3                    | (12.5%) | --              | --      | --                 | --      |
| 10 Hippurate hydrolysis         | 9                        | (4.4%)  | 24                   | (100%)  | --              | --      | --                 | --      |
| 11 Sucrose fermentation         | 15                       | (7.4%)  | 4                    | (16.6%) | 1               | (8.3%)  | --                 | --      |
| 12 Lactose fermentation         | 25                       | (12.3%) | 5                    | (25%)   | 1               | (8.3%)  | --                 | --      |
| 13 Glucose fermentation         | 15                       | (7.4%)  | 2                    | (8.3%)  | 1               | (8.3%)  | --                 | --      |
| 14 H <sub>2</sub> S             | 20                       | (9.9%)  | 2                    | (8.3%)  | --              | --      | 1                  | (14.2%) |
| 15 Indole                       | 10                       | (4.9%)  | 3                    | (12.5%) | 2               | (16.6%) | 1                  | (14.2%) |
| 16 Motility                     | 202                      | (100%)  | 24                   | (100%)  | 12              | (100%)  | 7                  | (100%)  |
| 17 Growth in 1% glycine         | 50                       | (24.7%) | --                   | --      | --              | --      | 4                  | (57.1%) |
| 18 Growth in 3.5% NaCl          | 10                       | (4.9%)  | --                   | --      | --              | --      | 1                  | (14.2%) |
| 19 Nalidixic Resistant (30µg)   | 60                       | (29.7%) | 3                    | (12.5%) | 2               | (16.6%) | 1                  | (14.2%) |
| 20 Cephalothin Resistant (30µg) | 160                      | (79.2%) | 3                    | (12.5%) | 2               | (16.6%) | 5                  | (71.4%) |

n = number of test positives  
% = Percentage frequency of test positives

**Table 4: Antibiotics susceptibility pattern of H. pylori**

| Antibiotics      | Disc Potency | Susceptibility Pattern |               |     |    |                  |         |     |                 |
|------------------|--------------|------------------------|---------------|-----|----|------------------|---------|-----|-----------------|
|                  |              | n                      | Resistant (%) | ZI  | n  | Intermediate (%) | ZI      | n   | Sensitivity (%) |
| 1 Amoxicillin    | 20µg         | 18                     | (8.9%)        | <17 | 4  | (2%)             | 19 - 17 | 180 | (89%)           |
| 2 Metronidazole  | 10µg         | 90                     | (44.5%)       | <28 | 12 | (5.9%)           | 31 - 28 | 100 | (49.5%)         |
| 3 Erythromycin   | 5µg          | 35                     | (17.3%)       | <19 | 10 | (4.9%)           | 25 - 19 | 157 | (77.7%)         |
| 4 Kanamycin      | 30 µg        | 30                     | (14.8%)       | <21 | 7  | (3.5%)           | 24 - 21 | 165 | (81.6%)         |
| 5 Clarithromycin | 5 µg         | 28                     | (13.9%)       | <15 | 8  | (3.9%)           | 17 - 15 | 166 | (82.1%)         |
| 6 Rifampin       | 5 µg         | 30                     | (14.8%)       | <23 | 12 | (5.9%)           | 25 - 23 | 160 | (79.2%)         |
| 7 Tetracycline   | 30 µg        | 8                      | (3.9%)        | <19 | 4  | (1.9%)           | 21 - 19 | 190 | (94%)           |
| 8 Ciprofloxacin  | 5 µg         | 10                     | (4.9%)        | <17 | 7  | (3.4%)           | 19 - 17 | 185 | (91.5%)         |
| 9 Vancomycin     | 30 µg        | 135                    | (66.8%)       | <13 | 20 | (9.9%)           | 14 - 13 | 47  | (23.2%)         |
| 10 Polymyxin B   | 300 : unit   | 80                     | (39.6%)       | <17 | 27 | (13.3%)          | 19 - 17 | 95  | (47%)           |
| 11 Trimethoprim  | 5 µg         | 125                    | (61.9%)       | <20 | 25 | (12.3%)          | 22 - 20 | 52  | (25.7%)         |

n = number of isolates  
% = Percentage frequency of isolates  
ZI= Diameter of Zone of inhibition (mm)

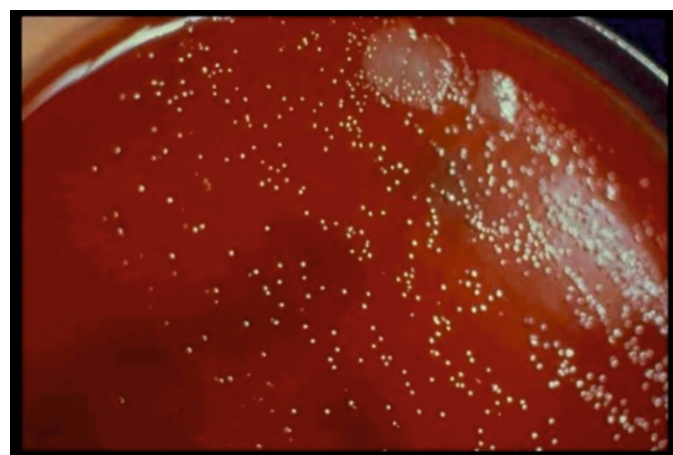


**Plate 1: Pure growth of H. pylori in blood agar plate [sample POAG 11]**

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