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ASSESSMENT OF *HELICOBACTER PYLORI* USING DIFFERENT IDENTIFICATION METHODS ON SUBFERTILE FEMALES IN A FERTILITY CLINIC IN AWKA

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ABSTRACT

Background: *Helicobacter pyroli* infection occurs when the bacteria infects the stomach lining and causes inflammation, peptic ulcer disease and certain types of stomach cancer. Women with *H.pyroli* infection have specific antibodies in cervical mucus.

Aim: This study was aimed at assessment of *H.pyroli* using different identification methods on subfertile female patients in Awka, Anambra State, Nigeria.

Methodology: This cross sectional study involved 101 females attending Life International Hospital, consecutively sampled aged 18 to 35 years. Stool samples were collected for culture and *H. Pyroli* stool antigen Elisa test (HpSA). Smears from isolated colonies were used for gram staining, urease test, catalase test and oxidase test. PCR products were separated on a 1.5% agarose gel and DNA bands were visualized with Ethidium bromide.

Results: Out of the 101 participants, 21.8% were positive in the culture method and 14.9% positive in the stool Antigen Elisa method. Among the participants, age group 31-35 years tested 100% for *H. Pyroli* in both methods. There were no positive cases in PCR method in all the age groups, with all cases being negative. There was significant difference in *H. Pyroli* among different age groups for both culture method (X^2 =9.751, P=0.002) and stool antigen Elisa method (X^2 =6.107, P=0.013). In the duration of subfertility, age groups 6-10 years had 54.5% positive in culture method and 73.3% positive in the stool antigen Elisa method with no positive in the PCR method. There was no significant difference among the different methods (p-values> 0.05).

Conclusion: The choice of test to detect *H.pyroli* infection depends on the prevalence and strains of *H.pyroli* on endemic areas, accessibility, advantages and disadvantages of each method as well as different clinical circumstances of each patient. To combine the results of two or more tests could be a reasonable strategy in routine clinical practice to achieve the most reliable result.

Keywords: *Assessment, H. pyroli, Subfertility, Identification methods.*

INTRODUCTION

Subfertility is failure to conceive after regular unprotected sexual intercourse for 12 months. Subfertility is a global health problem and of public health importance in Nigeria and many other developing nations because of its high prevalence, and due to its serious social implications[1]. Subfertility in women is reduced fertility with a prolonged time of unwanted non conception and includes many reversible causes like infections, tubal diseases, ovulation problems, fibroids, endometriosis, pelvic inflammatory diseases, etc[2]. It affects over 48 million women worldwide, one couple in seven has difficulty conceiving a baby[2]. Couples who have sex every two or three days have 84% chances of conception in a year and women who have regular sex without using any protection and do not get pregnant are considered subfertile[3]. Subfertility can be categorized as primary or secondary. Primary subfertility is the inability to achieve conception and to have a

successful live birth without ever having a child. On the other hand, secondary subfertility is the inability to achieve conception and have a successful live birth when individuals have had a previous biological child[4].

The burden of *Helicobacter pylori* infection globally estimated at 50% of the world's population undoubtedly makes the organism a pathogen of concern coupled with its classification as group I carcinogen by the World Health Organization[5]. In a study, 2.6% of Nigerian patients were reported to have gastric cancer resulting from *Helicobacter pylori* infection. Beyond the high prevalence of *Helicobacter pylori* infection and its associated clinical outcomes, emerging high antimicrobial resistance in Nigeria poses grave danger to the effective treatment and eradication of the pathogen. *Helicobacter pylori* is a Gram-negative, helical, micro aerophilic bacterium that colonizes the gut[6]. *Helicobacter pylori* has been implicated in various gastric disorders including peptic ulcer disease and chronic gastritis.

METHODOLOGY

The study was a cross sectional research on the analysis of *Helicobacter pylori* of sub fertile female patients attending Life International Hospital Awka (LIHA), Anambra State. It is a multi-specialist hospital with excellence in areas of endoscopy surgery and fertility care.

A consecutive sampling method was used to recruit 101 participants. Samples were collected from willing female patients clinically diagnosed for subfertility (primary and secondary subfertility) within the reproductive age range of 18-35 years of age.

Fresh stool samples were produced by subjects in Stericon containers avoiding any possible contact with urine or water. Stool samples were maintained at room temperature and culture and *H.pylori* stool antigen test (HpSA) were done within three hours of collection.

Helicobacter pylori stool antigen test method, HpSA.

The stool sample was first prepared for HpSA by emulsifying 1gram of stool into 4ml of phosphate buffer saline (PBS). 1ml of the mixture was collected into Eppendorfs tubes and spun at 6000rpm for 15minuites after which clear supernatants where separated carefully and used for HpSA analysis.In the Micro plate, the corresponding micro pores of the sample in sequence were numbered, two wells were left as negative control, two wells as positive control and one empty well as blank control. Negative and positive control in a volume of 50 µl were added to the negative and positive control wells respectively. In sample wells, 40µl sample dilution buffer and 10 µl sample were added (dilution factor is 5). Incubate was done for 30 min at 37°C after sealed with closure plate membrane. The concentrated washing buffer was diluted with distilled water. The wash solution was discarded after resting for 30 seconds. The washing procedure was repeated for 5 times. A volume of 50 µl HRP-Conjugate reagent was added to each well except the blank control well and incubated for 30min at 37◦C after which it was washed as described above. A volume of 50 µl Chromogen Solution A and 50 µl Chromogen Solution B was added to each well, it was mixed with gently shaking and incubate at 37°C for 15 minutes. Light was avoided during colouring.

Stop solution of a volume of 50ul was added to each well to terminate the reaction. The colour in the well changed from blue to yellow. Absorbance optical density (OD) was read at 450nm using a Microtiter Plate Reader. The OD

value of the blank control well was set as zero. Assay was carried out within 15 minutes after adding stop solution.

Culture Method

The stool sample was emulsified in phosphate buffered saline and 1gram of cholestyramine was added to the suspension. The emulsion was filtered using sterile muslin cloth to remove stool debris. Filtrate further filtered using membrane filter of pore size 0.45 µm as it is expected to retain *H. pylori* if present in the stool. The membrane filter was now cultured for a period of 3 to 12 days in a microaerophilic atmosphere (5% O_2, 10% CO_2 and 85% N_2) using the anaerogen gas pak (Oxoid-England) at 37^0C on a ready-to-use selective Columbia blood agar medium containing 10% sheep-defibrinated blood and 1% heat-inactivated fetal bovine serum, supplemented with Dent antibiotic supplement. The plates were incubated at 37 °C under microaerophilic conditions (10% CO_2, 5% O_2, and 85% N_2) with saturated humidity .Plates were checked intermittently for sub culture after the first 3 days through to the 5th day before discarded as no growth. Colonies appearing very tiny, dome shaped, pin head size, translucent with some weak haemolytic features were sub cultured for further testing to characterize *H. pylori*.

The isolates were Gram negative spiral rods and produce urease, oxidase, and catalase enzymes during preliminary biochemical characteristic reactions.

. **Polymerase chain reaction (PCR) method**

A volume of 12.5µl of One Taq Quick-Load 2X Master Mix with Standard Buffer (New England BiolabsInc.); 0.5µl each of forward HP-1 and reverse primers HP-2 specific primers (Vilber,Germany) (HP-1 5'-CTCAGTCAAGCGGTATCAGAAG -3' and 5'-HP-2 TCCCTCGCCAAGGAGTAATA -3'); 8.5µl of Nuclease free water and 3µl of DNA template was used to prepare 25µl reaction volume of the PCR cocktail. The reaction was gently mixed and transferred to thermalcycler. PCR products were separated on a 1.5% agarose gel and DNA bands were visualized with Ethidium bromide.

Agarose Gel Electrophoresis

A percentage of 1.5agarose gel was prepared by dissolving 1.5g of Agarose in 100ml of 1X TBE Buffer.

The mixture was heated to a clear solution using a microwave oven and allowed to cool to about 50 ºC. A volume of 3µl of Ethidium bromide was added into the solution and mixed thoroughly. The agarose preparation was carefully poured into a gel tray, with the gel comb in place and allowed to solidify. The tray was loaded into the gel tank and 1X TBE Buffer was poured into the tank, making sure that the gel was properly submerged. The gel comb was carefully removed. A volume of 5 µl of amplicon was loaded into the wells. The tank was connected to the power pack and set to run at 120 volts for 20 minutes after which it was viewed on a gel documentation system. Bands similar to that of 110 bp band of the positive control was accepted as positive.

Ethical approval

The ethical approval for the research was obtained from Nnamdi Azikiwe University Teaching Hospital, Nnewi, as well as from Life International Hospital, Awka.

Statistical analysis

Data was summarized and presented using frequency counts, percentage, while chi square was used to analyze data with level of significance set at 95% at 0.05 confidence interval.

Table 1: Determination and comparing the prevalence of positive *H.pylori* status using different identification method in relation to age range, occupation and duration of sub fertility

Variables	Category	Culture method		Stool antigen ELISA		PCR *H.pylori*	
		Positive	Negative	Positive	Negative	Positive	Negative
Age range	20-25 years	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	26-30 years	0(0)	26(32.9)	0(0)	26(30.2)	0(0)	0(0)
	31-35 years	22(21.8)	53(67.1)	15(14.9)	60(69.8)	0(0)	22(21.8)
	Total	22(21.8)	79(78.2)	15(14.9)	86(85.1)	0(0)	22(21.8)
	X^2	9.751		6.107		-	
	P-value	0.002*		0.013*		-	
Duration of sub-fertility	1-5 years	10(45.5)	38(48.1)	4(26.7)	44(51.2)	0(0)	10(45.5)
	6-10 years	12(54.5)	37(46.8)	11(73.3)	38(44.2)	0(0)	12(54.5)
	11-15 years	0(0)	4(5.1)	0(0)	4(4.7)	0(0)	0(0)
	Total	22(21.8)	79(78.2)	15(14.9)	86(85.1)	0(0)	22(21.8)
	X^2	1.350		4.547		-	
	P-value	0.509		0.103		-	
Occupation	Self employed	11(50)	46(58.2)	9(60)	48(55.8)	0(0)	11(50)
	Government employed	4(18.2)	17(21.5)	5(33.3)	16(18.6)	0(0)	4(18.2)
	unemployed	7(31.8)	16(20.3)	1(6.7)	22(25.6)	0(0)	7(31.8)
	Total	22(100)	79(100)	15(100)	86(100)	0(0)	22(100)
	X^2	1.309		3.379		-	
	P-value	0.520		0.185		-	

Key:-

*=Significant at $p<0.05$

X^2=Chi square

Table 2: Prevalence of *H.pylori* infected and non-infected groups according to gastroenterological guideline in relation to age groups and occupation.

Variables	Category	*H.pylori* infected n(%)	*H.pylori* non-infected n(%)	X^2	P-value
Age range	20-25 years	0(0)	0(0)		
	26-30 years	0(0)	26(30.2)		
	31-35 years	15(100)	60(69.8)	6.107	0.013*
	Total	15(100)	86(100)		
Occupation	Self employed	9(60)	48(55.8)		
	Government employed	5(33.3)	16(18.6)	3.379	0.185
	Unemployed	1(6.7)	22(25.6)		
	Total	15(100)	86(100)		

Key:-
*=Significant at $p<0.05$
X^2=Chi square

PCR *H. pylori* (110 bp)

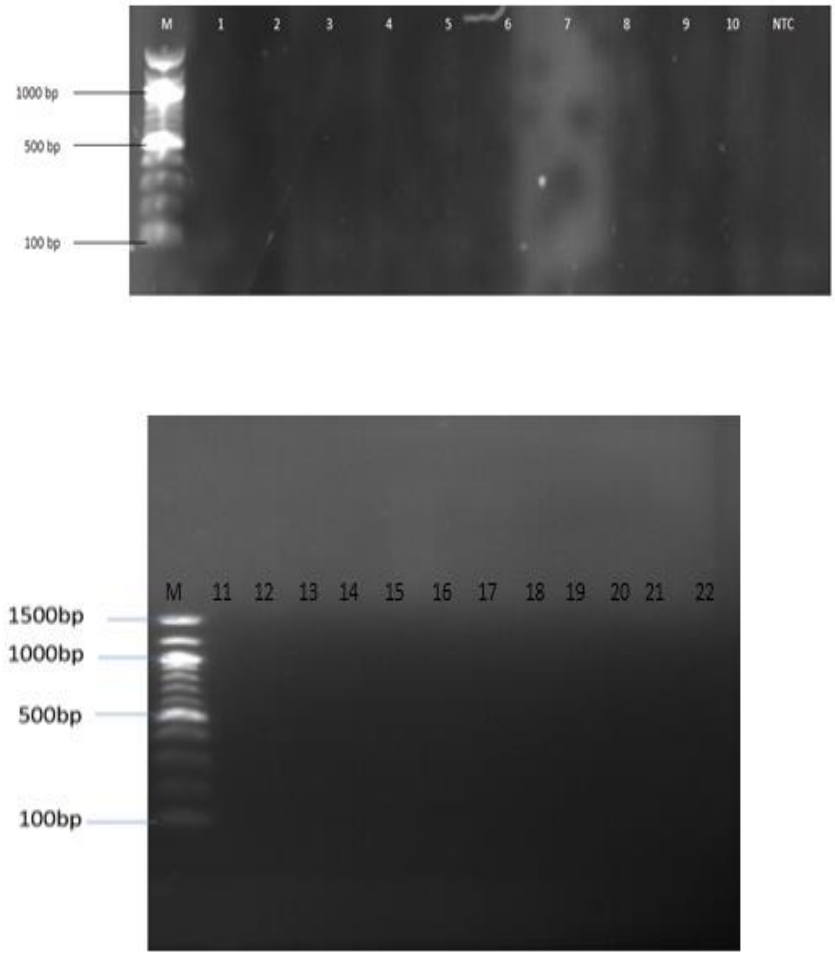

Plate 1: PCR results for positive *H.pylori* status on agarose gel electrophoresis stained with ethidium bromide using *H.Pylori* specific primers . M is a 100bp – 1500bp DNA ladder(molecular marker). Samples 1to 22 were negative bands for the expressed *H. pyroli*.

KEY:-

M= Marker

NTC= Non template control

110bp=Amplification band

Samples 1-22 = Bacteria DNA from stool of subjects with positive *H.pylori* culture isolates.

RESULTS

Table 1 shows the prevalence of positive *H. pylori* status using different identification methods in relation to age range, occupation, and subfertility duration. From the culture method used, out of the 101 participants, 21.8% were positive for *H.pyroli*, among the participants aged 31-35 years tested 100% positive for *H. pylori*. From the stool Antigen Elisa method, out of the 101 participants, 14.9% were positive and age 31-35 also tested 100% positive for *H. pyroli*. No participants in other age groups tested positive. There were no positive cases in PCR method in all the age groups, with all cases being negative. The chi-square test showed a significant difference in *H. pylori* prevalence among different age groups for both culture method ($X^2 = 9.751$, $p = 0.002$) and stool antigen Elisa method ($X^2 = 6.107$, $p = 0.013$). Regarding the duration of subfertility, 73.3% of participants with 6-10 years of subfertility tested positive for *H. pylori* using the stool antigen Elisa method (HpSA), compared to 45.5% for those with 1-5 years of subfertility. Using culture method, majority of the positive cases were among those with 6 -10years (54.5%) duration of sub-fertility. However, no positive cases were observed in the 11-15 years subfertility group. The chi-square test indicated no significant difference in *H. pylori* prevalence among different subfertility durations for any of the methods (p-values> 0.05). In terms of occupation, the prevalence of *H. pylori* was highest among self-employed individuals, with 50% testing positive using the culture method and 60% using the stool antigen Elisa method. However, no significant difference was found among the different methods (p-values> 0.05).

Table 2 shows the prevalence of *H. pylori* infection among different age groups and occupations according to gastroenterological guideline. The American College of Gastroenterology (ACG) Clinical Guideline recommends that patients with active peptic ulcer disease (PUD), a history of PUD (unless previous cure of *H.pylori* infection has been documented), low-grade MALT lymphoma, or a history of endoscopic resection of early gastric cancer (EGC) be tested for *H.pylori* infection. For the age range variable, 100% of *H.pylori* infected were of the 31-35 years category. Among those who were non-infected, 30.2% were 26-30 years, while 69.8% fell in the 31-35 years category. The chi-square (X^2) value was 6.107 with a p-value of 0.013, indicating a significant difference in *H. pylori* infection prevalence across age groups as explained in table 1. Regarding occupation, among the infected, 60% were self-employed, 33.3 government employed and 6.7% unemployed. The chi-square (X^2) value for occupation was 3.379 with a p-value of 0.185, indicating no significant difference in *H. pylori* infection prevalence across different occupations.

Plate 1 shows the electrophoretic pictures of DNA separation of products of *H.pylori* positive isolates from culture on agarose gel electrophoresis stained with ethidium bromide. Bacteria DNA 1-22 shows no migration at amplification band 110bp, indicating 22(100%) *H.pylori* negative.

DISCUSSION

Helicobacter pyroli infection occurs when the bacteria infects the stomach. *H. pyroli* attacks the lining that protects the stomach. The bacteria make an enzyme called Urease. From the culture method used, 21.8% were positive for *H.pyroli*

and among the participants aged 31-35 years tested 100% positive for *H. pyroli*. From the stool Antigen Elisa method, 14.9% were positive for *H. pyroli* and among the participants 31-35 years tested 100% positive for *H. pyroli*. From the PCR method, there were no positive cases for PCR *H. pyroli* in all the age groups with all the cases being negative. This may be because ageing has effect on fertility.

The chi-square test showed a significant difference in *H. pylori* prevalence among different age groups for both culture method (X^2 = 9.751, p = 0.002) and stool antigen Elisa method (X^2 = 6.107, p = 0.013). This corroborates with a study which showed a statistical significant relationship between the age of participants and their *H. pylori* infection status and also previously inferred where incidence of *H. pylori* was synonymous with increasing age of the studied population[7, 8]. This may be ascribed to increasing biomass of the organism as they multiply in their host with increasing age.

Moreso, for the duration of subfertility, age groups 6-10 years had 54.5% in culture method and 73.3% in the stool antigen Elisa method with no positive in PCR method. There was no significant difference among the different methods. Self-employed participants were more infected. This could be due to the possibility that they overwork in order to keep their businesses afloat thus more vulnerable to contracting *H.pyroli*.

The positive *H. pyroli* status on agarose electrophoresis using specific primers were all negative bands for the expressed *H. pyroli*. This presumably false negative outcome from the PCR testing which is in discordance with some co-positive *H.pylori* result from culture and *H.pylori* stool antigen test obtained in this study could be as a result of the fact that despite the high sensitivity of PCR and its suitability for diagnosis when an organism is present in low number, slow growing or difficult to identify is in doubt. The technique is susceptible to inhibition by contaminants present in clinical specimens, thus giving false negative results. Human faeces are known to contain PCR inhibitors, which should be removed from the specimen before target DNA amplification[9]. It has been suggested that the inhibition of PCR can be overcome by dilution of the faecal suspension[10], but this may make the assay less sensitive as fewer bacteria would be present in the diluted sample.

CONCLUSION

The choice of test to detect *H.pyroli* infection depends on the prevalence and strains of *H.pyroli* on endemic areas, accessibility, advantages and disadvantages of each method as well as different clinical circumstances of each patient. To combine the results of two or more tests could be a reasonable strategy in routine clinical practice to achieve the most reliable result.

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